PREFACE

This thesis is submitted to the Faculty of Health Science at the University of Copenhagen, Denmark in order to meet the requirements for obtaining the Ph.D.-degree. The work was carried out at the National Institute of Occupational Health, Lersø Park Allé 105, Copenhagen, Denmark. During my Ph.D. studies I received supervision from Professor Håkan Wallin, Associated Professor Ulla Vogel and Professor Steffen Loft. The project was funded by the National Institute of Occupational Health and the EU 5th Framework Programme, Key Action 4, Environment and Health, Quality of Life and Management of Living Resources, project no. QLRT-2000-00573.

First at all, I would like to express my gratitude to Håkan Wallin and Ulla Vogel for their excellent supervision during my studies and for their enthusiasm and daily guidance. I would also like to thank my supervisor, Steffen Loft at University of Copenhagen for his support and interest. Also very special thanks to the rest of the WOOD RISK collaboration who made this project possible by their enthusiasm and endless patience with me. Thanks to the pathology departments at hospitals all over Denmark for taking time out of their busy schedule to help me with the collection of tumour blocks and for lending me this priceless material. Especially thanks to Specialechef. Dr.Med. Torben Steiniche and Dr. Med. Annemarie Antonsen for your priceless help in selecting the tumour blocks and for making sure that I was not lost forever in the pathology archives.

During my project I have had the opportunity of working in a dynamic laboratory, surrounded by wonderful persons. Whom I would like to thanks for a lot of things; from their enlightening spirit on grey cold mornings to always being helpful even on a late afternoon. Especially Lourdes Petersen for technical assistance in the sequencing of the mutations. Most of all I would like to thank my dear office-mate Anne Saber for enlightening conversations, priceless teamwork during the cell exposures and her always pleasant spirit. I really enjoyed sharing our 8 m².

Last, but not least, I would like to thank my family for being there for me or not being there in the final stage of this thesis and a very special thanks to my mother for taking care of my children.

Copenhagen, April 2008
Jette Bornholdt Lange
SUMMARY

The present thesis focuses on the inflammatory, genotoxic and carcinogenic effects of exposure to different species of wood dust. The experimental work performed in this thesis consists of two parts. The first part of the study was carried out in an *in vitro* model with the human lung epithelial cell line A549 measuring inflammatory and DNA damaging effects. The second part consists of a molecular analysis of the K-ras gene for mutations in the hotspots codons in human sinonasal cancers. Design, calibration and validation of the assays were performed.

Cancer at the sinonasal cavities is rare with incidence rates between of 0.3 to 1.4 per 100,000 for men and 0.1 to 0.8 per 100,000 for women in Europe, depending on country. However, cancer at this site is associated with occupational exposures including wood dust. Especially the adenocarcinoma subtype is strongly associated with exposure to wood dust primarily from hard woods. Non-malignant symptoms like allergy, asthma, rhinitis and chronic bronchitis have also been associated with occupational exposure to wood dust in epidemiological studies. In most epidemiological studies hardwoods (e.g. oak and beech wood dust) seems to have greater association to both the malignant and non-malignant symptoms compared to softwoods (e.g. pine and spruce wood dust). Since, well standardized experiments of wood dust exposure are limited and difficult to execute in humans, knowledge of the cellular mechanisms underlying wood dust induced carcinogenicity and non-malignant symptoms are still poorly understood. Particulate induced inflammation as well as extractives are suggested to be involved in the carcinogenesis.

In this thesis wood dust potential to induce DNA damage and inflammation was investigated exposing the human lung epithelial cell line A549 to various species of wood dust and endpoints for inflammation and genotoxicity was evaluated. The experiments showed that the different species of wood dust vary in their ability to cause DNA strand breaks and inflammation. There was no apparent correlation between the species potential to initiate inflammation and their potential to cause DNA damage. Contrary to our hypothesis, we showed that pure wood dust is able to cause primary DNA damage, independent of inflammation as well as hardwoods had no higher inflammatory or genotoxic potential than softwoods.

To investigate the molecular mechanisms behind the wood dust induced carcinogenesis, we examined human sinonasal tumours for mutations in the *ras* genes. The mutational spectrum, which is the type, site and frequency of mutations, provides useful clues to etiological factors and identification of exposure related tumours. We examined all incident cases of sinonasal
adenocarcinoma and squamous cell carcinoma reported to the Danish Cancer Registry between 1991 and 2001. After a very careful inclusion process in order only to include sinonasal cancer of the nasal cavities and sinuses with correct histology, we included 174 cases. Wood dust exposure was assessed by interview and job/trade codes from the National Pension Fund and self reported job titles in the Central Personal Register. Among the cases wood dust exposure occurred in 21 percent of our patients and was 7 times more frequent in patients with adenocarcinomas than in squamous cell carcinoma (p<0.0001). In the mutational analysis, the K-ras gene was mutated in adenocarcinoma (13%) which is in the range reported in earlier studies, whereas the frequency was very low in the squamous cell carcinoma (1%). By analysis of all published K-ras mutations, the \text{GGT}^{\text{GLY}} \rightarrow \text{GAT}^{\text{ASP}} \text{ transition} was the most common K-ras codon 12 mutation. Despite that 65 percent of these patients had been exposed to wood dust, it was not significantly different from the patients without a G→A mutation.

The main conclusions based on the results obtained in this thesis are:

- All wood dust species cause inflammation. However, the species vary in their potential to induce the inflammation.
- Wood dust species vary in their potential to cause DNA damage. The DNA damage observed seemed to be caused by a direct genotoxic effect of the dust it selves.
- The current study provides evidence that the distinction between hard and softwoods dust may not be that clear cut in relation to health effects.
- The previously reported association between adenocarcinomas and exposure to wood dust could be confirmed by our study.
- Mutations activation of K-ras was restricted to a small subpopulation of the adenocarcinomas. The predominant mutations among these tumours were \text{G•C→A•T transitions}.
- Overall, the study suggests a limited role for K-ras mutations in development of sinonasal cancer.
DANSK RESUMÉ

Denne ph.d. afhandling omhandler inflammatoriske, genotoksiske og karcinogene effekter efter eksponering for træstøv fra forskellige træsorter. Eksperimenterne udført i forbindelse med dette projekt, var delt i to. Den første del omhandlede en **in vitro** model, hvor den humane lunge epitel celle linje A549 blev brugt, til at bestemme inflammatoriske og DNA skadende effekter. Den anden del bestod i en molekylær analyse af humane sinonasale tumourer for mutationer i det kræft relaterede gen **K-ras**. Mutationer i kodon 12, 13, og 61, såkaldte ’hotspot’ kodons blev bestemt. I forbindelse med disse forsøg blev kalibrering og validering af alle de brugt metoder udført.

Kræft i næse og bihuler er sjældent. I Europa er forekomsten af nydiagnosticerede tilfælde på mellem 0,3 til 1,4 per 100 000 indbyggere for mænd og mellem 0,1 og 0,8 per 100 000 for kvinder, afhængigt af hvilket land man ser på. Til trods for den lave hyppighed ses en stærk sammenhæng mellem kræft i næse og bihuler og erhvervsmæssig udsættelser for især træstøv. Professionel udsættelse for træstøv, især fra hårde træsorter, er blevet stærkt associeret med histologi typen adenokarcinom. Ikke maligne symptomer så som allergi, astma, rhinitis og kronisk bronkitis kan ligeledes i epidemiologiske undersøgelser relateres til en professionel udsættelse for træstøv. I de fleste studier er træstøv fra hårde træsorter (f.eks. eg og bøg) mere karcinogene og har en stærkere association til de ikke maligne symptomer sammenlignet med støv fra bløde træsorter (f.eks. fyr og gran). Da standardiserede og kontrollerede forsøg med hensyn til eksponering er svært at udføre på mennesker, er de underliggende mekanismer bag træstøvs effekter mangelfuld kortlagt. Det er blevet foreslået at partikel induceret inflammation, så vel som de indholdsstoffer der er i selve træt kan virke karcinogene.

I dette projekt eksponerede jeg den humane lunge epitel celle linje A549 for træstøv fra forskellige træsorter og målte markører for inflammation og genotoksicitet. Resultaterne viste, at de forskellige træsorter varierer i deres potentiale til at give DNA streng brud og til at kunne inducere en inflammations proces. Der var umiddelbart ingen sammenhæng mellem en træsorts evne til at forårsage inflammation og dets genotoksiske potentiale. I modsætning til vores hypotese, var inflammation ikke den direkte årsag til DNA streng brudene, ligesom træstøv fra hårdt træ ikke var mere inflammatoriske eller genotoksiske i forhold til støv fra blødt træ.


Hovedkonklusioner, der kan blive dragtet ud fra resultaterne i denne ph.d. afhandling:

- Alle testede træ sorter kan forårsage inflammation, men variere i deres potentiale til at inducere inflammationen.

- Træsorter varierer i deres potentiale til at forårsage DNA skade. Den observerede DNA skadende effekt, var forårsaget af en direkte genotoksisk effekt af selve træstøvet.

- Resultaterne fra dette studie tyder på, at brugen af kategorierne hårdt og blødt træ ikke er de mest optimale, når det gælder helbreds effekter.

- Sammenhængen mellem adenokarcinomer og erhvervsmæssig udsættelse for træstøv blev bekræftet i dette studie.
• Mutationer i K-ras genet er begrænset til en lille population af adenokarcinomer. Den hyppigst forekomne mutation blandt disse tumorer var G•C→A•T mutationer.

• Alt i alt, viser studiet kun en begrænset rolle for K-ras mutationer i udviklingen af kræft i næse og bihuler.
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INTRODUCTION

Money is worthless if one loses the most valuable of everything; one's health. This sentence was phrased back in the eighteen century by the famous Italian physician Bernardino Ramazzini (1633 - 1714), who is considered the father of occupational medicine [1] but it is still true and relevant even today.

Health effects of both non-malignant and malignant character have been observed in the respiratory tracts of workers occupationally exposed to wood dust. One of the first observations was made by Ramazzini himself. He observed eye symptoms and headache in carpenters and saw mill workers [1]. But it was not until later, in 1968 an association between adenomas of the nasal cavity and employment in the furniture industry was found [2]. Since that time many epidemiological studies have been conducted, showing an association between wood dust and sinonasal carcinomas, especially the adenocarcinoma subtype. Sinonasal cancer is a rare type of tumour with standardised incidence rates between 0.3 and 1.4 per 100,000 for men in European countries [3]. However, odd ratios of up to 45-fold [4], for furniture workers with high wood dust exposure, have lead to the knick name; carpenters nose.

Besides the malignant symptoms, workers employed in occupations with wood dust exposure also suffer from non-malignant symptoms. These symptoms may not have as high personal consequences as the malignant. However, the non-malignant symptoms are more frequent and still diminish the quality of life. Of non-malignant symptoms can be mentioned asthma, rhinitis, chronic bronchitis and eye symptoms [5-8]. Most of these symptoms are suspected to be induced by inflammation, for instance caused by the wood dust.

Despite the many epidemiological studies of wood workers and the health effects of wood dust exposure, little is known about the mechanisms behind the effects of wood dust. The evaluations of wood dust as a carcinogen [7] and most legislative aspects like threshold limit values are based on epidemiological data. In year 2000, the EU funded research project WOOD RISK was launched. The aim of this project is to add knowledge about the mechanisms behind wood dust induced health effects and exposure levels in the European countries in order to provide information for a risk assessment of wood dust. This thesis is a part of the project.
The overall aim of this thesis is to contribute to the knowledge about the mechanisms related to the inflammation, genotoxicity and carcinogenicity of wood dust. More specifically, this study investigated the inflammatory and genotoxic potential of different species of wood dust \textit{in vitro}. These effects are measured as the release of pro-inflammatory cytokines and DNA strand breaks. The carcinogenicity of wood dust is investigated in human tumours by searching for a link between exposure to wood dust and patterns of mutations in the sinonasal cancers that epidemiologically are associated with wood dust exposure.

The thesis is organised as follows: First a chapter of background material is provided. Some of the sections are meant as a help for the reader to understand the abundance of wood dust exposure and the adverse health effects exposure to wood dust can have. The other sections are more specifically aimed towards this project. Secondly, a description of the aims and hypothesis for this thesis is presented. Thirdly, the models and methods used in this thesis are described. Finally, the obtained results in this thesis is described and discussed, and summarized in a conclusion. The two manuscripts this thesis is built upon can be found in the end of the report.
BACKGROUND

The wood industry
Wood is one of the world’s most important resources, as raw material for industries, for construction and as fuel. Today, forests cover an estimated 3,952 million hectares or 30 percent of the total land area. In figure 1 the distribution of forest in the world is shown. The forests are unevenly distributed in two belts around the planet. Europe accounts for 1,001 million hectares of forest, which equals about 25% of all forests. [9].

Figure 1. The world’s forests cover [10].

In 2003 the worldwide annual harvest was 3,342 million m³ of round wood, 1,754 million m³ (52%) of which was used as fuel. The consumption of industrial round wood (table 1) was worldwide 1,592 million m³. The term “industrial round wood” covers the use of wood for saw logs, veneer logs, pulp wood (round wood and split wood) and other types of industrial wood like fibreboards. As can be read in table 1, the majority of the wood processed in Europe is coniferous, except in Italy where it is approximately half coniferous and half non-coniferous [11].

The wide use of wood makes it one of the most commonly seen occupational exposures. In year 2000 approximately 13 million people were occupied in the forestry sector worldwide and additionally about 3.5 million people in the furniture industry. This means that about 0.4% of the total labour force worldwide is occupied in the forestry sector [12]. In the European Union (EU-25) it was recently estimated that approximately 3.6 million workers are exposed to wood dust, i.e. about 2.0% of the work force within the EU [13]. The global trade in wood products was in 2003 estimated to about 150 billion US dollars [10].
Table 1. Consumption of industrial round wood in year 2003. Data obtained from FAO’s Yearbook of forest products 2003 [11]

<table>
<thead>
<tr>
<th></th>
<th>Total (1000 m³)</th>
<th>Coniferous (%)</th>
<th>Non-coniferous (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Worldwide</td>
<td>1,591,896</td>
<td>1,011,474 (64%)</td>
<td>580,423 (36%)</td>
</tr>
<tr>
<td>Europe</td>
<td>472,413</td>
<td>348,865 (74%)</td>
<td>123,548 (26%)</td>
</tr>
<tr>
<td>Denmark</td>
<td>784</td>
<td>719 (92%)</td>
<td>65 (8%)</td>
</tr>
<tr>
<td>Finland</td>
<td>61,683</td>
<td>48,738 (79%)</td>
<td>12,944 (21%)</td>
</tr>
<tr>
<td>France</td>
<td>32,214</td>
<td>21,701 (67%)</td>
<td>10,513 (33%)</td>
</tr>
<tr>
<td>Germany</td>
<td>43,744</td>
<td>35,805 (82%)</td>
<td>7,939 (18%)</td>
</tr>
<tr>
<td>Italy</td>
<td>6,986</td>
<td>3,161 (45%)</td>
<td>3,825 (55%)</td>
</tr>
</tbody>
</table>

The term wood industry covers industries, which predominately carry out mechanized processing of wood. Examples of such industries are furniture making, carpentry and joinery, sawmilling, manufacture of other wooden products e.g. wooden containers, manufacture of veneer sheets, plywood, particle fibre board and panels. The wood dust is produced during the processing of the wood by hand or machinery. Some of the most dusty work tasks are manual sanding in the furniture making, sawing and drilling. However, the dust is often reduced by adding filters on the machines or local ventilation on the machines. Despite this effort a part of the wood dust will always escape the filtering systems causing an exposure of the personnel working in the facility.

**Exposure Levels to Wood Dust by Legalisative and Measurements**

Exposure to dust is defined by various methods; total dust, inhalable dust, and respirable dust. Total dust is collected by a passive dust collector and is considered less relevant with respect to health effects, because it is without size specifications. The inhalable dust is defined as those particles captured by samplers regardless to orientation with an aerodynamic diameter between 0 and 100 µm. Respirable particulate mass is the mass, collected by a 10-mm nylon cyclone at a flow rate of 1.7 litre per minute. The particle fraction collected by this method has a aerodynamic diameter between 0-100 µm and a median cut point of 4 µm[14]. Nevertheless historically, total dust is often used in the regulation of wood dust.

The threshold limit value or recommendations for wood dust exposure, varies between countries and type of wood. Examples from different countries are listed in table 2.
Table 2. Threshold limits for wood dust exposure in different countries.

<table>
<thead>
<tr>
<th>Country</th>
<th>Institution</th>
<th>Time weight average</th>
<th>Limitations</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>The Danish Working Environment Authority</td>
<td>2 mg/m³</td>
<td>All types of wood dust</td>
<td>[15]</td>
</tr>
<tr>
<td>Sweden</td>
<td>Arbetsmiljöverket</td>
<td>2 mg/m³ *</td>
<td>All types of wood dust</td>
<td>[16]</td>
</tr>
<tr>
<td>Finland</td>
<td>Social- och hälsovårdsministeriet</td>
<td>5 mg/m³ *</td>
<td>Organic dust</td>
<td>[17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(inhalable)</td>
<td>Especially some wood dust</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>Institut National de Recherche et de Sécurité</td>
<td>1 mg/m³ *</td>
<td>All types of wood dust</td>
<td>[18]</td>
</tr>
<tr>
<td>Spain</td>
<td>Instituto Nacional de Seguridad e Higiene en el Trabajo</td>
<td>5 mg/m³</td>
<td>Hardwood ¤</td>
<td>[19]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/m³</td>
<td>Softwood</td>
<td></td>
</tr>
<tr>
<td></td>
<td>U.S. Department of Labor, Occupational Safety &amp; Health</td>
<td>15 mg/m³</td>
<td>All types of dust</td>
<td>[20]</td>
</tr>
<tr>
<td></td>
<td>Administration</td>
<td>5 mg/m³</td>
<td>Respirable fraction of wood dust (all species)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACGIH</td>
<td>1 mg/m³</td>
<td>Non-allergenic species</td>
<td>[21]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.5 mg/m³</td>
<td>Western red cedar</td>
<td></td>
</tr>
<tr>
<td>Canada</td>
<td>Occupational Health and Safety</td>
<td>1 mg/m³</td>
<td>allergenic wood dust</td>
<td>[22]</td>
</tr>
<tr>
<td></td>
<td>British Colombia</td>
<td>1 mg/m³</td>
<td>non allergenic hardwood non</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 mg/m³</td>
<td>allergenic softwoods</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Alberta</td>
<td>2.5 mg/m³</td>
<td>allergenic wood dust</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/m³</td>
<td>non allergenic wood dust</td>
<td>[23]</td>
</tr>
</tbody>
</table>

* inhalable fraction. ¤ classified as a carcinogenic compound. For hardwood, the threshold limit applies for the whole dust fraction in mixtures between hardwoods and softwoods.

In Denmark, the wood industry can be divided into five subgroups; sawmills and wood preservation, manufacturing of plywood and boards, manufacturing of construction materials, wood packing factories and manufacturing of other wooden products like wooden kitchen utensils and coffins. The furniture industry is classified in a category on its own [24]. Between the subgroups, work tasks and products vary and therefore the exposure level to wood dust varies. The exposure has been determined in extensive studies in the Danish furniture industry. The average concentration of total dust was 0.60 mg/m³ [25].

A review on exposure measurements from the furniture industry in different countries performed by Mikkelsen et al [26] shows an average concentration of total dust about 1 mg/m³. In a comparison with measurements made in 1988 the wood dust level had in 2000 decreased by 52 percent in the furniture industry mainly due to increased implementation of local ventilation.
on machines [25]. In a study of inhalable dust in 12 Swiss sawmills the average concentration was 1.7 (0.2-8.5) mg/m³ [27].

A very recent study [13], which includes data from 25 countries in the European Union, estimates the numbers of exposed workers, levels of exposure and the use of different wood species. The exposure assessment was based on industrial hygiene measurements, labour force statistics, country questionnaires and expert judgements. The overall conclusions were that 2.0 percent (3.6 million workers) of the labour force in the European Union is exposed to wood dust. Of these exposed workers, 21 percent were exposed to a wood dust level below 0.5 mg/m³, whereas 17 percent were exposed to between 0.5 and 1 mg/m³, 21 percent between 1-2 mg/m³, 25 percent between 2-5 mg/m³ and 16 percent were exposed to 5 mg/m³ or more, which is a very heavy exposure. Subgroups in the forestry industry had a very low level of exposure as 93 percent of the employees were exposed to levels below 0.5 mg/m³ wood dust. In the opposite end of the scale, in the ship and boat building industry, the majority (64 percent) of the workers were exposed to 2 mg/m³ wood dust or more. Industries like the furniture industry, manufacturing of wooden containers and wood products, and the construction industry have medium to high exposure levels. In these industries between 32% and 54% of the workers were exposed to 2 mg/m³ wood dust or more. In the company survey, Finland, France, Germany and Spain were selected to represent the European Union with respect to the use of wood species.

The use of wood species varies between countries as well as between industries. The below mentioned numbers refer to the frequency in the use of the different species and are not necessarily well related to the consumption of wood. Pine and spruce were popular species in all four countries and in all industries especially in the manufacturing of builders’ carpentry, on average 68 percent of the factories used pine and 54 percent used spruce. Beech and oak were less popular. Beech, oak and birch were less popular and mainly used in the making of furniture and other wood products. In the furniture industry, pine and spruce are used in about 30 percent of the companies, whereas beech or oak were used by on average 40 percent of the companies. The most frequently used type of wood in the furniture industry is the wooden board. On average 67 percent of the companies used wooden boards. [13].
Particle size of wood dust varies depending on the work task from which they are generated. The distribution of particle size after two typical wood working processes, sanding with an 80 grit paper and sawing has been investigated for pine and oak wood. When sanding, the median diameter in size distribution based on the number of particles was 4.7 µm for pine and 9.5 µm for oak and respectively 44.4 µm and 25.6 µm based on the mass distribution. When sawing the median diameter based on the number distribution was respectively 20.0 µm and 5.7 µm for pine and oak. For the mass distribution the median diameter was 72.2 µm for pine and 32.9 µm for oak. The size distributions were somewhat similar. However, oak dust had a smaller particle size compared to pine dust, especially when the dust were generated by sawing [28].

**Classification and composition of wood**

There is a very wide selection of species of trees, each having its own characteristics. Trees are botanically classified by the type of seed they produce: There are two different classes, gymnosperms and angiosperms. Gymnosperms, literally meaning “naked seed”, are characterised by having the seeds exposed to the surface. In this class there are four divisions with living representatives, the most familiar being the Coniferophyta which includes conifers like the pines, firs and spruces etc. [29]. Angiosperms make up most of today’s plants and trees and are characterised by having encapsulated seeds with indirect pollination [29]. Beside the structure of the seed, on which the classification is based, the two classes differ in the anatomical structure of the stem.

![Figure 2](http://www.olympusconfocal.com/gallery/plants/pinewood.html). The picture to the left shows a confocal microscopy of a pine stem [From http://www.olympusconfocal.com/gallery/plants/pinewood.html]. The picture to the right shows an electron micrograph of hardwood, showing vertical fibers, large vessels and horizontal rays [From NC. Brown Center for Ultrastructure studies, Syracuse. NY].

Wood from conifers, also called softwood, consists of only one kind of cells; the tracheides, which are elongated, fibre-like cells with a square or polygonal cross-view (see figure 2). Depending on the time of the season during which the cells are formed, they can either have a
wide lumen with thin walls (early wood) or a small lumen with thick walls (late wood). Early wood are mainly for transport of nutrients, while late wood have a stabilising function [7].

Trees from angiosperms, is termed hardwoods and have more complex structures in the stem. Here the stem consists of several differentiated cells located in between each other (see figure 2). Some are specialized for water transport in shape of vessels, while others are specialised for stabilisation and storage. Stabilizing tissues contain libriform fibres and tracheide shaped fibres (having thick polygonal walls and small lumens like the late wood) while the conduction system consists of joined vessel cells (having big lumen and thin walls like the early wood). Hardwoods consist of several additional cell types, which are not described here [7].

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Gymnosperms/ conifers/ softwoods</th>
<th>Angiosperms/ deciduous/ hardwoods</th>
<th>Angiosperms/ deciduous/ tropical hardwoods</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibres</td>
<td>Long (1.4 -4.4 mm)</td>
<td>Short (0.2 -2.4)</td>
<td>Short (0.2 -2.4)</td>
</tr>
<tr>
<td>Cell type</td>
<td>One (tracheids)</td>
<td>Various</td>
<td>Various</td>
</tr>
<tr>
<td>Cellulose content</td>
<td>~ 40-50%</td>
<td>~ 40-50%</td>
<td></td>
</tr>
<tr>
<td>Polyposes</td>
<td>~15-30%</td>
<td>~25-35%</td>
<td></td>
</tr>
<tr>
<td>Lignin</td>
<td>~25-35%</td>
<td>~20-30%</td>
<td></td>
</tr>
<tr>
<td>Extractive content</td>
<td>up to about 10%</td>
<td>1-10%</td>
<td>Up to 30%</td>
</tr>
<tr>
<td>Non-polar</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Polar</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

In both hardwoods and softwoods the stem is divided into two zones, the sapwood and the heartwood. The sapwood consists of alive and metabolic active cells which are responsible for the synthesis and storage of secondary metabolites. The heartwood is the dark-coloured wood found in the interior of the stem and consists of non-metabolic active cells. The heartwood functions as long-term storage for extractives, which provides protection of the wood. These extractives are responsible for many of the large-scale characteristics for the wood, like stability, resistance to fungi and water. In the wood industry the sapwood is often less valued, because it lacks the desirable properties from the heartwood extractives [9]. Characteristic properties for softwoods, hardwoods, and tropical hardwoods are summarized in table 3. Softwoods have long
fibres while hardwoods (including exotic hardwoods) have short fibres. The extractive content is similar for both soft and hardwoods but elevated in exotic hardwoods.

In this thesis seven commonly used wood species were selected for investigation, in an attempt to cover all tree classes of wood. Oak (*Quercus robur*), beech (*Fagus silvatica*) and birch (*Betula pendula*) are thought to represent hardwoods; where as teak (*Tectona grandis*) represents exotic hardwoods. Softwood is represented by pine (*Pinus sylvestris*) and spruce (*Picea abies*). To represent artificial wood, medium density fibreboard with oak surface (MDF) was chosen. In table 4 characteristics and secondary metabolites of the woods are listed for the seven species.

**Secondary metabolites and their biological effects**

The secondary metabolites are a wide range of substances with different chemical, physical and biological properties. They consist of many classes of compounds like: flavonoids, lignans, terpenes, alkaloids, sterols, waxes, tannins resin acids and carotenoids. So far the total number of secondary metabolites exceeds 100,000 [30]. Secondary metabolites are not only present in the stem of plants, but in all parts including seeds and fruits. Dietary intake of these is the major route of exposure in humans.

The terpenens (Terpenoids) represent the largest class of secondary metabolites being present in almost all plants [31]. The terpenes are derived from isoprene (C₅) building blocks and are often found in the resins. Terpenes extracted from softwoods contain all classes, whereas terpenes from hardwoods mainly contain higher terpenes. Examples of terpenenes are α-terpinene (*P. sylvestris*), limonene (*P. pinea*), squalene (*Fagus silvatica*) and betulinic acid (*Tectona grandis*). In the class of terpenoids exists a subgroup of sterols (also called phytosterols), examples of such phytosterols are campesterol (*P. sylvestris*), stigmasterol (*Fagus sylvestris*), and β-sitosterol (*P. sylvestris*).

Another major class is the phenolic compounds, characterized by the presence of a hydroxyl group attached to an aromatic ring structure, and includes phenols, tannins, stilbenoids, and flavonoids. Over 4000 different flavonoids have been isolated from plants and they commonly occur in the heartwood of trees, leading to the coloration of the wood [32]. The flavonoids are present in both soft- and hardwoods, but occur more frequently in hardwood than softwood [7]. Examples of flavonoids from woods are quercetin (*Quercus spp.*), pinocembrine (*P. sylvestris*) and kaemferol (multiple occurrences in plants). Examples of other phenolic and aromatic compounds are resorcinol (Quercus robur), matairesionol (*Picea abies*), and furfural (*Betula pendula*).
<table>
<thead>
<tr>
<th>Characters</th>
<th>SPRUCE</th>
<th>PINE</th>
<th>OAK</th>
<th>BEECH</th>
<th>BIRCH</th>
<th>TEAK</th>
<th>MDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wood type</td>
<td>Soft wood</td>
<td>Soft wood</td>
<td>Hard wood</td>
<td>Hard wood</td>
<td>Hard wood</td>
<td>Tropical hard wood</td>
<td>Artificial wood</td>
</tr>
<tr>
<td>Cell types</td>
<td>Tracheides</td>
<td>Tracheides</td>
<td>Tracheides</td>
<td>Tracheides</td>
<td>Tracheides</td>
<td>Tracheides</td>
<td>Varies</td>
</tr>
<tr>
<td></td>
<td>Fiber tracheides</td>
<td>Fiber tracheides</td>
<td>Vessels</td>
<td>Fiber tracheides</td>
<td>Fiber tracheides</td>
<td>Fiber tracheides</td>
<td>depending on</td>
</tr>
<tr>
<td></td>
<td>Libriform fiber</td>
<td>Sieve cells</td>
<td>Sieve cells</td>
<td>Libriform fiber</td>
<td>Libriform fiber</td>
<td>Libriform fiber</td>
<td>the type of wood</td>
</tr>
<tr>
<td>Formaldehyde</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Examples of secondary metabolites</td>
<td>Astrign</td>
<td>Campesterol</td>
<td>Butyric acid</td>
<td>Acetyl methyl betulinitete</td>
<td>1-arabinose</td>
<td>1-hydroxy-2-</td>
<td>Varies</td>
</tr>
<tr>
<td></td>
<td>Isorhapontin</td>
<td>Copahoneol</td>
<td>Coniferyl aldehyde</td>
<td>B-Amynin acetate</td>
<td>Fural</td>
<td>methylanthraquinone</td>
<td>depending on</td>
</tr>
<tr>
<td></td>
<td>Mataireinon</td>
<td>Muurolene</td>
<td>Quercus lactone (A+B)</td>
<td>Qualeine</td>
<td>β-Benzulenol Acetate</td>
<td>Tectoquine</td>
<td>the type of wood</td>
</tr>
<tr>
<td></td>
<td>Pictannol</td>
<td>Phenol</td>
<td>Valeric acid</td>
<td>Stigmastrol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Limonene</td>
<td>Pinorebrin</td>
<td>Resorcinol</td>
<td>Syringic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pinosylin</td>
<td>Sinupaldehyde</td>
<td>Vanillic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sitostrol</td>
<td>Quercetin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>A-Long-pinene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-Terpineol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-pinene</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hardness of wood in brinell</td>
<td>1.3</td>
<td>2.0</td>
<td>3.5</td>
<td>3.4</td>
<td>2.6</td>
<td>3.0</td>
<td>Variable</td>
</tr>
</tbody>
</table>
Secondary metabolites have many adverse biological effects in humans as well as in an ecologically context. Secondary metabolites vary in their effects from being anti-mutagenic to promoting carcinogenesis. These effects can only to some extent be predicted by the chemical class to which they belong. The flavonoids are for instance known for their pharmacologically useful antioxidant properties and other main properties like being anti-inflammatory, antihistaminic and antimicrobial/viral. Most terpenes affect the fluidity of membranes, while some have cytotoxic potential [30]. However, it is the chemical structure of the individual compound which determines the biological effects. Examples of beneficial properties in regard to tumour development among the species chosen in this study are; The phytosterols β-sitosterol (P. sylvestris) and campesterol (P. sylvestris) have shown to lower the incidence of colon cancer in rats [34]. Also anti-mutagenic properties have been detected by the flavonoid pinocembrin (P. sylvestris) [35]. The terpenoid betulinic acid (Tectona grandis) has been shown to be cytotoxic towards head and neck squamous cancer cells [36]. Also the terpenoid squalene (Fagus silvatica) has anti-tumours properties on chemically-induced colon, lung and skin tumourgenesis in rodents [37].

Other secondary metabolites exhibit a genotoxic and/or a carcinogenic potential. The flavones quercetin and kaempferol contains besides an antioxidant activity also a genotoxic potential [30]. Compounds like abietic acid (pinus species, acid from resins), ellagic acid (many hardwoods in tannin acids), and camphene (cypress oil) have tested positive in Ames test. Other secondary metabolites act as tumour promoters in animal experiments. Examples are the terpene α-pinene (P. sylvestris) and the naphthol chrysarobin from Quassia wood [38]. Among the secondary metabolites listed in table 4, phenol is classified as a group 3 carcinogen by IARC (not classifiable as to its carcinogenicity to humans) acting as a tumour promoter for skin cancer and induction of leukaemia at low doses in mice [39].

Wood dust particles deposition in the respiratory tract.
Working in an environment with wood dust leads to inhalation of the particles though the respiratory tract, causing health effects when the wood particles are deposited and come in direct contact with the tissue covering the respiratory tract. Upon inhalation, particles are deposited and retained in the respiratory tract according to their size, shape, solubility, and surface chemistry [40]. The most important mechanisms for the deposition of particles are impaction, sedimentation, and diffusion [41]. The particle size of wood dust varies depending on the type of wood and work task by which they are formed (described in the section of exposure levels). For example, dust generated by sanding with an 80 grit paper has, a mass distribution median diameter of 41.9 µm for pine and 24.5 µm for oak [28].
For dosimetry purposes the respiratory tract can be divided into three regions, the extrathoracic region, the tracheobronchial, and the alveolar region. The extrathoracic region covers the nasal passage, nasopharynx and the oral part of the pharynx down to the larynx. The cell populations which cover these areas are keratinising, respiratory and stratified squamous epithelial cells. The tracheobronchial region, which covers trachea and the bronchia, are covered with respiratory epithelium characterised by a ciliated surface. It is composed of ciliated cells and basal cells with mucus-producing goblet cells occasionally dispersed [42]. The alveolar region consists of bronchioles and alveoli. The bronchioles are covered by ciliated bronchiolar epithelium and the alveoli consist of Clara cells, endothelial cells and epithelial cells type I and II [40]. For a schematic presentation, see figure 3.

Figure 3. Schematic presentation of the respiratory tract. Modified from [40] and [45]

The nasal region of the extrathoracic region consists of a bony and cartilaginous skeleton with associated soft tissues. This makes it possible for the nose to adapt to environmental changes such as exercise and climatic changes. The main route for inhalation (besides during exercise) is through the nose. Impaction and filtration of the inhaled air is therefore an important function of the nose. Nasal hairs that are projected into the lumen of the nasal vestibule participate in the filtration of the inhaled air. The air is additionally filtered, warmed up and humidified in the
nasal cavity, whose internal dimensions are 8-11 cm in length and about 5 cm in height [41,43]. The surfaces are partly covered with pseudo-stratified columnar ciliated epithelium and partly with stratified cuboidal epithelium. The ciliated cells are located in patches of the respiratory epithelium that is covered by a 0.5-10 µm-thick mucus blanket to catch the inhaled particles [44].

Humans are oronasal breathers (we inhale through both the nose and the mouth), contrary to most laboratory animals that are obligated nose breathers. The deposition pattern of particles differs, depending on whether the air is inhaled through the mouth or the nose. The estimates of particle deposition versus particle size in nose and mouth breathing males are shown in figure 4. Particles >1 µm in diameter are mainly deposited in the extrathoracic region. When breathing though the nose 75% of inhaled particles with a diameter of ~2.5 µm are deposited in extrathoracic region. During mouth breathing the deposition pattern is changed with less particles deposited (~ 40%) in the extrathoracic region and more in the tracheobroncial and alveolar region, as the filtration capabilities of the nose are bypassed [40,41].

The deposition pattern of wood dust particles in the nasal cavity has been simulated in a computer model for pine and oak dust generated by sawing as representatives for soft- and hardwoods [45]. The model showed a large regional deposition of especially pinedust in the anterior nasal segment (figure 3, segment 2 in the extrathoracic region) and in the anterior part of the middle turbinate (figure 3, segment 6). The wood particles mainly deposited in the anterior part of the nose (figure 3, segment 1-6) and with a higher frequency for pine dust than oak dust, explained by larger particle size for pine dust. The model indicated that wood dusts which contain large amounts of fine particles, such as dusts generated by fine sanding, may travel beyond the nasal cavities and into the lungs[28,45].

Figure 4. Deposition of particles during nose breathing (to the left) and mouth breathing (to the right) in the ICRP model from [41].
Clearance of wood particles from the respiratory tract

There are several mechanisms by which the respiratory tract keeps the mucosal surfaces free from foreign materials e.g. wood dust. These mechanisms are either absorptive or non-absorptive and vary between the different regions of the respiratory tract.

In the extra thoracic region, the poorly soluble particles (i.e. wood dust) are transported by mucociliary transport. Particles deposited in the posterior parts of the nasal passage are moved towards the nasopharynx. The average flow rate in healthy adults is about 5 mm/min, resulting in a mean transport time of about 20 minutes. In the anterior part of the nasal passage, particulate matter is directed forward and removed most effectively by sneezing, wiping or blowing. Soluble compounds deposited on the nasal epithelium are translocated rapidly to the bloodstream or are metabolized in the nasal epithelium [40]. In studies of ultra fine particles (diameter less than 100 nm) translocation from the nose into the central nervous system and other parts of the brain have been observed [46,47].

In the thracheobronchial region, the poorly soluble materials are cleared mainly by mucociliary transport towards pharynx and then swallowed. The movement of the mucus varies throughout the thracheobronchial tree; the fastest clearance occurs in trachea and becomes progressively slower in more distal bronchi. The average rate for the trachea has been estimated to be between 4.3-5.7 mm/min for healthy non-smoking adults, whereas in the medium bronchi the rate is between 0.2 and 1.3 mm/min. Coughing is also an important mechanism by which the mucus is moved up the respiratory tract. The clearance time of non-soluble particles is estimated to be 24 hours on average. Soluble particles can be absorbed into the surrounding blood-stream or lymph nodes [40].

In the alveolar region, the ciliary clearance system is not present. Instead the particles have to be phagocytized by macrophages and in healthy adults, this occurs within 24 hours after deposition. The particle-loaded macrophages are cleared from the alveoli by migration to the distal end of the mucus blanket, followed by mucociliary transport. The macrophages can also translocate to the lymphatic system or the bloodstream. By these routes they can circulate to other organs. Soluble particles are dissolved in the epithelial cell lining fluid, and may diffuse into the blood or lymph. When the number of particles is high, the capacity of the macrophages is easily
exceeded, which results in an overload situation. In the overload situation, interstitial accumulation of the particles and inflammation occurs [48].

**Effects of the deposition of wood dust particles in the nose and lung**

Particles deposited in the nose are generally effectively removed by the mucociliary system. If the effectiveness of the mucociliary system is reduced or impaired e.g. by infections, cigarette smoke and traumas [44], the particles can be taken up by specialized epithelial cells and exposed to the underlying nasal-associated lymphoid tissue [49]. Here a non-specific and a specific immune response can occur at the same time. Antigenic stimulation can induce a local immune response primarily involving secretion of the immunoglobulins IgA and IgG. This response should not be considered as a local event, but influences the mucosa of the eyes, ears, and lungs as well. Heavy exposure of foreign materials to the nasal mucosa and chronic inflammation is harmful to the body, to avoid “overresponsiveness” to especially environmental antigens, mechanisms for development of tolerance exists [49]. The mechanisms behind induction of nasal tolerance may vary from antigen to antigen, and with the received dose.

Deposition of a large number of particles in the alveoli leads to a state of overload in the macrophages that removes particles by phagocytosis. The overload results in impaired phagocytosis which leads to interstitial accumulation of particles and inflammation [50]. The inflammation is initiated by the release of inflammatory mediators like cytokines from the overloaded macrophages. In the overloaded alveoli, a second route of clearance is suggested and partially confirmed. It is suggested that the smallest particles can translocate into the bloodstream themselves. The particle size and surface characteristics could be determining factors for the translocation. Particles have been found in lymph-nodes in both animals and humans several months after exposure [51].

**Particle induced inflammation**

In the inflammation process, recruitment of leucocytes requires a communication network between the infiltrating cells and the affected tissue. An important actor in this network is the cytokines. Cytokines are historically termed interleukins (IL), interferons (IFN), tumour necrosis factor (TNF), growth factor (GF) and hemopoietic growth factor (HGF), but are now all considered to be cytokines [52].

Cytokines are small secreted proteins that act on the secreting cells themselves (autocrine action), on nearby cells (paracrine action), or in rare cases on distant cells (endocrine action).
The way of action for the cytokines is through binding to specific membrane receptors, which leads to altered patterns of gene expression. The function of cytokines is often redundant, which means that similar functions are stimulated by more than one cytokine and a cytokine can act on different cell types. The regulation is usually controlled by transcription. The cytokines are therefore produced de novo in response to immune stimulus and their action is generally paracrine, over a short time span and at low concentration [52]. Concentrations as low as $10^{-15}$ and $10^{-10}$ mol/l induce responses by acting via highly specific receptors [53]. The cytokines are important in cellular events like growth, differentiation, wound healing and inflammation by inducing cytolytic, chemotactic, hemopoiesis, and systemic responses [52,54].

Cytokines are divided into subgroups on the basis of their role in the inflammation process (the pro-inflammatory, the immune-regulatory and those with growth and differentiation functions). In the selection of a marker for the inflammatory potential of a substance, pro-inflammatory cytokines are normally chosen. Pro-inflammatory cytokines are important in the initiation of the inflammatory process (e.g. IL-1, IL-6, IL-8, IL-10, IL12, TNF-$\alpha$, and TGF-$\beta$). An overview of the functions of some of the pro-inflammatory cytokines is shown in figure 5.

**Figure 5.** Overview of the cytokines produced by activated macrophages and the local and systemic effects of these cytokines. The arrows point the boxes relevant for the cytokine written next to the arrow. The effects can either be local or systemic (read horizontal for the same cytokine). The cytokines presented are IL-1, IL-6, TNF-$\alpha$, IL-8, and IL-12 from [55].
**Interleukin 6**

IL-6 is a multifunctional cytokine which is produced by both lymphoid and non-lymphoid cells. IL-6 is critical to the regulation of immune and hematopoietic systems. It was previously called interferon β2, hybridoma/plasmyctoma growth factor, B-cell stimulatory factor 2, hepatocyte stimulating factor, hematopoietic colony stimulating factor, and cytotoxic T-cell differentiation factor [52,56,57].

IL-6 is a glycoprotein with a molecular mass ranging from 21 to 28 kDa and consists of 211-212 amino acids. It is composed of four helical bundles and contains three sites for receptor binding, one binding site for the IL-6-Rα receptor and two binding sites for the gp130 receptor [52].

On target cells, IL-6 first binds to the IL-6 receptor (IL-6R) and then the complex associates with the gp130 membrane protein initiating intracellular signalling. The gp130 membrane protein is expressed by most cells, whereas IL-6R is mainly expressed by hepatocytes, neutrophils, monocytes/macrophages, and some lymphocytes. The IL-6R is also found naturally occurring in a soluble form in various body fluids, ready to form an IL-6/IL-6R complex, which can interact with the gp130 membrane protein of cells not expressing the IL-6R themselves [58,59]. Since the gp130 membrane protein also binds other cytokines, the functional redundancy of IL-6 can be explained in part by the sharing of the gp130 receptor [60].

![Figure 6. Functions of IL-6 from [57].](image)
IL-6 is expressed by a variety of cell types e.g. T-cells, mast cells, monocytes, macrophages, fibroblasts, endothelial cells, keratinocytes, and many cell lines and it acts on a wide range of tissues. In figure 6 some of the effects of IL-6 are shown. Some of the actions are: induction of B-cell differentiation, induction of acute phase proteins in liver cells, induction of IL-2 and IL-2 receptor expression, proliferation and differentiation in T-cells [56,57]. IL-6 is involved in diseases like asthma (increased levels of soluble IL-6R was found in the airways of asthma patients), Crohn’s disease (T cells of these patients produce large amounts of IL-6), and other autoimmune diseases [57,58].

**Interleukin 8**

IL-8 belongs to a group of cytokines called chemokines. As the name chemokine indicates, IL-8 is responsible for inducing chemotaxis, which is the directed migration of cells to the site of inflammation [52,61]. Chemokines are classified into four groups on the basis of the position of the first two cysteins adjacent to the aminoterminus of the protein: CXC, CC, C and CX3C [62]. IL-8 is a CXC chemokine and consists of a dimer of two identical subunits (see figure 7). However, the monomer is probably the active form. IL-8 is stable under various conditions like: different pH (pH=2 and pH=9) and under mild oxidising and reducing conditions: Even freezing or heating (to 100 °C) does not alter the activity [52].

IL-8 is important in the regulation of an acute inflammatory response. It is rapidly synthesised at the site of inflammation where it will fulfil its function to recruit and activate acute inflammatory cells, especially neutrophils. The cellular recruitment occurs through the development of a gradient of IL-8, which makes the inflammatory cells move towards the site of inflammation. IL-8 acts by binding to membrane bound specific cell surface receptors coupled to G regulatory proteins [61].

![Figure 7. Structure of the IL-8 protein. From [61].](image-url)
IL-8 is synthesised early in the inflammatory response, and it persist for a prolonged period of time, even days after initiation. Most other cytokines are typically synthesised and cleared in a matter of a few hours. In contrast to the other CXC chemokines, IL-8 is secreted by almost all nucleated cells e.g. endothelial cells, epithelial cells, smooth muscle cells, and even cancer cells [52]. However, the principal secreting cells are the monocytes and macrophages [61]. The above characteristics make the IL-8 a stable indicator of an inflammatory response.

**Inflammation and cancer.**

The idea of a connection between cancer and inflammation is not new. Already in 1863 the German physician Rudolf Virchow notes a connection between inflammation and cancer. He suggested that the “lymph reticular infiltrate” reflected the origin of cancer at the sites of chronic inflammation. It is estimated that about 15% of all global cancers are initiated by inflammation, especially chronic inflammation. Inflammation probably plays a role in later stages of the carcinogenesis in many more tumours [63].

Chronic inflammation can be caused by infectious agents, chemical and physical agents and autoimmune reactions of uncertain aetiology. The link between inflammation and cancer is partly based on epidemiological findings. It is well-known that the bacteria H. pylori induces gastritis and can induce gastric cancer, that ulcerative colitis and Crohn’s disease can cause colon cancer and that chronic viral hepatitis (caused by hepatitis B and C) can cause liver tumours [64-66]. Chemical and mechanical irritation is also associated with cancer. Well-known examples are the conditions silicosis and asbestosis, caused by inhalation and deposition of silica and asbestos fibres. These two conditions are strongly associated with lung cancer and mesotheliomas [67].

Animal experiments have also shown linkage between inflammation and mutations. In a study of carbon black particles, the increase in number of polymorphonuclear granulocytes in bronchiolar alveolar lavage fluid was positively correlated with the number of mutations detected [68].

Neutrophils are an important part of the immune system and may be responsible for the mechanism by which inflammatory processes and carcinogenesis are related. In the inflamed lung, neutrophils produces reactive oxygen species (ROS) like $\text{O}_2^*$, $\text{H}_2\text{O}_2$, $\text{HOCl}$ and $\text{NO}^*$ as part of their defence activities. These products can cause the formation of vide selection of
oxidants which are capable of reacting with the DNA. Neutrophils are simultaneously capable of metabolic activation of chemical carcinogens [69]. Recent literature suggest that inflammation of epithelial cells may cause increased activity of cyclooxygenase-2, and thereby generation of reactive oxygen species, which may be a source of DNA damage [70].

Cancer development
Carcinogenesis is the process by which a normal cell transforms into neoplastic cells. The main theory on carcinogenesis is the multi-step theory which is derived mainly from observations on the effects of chemical agents on laboratory animals. The carcinogenesis has been subdivided into initiation and promotion, where the initiation phase is considered to be driven by genotoxic events and the promotion phase is characterized by changes that affect proliferation.

Genotoxicity of wood particles
Particles are believed to induce genotoxicity in two ways; by primary and secondary genotoxicity. The mechanisms behind the primary genotoxicity are proposed to be generation of oxidants by the particle itself, probably related to the surface properties of the particle. The size and shape of the particles when ingested by cells e.g. asbestos fibres are found to directly interact with the mitotic spindle cell apparatus. Particles can also function as carriers for possible mutagens which are attached to the surface of the particles. The secondary pathway of genotoxicity is linked to excessive and persistent formation of ROS from inflammatory cells during particle-induced inflammation in in vivo models [71]. Wood dust induced genotoxicity could by the primary mechanism be related to the particle or fibre structure itself or to the release of secondary metabolites from the wood dust, which are known to possess adverse biological effects. Secondary genotoxicity is also plausible since an inflammatory response have been observed after in vivo installation of wood dust [72,73]. However, no genotoxicity tests were performed on the animals.

Tests for the assessment of the genotoxic potential of compounds have been developed and international guidelines have been established to provide a more reliable assessment. However, these protocols are designed for the evaluation of soluble compounds and not particles and fibres. The tests are generally applied to investigate gene, chromosome and genome mutations, which are considered to be essential in the carcinogenesis. Examples are the micronucleus test and the chromosome aberration test, which demonstrate induction of structural chromosome
aberrations [74]. However, induction of mutagenic and DNA damage can also be investigated in the sister chromatide exchange (SCE) test, detection of adducts by the use of spectrophotometry, Ames tests, the HGPRT test, the TK⁺/⁻ mouse lymphoma test, the use of transgenic cell lines/animals and the single cell gel electrophoresis assay (comet assay).

The comet assay seems well suited for the detection of primary DNA damage induced by fibers. The comet assay can be used as an indicator assay, since positive results indicate a direct or indirect interaction between test material and the genome [74]. The assay is technically simple, relatively fast and cheap and virtually all mammalian cell types can be used [75]. If the comet assay is used in combination with endonucleases specific for oxidized bases additional information on the contribution from oxidative damages is obtained [74]. In a large evaluation of the comet assay against results from 208 chemicals selected from the IARC and The National Toxicology Programme database, the comet assay was found suitable for prediction of potential carcinogens [76]. The sensitivity of the assay as an indicator for carcinogens was found to be between 79-88%, where as the specificity was 64-76% [76,77]. In one study, the time at which the samples were taken, was found to be of great importance for the comet assay, and optimal detection was found to be after one to four hours after chemical exposure [78].

**Carcinogenesis**

Animal experiments are a major source of information about the mechanisms behind carcinogenesis. In animals it has been demonstrated that carcinogenesis is a multi-step process (See schematic presentation in figure 8). Initiation, the first step in the carcinogenesis, is an irreversible change which we today think is equivalent to mutagenesis. As a consequence of genotoxicity there is a possibility for the development of a tumour. This is the event that induces a change of the genetic code in the cells, which increase the likelihood for neoplastic development. The next step, promotion, involves stimulation of clonal proliferation of the initiated cell. Finally, during the stage of persistence, earlier events enable the transformed cells to grow by autocrine stimulation [79]. At this stage the tumour is able to break through tissue boundaries and metastasize. During the stages of initiation and promotion, genetic alterations occur in caretaker genes (e.g. mismatch DNA repair genes like the MSH2 and MLH genes) and gatekeeper genes (e.g. transcription factors like p53 and transcriptional regulators like the Rb1 gene) leading to an increase of the genetic instability [80]. With defective gatekeeper genes, cells with mutations are allowed to proceed into S-phase and the mutation is passed on to the daughter
cells, possibly leading to the formation of a tumour. Also the proto-oncogenes are important players at these stages, as they are involved in the regulation of cellular proliferation and intracellular signalling e.g. the myc, src and ras genes. Abnormal expression of these genes leads for instance to independence of growth factors, increased production of proteases as well as reduced cell cohesiveness. Inactivation of the recessive inhibitory tumour suppressor genes and constitutive expression of the dominant stimulatory oncogenes are events that are crucial in the transformation into a neoplastic cell, and thereby the progression of cancer [79].

Figure 8. Illustration of chemically induced carcinogenesis by the multi-step theory from [79].

The Ras genes
The RAS proteins identified in the 1970’ies, were some of the first proteins to be identified, that posses the ability to regulate cell growth. The proteins are a family of 21 kDa-polypeptides [81]. The name ras derived from the words “rat sarcoma” because these genes were first identified as the transforming principle of the Harvey and Kirsten strains of rat sarcoma viruses. The ras family consists of three members, Kirsten-ras-2 (K-ras), Harvey-ras-1 (H-ras) and
Neuroblastoma-ras (N-ras) named after the virus strains and the tumour in which they were discovered [82]. Additionally, there are two pseudogenes designated H-ras-2 and K-ras-1. The chromosomal location of the ras genes has been determined in several species. In humans, the K-ras gene is allocated to the short arms of chromosome 12 at position 25,259,624-25,295,121 [83] whereas the K-ras-1 pseudogene is located at chromosome 6 [82]. The H-ras gene is located at chromosome 11 at position 522,243-525, 550 (location 11p15.5) [84], whereas the pseudogene H-ras-2 maps to the X chromosome. The N-ras gene has been assigned to the short arms of chromosome 1 at position 114,961,627-114,971,557 [85]. The transcripts from all tree genes code for about 189 amino acids and are distributed in between 5 exons (K-ras), 6 exons (H-ras) and 7 exons (N-ras) [83-85].

The three ras members are closely related as the first 85 amino acids are identical and the next 80 amino acids are 85% identical. However the sequence between amino acid 165 and 185 differ greatly, suggesting that each species of protein has a unique activity [86]. The structural and functional domains of ras are shown in figure 9. The GDP/GTP binding sites, ras effector domain and antibody binding site are distributed along the first 165 amino acids, which are highly conserved. The carboxy terminus of the poly-peptide varies much more among the ras genes and contains the membrane binding site [82]. This suggests that the difference in activity is related to the cellular localisation of the ras protein. Differences in function were seen in mouse knock out studies. The N-ras and H-ras gene were found not to be required for normal development, whereas K-ras was found to be essential [87].

Figure 9. Graphical illustration of the interaction between ras and GDP. The protein is shown as a green ribbon with purple, orange and yellow cylinders indicating the GDP binding domains with the corresponding cloured parts of GDP. GDP is shown as the purple rectangle representing the guanosine, the yellow pentagon the ribose, and the orange circles the phosphates of GDP. From [88]
Only a single point mutation is required for mammalian \textit{ras} genes to transform into oncogenes. Thus, point mutations have been registered in codon 12, codon 13, and around codon 61 in a variety of tumours. \textit{In vitro} studies have shown that codon 63 and 119 also possess transforming properties. The codon 12 and 13 are located in exon 1, whereas codon 61 is located in exon 2. Substitution of the glycine residue at position 12 with any other amino acid (except proline) leads to constitutive activation of the \textit{ras} protein. A similar effect is observed if the glycine residue is deleted or if another amino acid is inserted after the 11th amino acid. The neighbouring glycine at codon 13 is also sensitive for substitution except with serine. Substitution of the glutamine at codon 61 to any other amino acid, besides proline, glutamic acid and lesser extent glycine, also leads to transformation of the \textit{ras} protein [82].

\textbf{Function of Ras proteins}

The \textit{ras} proteins act as switches in the signalling pathways that modulate different aspects of cellular behaviour, for instance cell proliferation, cell differentiation, and apoptosis. Only one splice variant of the N-\textit{ras} and H-\textit{ras} proteins exists, whereas K-\textit{ras} is expressed as two splice variants, called K-\textit{ras}4A and K-\textit{ras}4B. However, in humans the expression of K-\textit{ras}4A is very low so when mentioned, K-\textit{ras} refers to the splice variant K-\textit{ras}4B [89]. The Ras proteins are synthesised as a cytosolic protein and are then post-translationally modified to anchor the inner face of the plasma membrane. Attached to the inner surface of the plasma membrane, the Ras-proteins in normal cells are controlled by the GDP/GTP ratio. The upstream regulation of Ras is shown in figure 10. The activation cascade is initiated by the binding of a receptor agonist to one of several plasma membrane receptors (e.g. epidermal growth factor receptor). This activates the guanine nucleotide exchange factors (GEFs) through one of several different adaptor proteins. The GEFs, of which there are different kinds, exchange the Ras bound GDP with GTP, causing Ras to be switched into an activated state. To “turn off” the Ras protein the GTP is hydrolysed to GDP by one of the several GTPase activating proteins (GAPs). Thus, the function of Ras is determined by the ratio of activated GEFs versus GAPs. When Ras is mutated in codon 12, 13, or 61, the protein becomes insensitive to GAP activity and remains in a constitutively activated state. The downstream signalling of Ras therefore remains in a stimulated state.
The main downstream pathways are shown in figure 11. The four main pathways stimulated by Ras are controlled by RAF (a protein serine/threonine kinase), PI3K (phosphoinositide 3-kinase), RALGDS (a guanine nucleotide exchange factor for the RAS related protein RAL) and an activation of phospholipase Cε (PLC).

The activation of RAF causes activation of a cascade of kinases involving the mitogen-activated protein kinase. This results in cell-cycle progression because of increased transcription of the transcription factors FOS and JUN. The PI3K induced pathway is a result of RAS interacting directly with the catalytic subunit of PI3K. PI3K is activated by translocation to the membrane and a change in conformation. Effects of activated PI3K are increased transcription of transcription factors e.g. nuclear factor-κB, increased survival of the cells (caused by anti-apoptotic signals), and activation of cytoskeletal signalling. The RALGDS pathway results in inhibition of the transcription of the Forkhead transcription factors. Normally these transcription factors promote pathways leading to cell cycle arrest and apoptosis. Activation of the RALGDS pathway therefore leads to increased vesicle transport and progression of the cell cycle. The PLC pathway mediates release of Ca^{2+} from intracellular compartments leading to activation of protein kinase C (PKC). PKC regulates DNA transcription and cellular metabolism among many things [81] [89] [90].

The pathways mentioned above are the best understood so far. The Ras proteins act in a network and are involved in many of the functions that regulate cell maintaining, growth, and survival.
However, despite many years of research the full function of the Ras proteins have not been fully established yet [91].

**RAS as human oncogenes**

The first link to the identification of the *ras* genes was done by the identification of retrovirals isolates in the 1960’s. However, the human oncogenes were not related to the *ras* genes until about twenty years later in 1982. Already later that year it was discovered that the activated oncogene of H-*ras* was caused by point mutations [92]. Later it was found that point mutations in codon 12, 13, and 61 are associated with cancer. Mutations in these codons result in constitutively active Ras proteins. When screening human tumours for these mutations an overall mutation frequency of 20-30 % was found [93]. There is however, a great difference in mutation frequency between tumour type and tissue location. The highest incidence is found in adenocarcinomas of the pancreas with a mutation rate of 84 % [93,94]. Other frequently mutated tumour types like thyroid carcinomas (~60-50 %), colon adenocarcinomas (~50 %), and lung adenocarcinomas (~30% and nearly all K-*ras* mutations)[93]. Generally, K-*ras* mutations seem
to be more frequent in adenocarcinomas than in other types of histology. For sinonasal cancers several small studies have been performed, the results from these studies are presented in table 5. The overall mutation frequency is between 3 and 50% for K-ras [95]. The mutations are located mainly in codon 12, but the studies are of small scale, different detection methods have been used and there are differences in the type of exposure.

Table 5. Ras mutation studies in codon 12, 13, and 61 performed on sinonasal adenocarcinomas.

<table>
<thead>
<tr>
<th>Histology</th>
<th>Number of patients</th>
<th>K-ras mutations</th>
<th>H-ras mutations</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenocarcinoma</td>
<td>28</td>
<td>4 (14%)</td>
<td>Not done</td>
<td>Saber et al., 1998 [95]</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>31</td>
<td>1 (3%)</td>
<td>5 (16%)</td>
<td>Pérez et al., 1999 [96]</td>
</tr>
<tr>
<td>Intestinal type adenocarcinoma</td>
<td>21</td>
<td>Not done</td>
<td>0 (0%)</td>
<td>Perrone et al., 2003 [97]</td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>15</td>
<td>2 (13%)</td>
<td>Not done</td>
<td>Yom et al., 2005 [98]</td>
</tr>
<tr>
<td>Intestinal type adenocarcinoma</td>
<td>20</td>
<td>9 (50%)</td>
<td>Not done</td>
<td>Frattini et al., 2006 [99]</td>
</tr>
</tbody>
</table>

**Ras mutations as a result of exposure to chemical agents?**

Mutagenic carcinogens can leave fingerprints in form of special mutation patterns, e.g. increased frequency, a special type of mutation, changes in the composition among observed mutations, which may help to identify environmental risk factors [100]. Analysis of mutational spectra from experimental animal tumours often reveals if the DNA changes are caused by endogenous or exogenous carcinogens, providing knowledge about the underlying mechanisms for the carcinogenesis [101]. The mutational patterns can also be recognized in workers exposed to a suspected carcinogen, and thereby provide evidence for a link between a chemical exposure and carcinogenesis. Tumour suppressor genes and oncogenes are involved in carcinogenesis and have therefore been studied for special mutational patterns after exposure to carcinogens. The ras genes are often mutated in animal tumours after exposure to a variety of carcinogens (e.g. methyl(methoxymethyl)nitrosamine, dimethylbenz(a)anthracene, and tetranitromethane [82]. The pattern in base changes are often the same as the ones found in simple mutagen assays e.g. Ames test. This implies the direct interaction with the DNA or an indirect mutagenic interaction [100]. The question is, if we can see the same patterns after environmental exposures. This would be a good evidence for risk assessment of potential carcinogens.
One of the most well established links between a pattern of mutations and environmental exposure is the UV-induced mutations. Most ultraviolet light (UV) induced DNA lesions are produced between the formation of dipyrimidine adducts between two adjacent pyrimidines. This adduct produces a unique UV induced transition, the CC → TT tandem mutation. This mutation pattern is like a fingerprint of UVB induced damage and can be found at high frequencies in human skin cancer [102-104]. This confirms epidemiological and experimental data that demonstrate that UV-light is the major human skin carcinogen, at least for persons with lightly pigmented skin in the Western world. Another example is the high frequency of G→T transversions at the third base of codon 249 in the p53 tumour suppressor gene of hepatocellular carcinomas which are thought to be induced by aflatoxin contaminated food. There are some experimental data that support that such a mutation spectrum can be induced by the most important aflatoxin, namely aflatoxin B1 [94,105].

Mutations in the activating codons 12, 13, and 61 of ras have been studied trying to link specific DNA adducts or chemical exposures to special mutational patterns. In lung cancers from smokers the main type of mutations is G → T transversions in codon 12. The presence of the adduct benzo(a)pyrene diol-epoxide-guanine has been identified in lung samples from smokers, which is in accordance with the type of adduct it gives (G·C to T·A transversion) [106]. In lung adenocarcinomas from mice exposed to the tobacco-specific compound 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanon linkage to G·C → A·T transition were seen in codon 12[105]. However, in humans the G·C → A·T transition in codon 12 was also observed in lung adenocarcinomas from both smokers, passive smokers, and never smokers [107,108]. Although there are not many studies on genetic changes in sinonasal adenocarcinomas, a few reports on a limited number of patients indicated that ras mutations are observed in about 15% of the tumours [95,96]. This suggested that wood dust exposure might be correlated to a ras mutation pattern.

**Epidemiological studies of the biological effects of wood dust exposure- non malignant**

Since the early eighteenth century, wood dust has been known to affect the health of workers [1]. Until recently the main focus has been on the association to malignancies in the sinonasal region, but recently the focus has also turned to the non-malignant symptoms which affect a larger part
of the population, although the personal consequences are less serious. Allergy, asthma, rhinitis and chronic bronchitis are the most frequent non-malignant symptoms.

The term wood worker covers a wide range of job functions and thereby many different exposures which can contribute to the health effects. Employees at sawmills, working with wet or dry wood, may be co-exposed to biohazards like fungi and bacteria, which also causes lung diseases [109]. Furniture workers are often simultaneously exposed to lacquers and glues containing organic solvents and reactive chemicals like formaldehyde, which are known to cause respiratory symptoms [7].

Nasal symptoms among wood dust exposed are found in a substantial numbers of studies. A mean wood dust exposure of around 1 mg/m³ is believed to be a mucous membrane irritant [110] and rhinitis is common after wood dust exposure. The frequency may be increased by 4-53% in wood workers compared to unexposed [5,6]. Wood dust was the second most common cause of occupational rhinitis (mainly allergic) from 1980 to 1987 in a Finnish survey [111]. Sensitization to wood dust, which results in IgE mediated allergy, can be indicated by prick-test. Such testing of allergic individuals indicated that oak, beech, ash, pine, and several exotic woods can be allergenic [6]. Self-reported nasal itching, nasal congestion and sneezing have been reported to be more frequent among the wood dust exposed workers than in unexposed workers [6]. The function of the nasal membranes in exposed versus non-exposed workers has been measured and the clearance of the mucous membranes was impaired after exposure to levels above 1.5 mg/m³ of total dust [112-114].

Not only the nasal region, but also the lungs of wood exposed workers are affected. Symptoms like, impairment of lung function, asthmatic symptoms, chronic bronchitis and coughing have been found in excess among wood workers [7]. Especially the species Western Red Cedar (mainly used in Canada, United States and Japan) has been reported to increase the prevalence of asthma among exposed workers. Between 1 and 13% of employees who work with Western Red Cedar develop occupational asthma [25]. Certain other species, such as oak, iroko, pine, and mahogany can cause asthma as well. However, no strong dose-response relationship has been detected and the data are mainly based on case reports [115]. Asthma has also been related to employment in the furniture industry. In a recent Polish study on 48 furniture workers
occupational asthma was recorded in 6.2% of the workers [5], and in a study of the Danish furniture industry, wood dust exposure was associated with clinical asthma especially among atopic workers with an odds ratio (OR) of 3.32 and a confidence interval (CI) with 95% confidence of 1.81-6.06 [115]. Impairment of the lung function has also been described in wood workers and in several studies a dose-response relationship has been found. Lung function, measured as forced expiratory volume in one second (FEV₁), was negatively correlated with increased concentration of wood dust in the working environment [5,6,116]. Other studies report that chronic bronchitis and coughing were more frequent in employees exposed to wood dust than in the rest of the population [117-119].

In addition to the respiratory tract, also the mucous membranes of the eyes may be affected by wood dust exposure. Most data on eye symptoms are on self-reported irritation of the eye. An increased frequency of eye discomfort (13-46%) was concluded, in reviews of the literature [6,120].

**Epidemiological studies of the biological effects of wood dust exposure- malignant**

Occupationally related cancer is a well known phenomenon. Today, sinonasal cancer is a well recognized occupational related cancer type, and Danish patients are usually acknowledged compensation from The National Board of Industrial Injuries when diagnosed with cancer at this site [121].

**Sinonasal cancer**

Malignant tumours of the nasal cavity and paranasal sinuses (ICD-160, 7th Revision of the International Classification of Diseases) fortunately are rare. Nasal tumours may arise from the surface epithelium, the olfactory neuroepithelium, and the mucosal glands, or have vascular, lymphoreticular, skeletal, or fibrous origin. In the nasal cavity and paranasal sinuses, several different kinds of tumours exist [122] but only the adenocarcinomas and squamous carcinomas have been associated with chemical exposures [8].

Squamous carcinomas, the most common form, typically originate from the lining epithelium of the nasal cavitiy. Squamous cell cancer is characterized by the presence of epithelial cells with pleomorphic nuclei, varying amount of cytoplasm, and mitoses are also often found.
Differentiation in the squamous type is characterized by formation of intercellular bridges and keratin pearls [8,123]. The adenocarcinoma type, which is associated especially with hardwood dust exposures, is composed of columnar epithelium forming papillae and glandular structures. These adenocarcinomas are often called intestinal type because of their resemblance to the adenocarcinomas in the large bowel [8]. Other types of adenocarcinomas arise from glands in the mucous membrane and are composed of cylindrical epithelium that grows in imitations of glands [123]. The symptoms of sinonasal cancer are nasal obstruction, nasal bleeding, unilateral stenosis, or neurological complaints like facial insensitivity and hearing loss [123,124]. Sinonasal cancers are located in the areas shown in fig 12. Because of their proximity to the central nervous system and the orbit, they present a therapeutic challenge for both the surgeons and radiation oncologists. Most of these tumours are removed surgically in combination with either preoperative or postoperative irradiation. In Denmark, the tendency in the treatment strategy has been changing towards surgery, followed by high doses of radiotherapy. However, the choice of treatment is made individually, taking into consideration the extent of the tumour, histology, age and general condition of the patient [124]. The treatment has risks of traumas and blindness [125].

![Figure 12. The sinonasal region from [126].](image)

The sinonasal cancers account for only about 0.2 percent of all newly diagnosed cancers in Denmark (2000), the Danish annual standardized incidence rate were 1.05 per 100 000 person
years for men and 0.45 for women in year 2000 [45] and 0.46 per 100,000 person years for men and 0.62 for women in year 2001[127]. In figure 13 the number of newly diagnosed cases in Denmark over the last decade is shown. The incidence rate seems to be slightly increasing for men, while stable for women. In Europe the standardised incidence rate is between 0.3 to 1.4 per 100,000 for men and 0.1 to 0.8 per 100,000 for women, depending on country. The incidence varies markedly from one country to the next, even from one region to another [3]. Cancer at this site arises relatively late in life, with an average age between 57 to 65 years for the first diagnosis [45,124,125]. The tumours arise in air-filled cavities, which usually become infiltrated with tumour tissue before the patient gets symptoms. Most patients not diagnosed until the tumour is at advanced stages, and that affects the survival rate. The survival rates for nasal cancer depend on the stages of the tumours at the time of diagnosis, localisation, and histology type. For all types of histology the overall 5-year survival rate is 41 percent [124], whereas for the adenocarcinomas the 5-year survival rate is about 30 percent [124,128], and only a few percent greater for squamous carcinomas [124].

**Figure 13.** The numbers of newly diagnosed sinonasal cancers (ICD code 160) in Denmark. Data from 2001-2003 (in hatching) are preliminary estimates. Data from [45] and [129].

**Sinonasal cancer and wood dust exposure**

One of the first observations of sinonasal cancer being linked to occupational exposure, was made in 1938, when it was noted that an abnormal number of workers from the factory Mond Nickel Works in South Wales, UK had died from squamous cell cancer in the nose, sinuses and bronchi (reviewed in [2]). In 1968 the association between the adenocarcinoma histology type of sinonasal cancer and employment in the furniture industry in Buckinghamshire, UK was noted by Acheson and colleagues [130]. In 1969 this cancer form became a “prescribed disease” called
the furniture workers cancer in the UK, giving rights of afflicted workers to Industrial Injuries Disablement Benefits [2]. Today, nasal cancer in wood dust exposed personnel still entitles to disablement benefits in UK [131] and Denmark [132] as well as in many other western countries. Since these first observations, many independent epidemiological studies and surveys have been conducted in Europe, America, and in Asia. Associations between sinonasal cancer and wood dust, leather dust, formaldehyde, chromium, and nickel are some of the results. It has been estimated that 60 % of nasal cancers in males could be attributed to occupational exposures [133]. Many of the studies conducted on sinonasal cancer are small case-control studies which lack the power to examine the relative risk of adenocarcinoma and squamous cell carcinomas in association with specific jobs or exposure levels. This is due to the rare occurrence of sinonasal cancer.

In 1995 a pooled analysis of 12 case-control studies in relation to wood dust was performed by Demers et al [4]. They found a high risk of adenocarcinomas among males employed in wood-related occupations, with an odds ratio of 13.5 [95% CI= 9.0-20.0]. Among men with the highest exposure (< 5 mg/m³ over an 8-hr time-weighted-average in the 1970’s) the odds ratio was 45.5 [95% CI= 28.3-72.9] for adenocarcinomas and lower when all the histologies were considered (OR= 5.8 [95% CI = 4.2-8.0]). No association was found to squamous cell carcinomas (OR= 0.8 [95% CI =0.4-1.6]).

The above analysis was performed in connection with an evaluation of wood dust by the International Agency for Cancer Research (IARC) in 1995 [7]. The Agency publishes monographs with the objective to collect existing evidence and evaluate it with special reference to carcinogenicity. This is done by the help from an international working group of experts. In the wood dust monograph, the epidemiological studies were summarized in three tables (the three studies are listed in appendix A). The main conclusions were that most of the available cohort and case-control studies showed increased risks of sinonasal cancer. The greatest risk associations between wood dust and cancer were found for the adenocarcinoma subtype, and mainly in the European studies. The adenocarcinoma subtype was found to be clearly associated with exposure to hardwood dust; the finding was confirmed in several case-control studies. The risk for sinonasal cancer after exposure to softwood was considerably lower. However, in these epidemiology studies simultaneous exposure to both wood types is very common, as employees
often work with several wood species through-out their working life. The relative risk of squamous cell carcinomas for wood exposed persons was smaller than for adenocarcinomas[7]. In the pooled study by Demers et al [4], no association between the risk of squamous cell carcinomas and exposure to wood dust was observed. However, the risk for squamous cell carcinomas was found to be associated with exposure to softwood in a Norwegian study from 1983 with 70 cases [134].

Since the publication of the monograph, a few studies have been published on the association of sinonasal cancer and wood dust exposure. In table 6, studies which could be identified by searching the PubMed database with the keywords; wood dust, sinonasal cancer, cancer of the nasal cavities, nasal cancer in combinations is listed. A few case-control studies have been published in the Italian and Spanish language: These have not been included in this overview. The listed studies are mainly cohort studies based on self reported job titles stated in censuses in the 1970 and data from the national cancer registries of the respective countries. These cohort studies show an increased risk among men exposed to wood dust, for sinonasal cancer with an odds ratio of approximately 1.9 for all histological types. For women, no statistically significant increase in the risk for sinonasal cancer after wood dust exposure could be established. A small non-statistically significant increase in the risk for sinonasal cancer was found in all studies including women (OR=1.17 [95% CI =0.31-4.47] [135], SIR=1.88[95% CI =0.05-10.48][136] and SIR=1.74[95% CI =0.04-9.69][137]).

**Sinonasal cancer and other occupational exposures than wood dust.**

Cancer in the sinonasal region has also been linked to other occupational exposures mainly dust related occupations and metal manufacturing/processing. However, the results from the available studies are not consistent. Increased rates of sinonasal cancers have been observed among several occupational groups besides the wood dust-related. One of the first observations for nasal cancers in relation to occupational exposures was related to employment at a nickel refinery [2]. A very early cohort study back in 1958 showed an excess of 196:1 in nickel workers compared to the normal population [138]. In a more recent Finnish cohort study with workers from a nickel refinery, a SIR of 8.79 [95% CI=1.06-31.7] was found between nickel exposure and nasal cancer (ICD7 code 160). Taking a latency time of 20 years into consideration and narrowing the cohort to only nickel refinery workers a SIR ratio of 67.1 [95% CI=8.12-242] was reported [139]. Nickel has been evaluated by IARC and was placed in group 1 (carcinogenic to humans) and
Table 6. Lists studies after the publication of the IARC monograph on wood dust [7], there could be identified searching the Pubmed database. [135-137,140,141]

<table>
<thead>
<tr>
<th>Reference</th>
<th>Country</th>
<th>Study type</th>
<th>Source of information on exposure</th>
<th>Conditions for which the relative applies</th>
<th>Sex (persons in the study)</th>
<th>OR/RR [95% CI or p]</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Innos et al, 2000 [137]</td>
<td>Estonia</td>
<td>Cohort study of employees at two furniture factories between 1968 and 1988 with 6 months of employment.</td>
<td>Employment records of the companies and questionnaires sent to the person.</td>
<td>Cancer in nose and sinuses according to the ICD 9 code 160</td>
<td>M (3,723) F (3,063)</td>
<td>SIR = 1.86 [0.23-6.73] SIR = 1.88 [0.05-10.48]</td>
<td>Males employed Male employees</td>
</tr>
<tr>
<td>Hemelt et al, 2004 [138]</td>
<td>Sweden</td>
<td>Cohort study</td>
<td>Persons from the Swedish cancer family database who were economically active in the 1960 or 1970 census and born after 1931. SIR adjusted for socioeconomic status and age. ICD code 160 was used to select nasal cancers.</td>
<td>M (87,028)</td>
<td>SIR = 1.91 [1.53-2.36]</td>
<td>All male woodworkers</td>
<td></td>
</tr>
</tbody>
</table>

**Exposure level:**
- None (0)
- Low (<3 mg/m³·y)
- Medium (3-50 m³·y)
- High (>50 m³·y)
- None (0)
- Low (<3 mg/m³·y)
- Medium (3-50 m³·y)

* consisted of one French, one German, one Swedish, one Dutch, and four Italian studies.
metallic nickel in group 2A (probably carcinogenic to humans). In the IARC evaluation an increased risk for nasal cancers was found associated to the exposures during refining of the nickel [142].

Employees in the basic metal industry have increased risks for nasal cancer [143]. Wood dust and chromate exposure are often found together. This is due to the wide use of the wood preservative chromate copper arsenate (CCA), which can be applied by non-pressure or pressure treatment [9]. Chromium exposure is often related to welding in stainless steel, which contains up to 30 % chromium [144]. Lung cancer is the most frequent cancer form related with exposure to chromium [145,146]. However, nasal cancer have been found among primary chromate production workers, chromate pigment production workers, stainless steel welders, and chromium platters [142,144,146]. In a recent study of former chromium smelter workers, greater than a five fold risk of nasal cancer was found [146]. Chromium [VI] was in 1990 classified into group 1 by IARC (carcinogenic to humans) [142].

Formaldehyde is another very common co-exposure to wood dust, due to its use in glues used in furniture making and particle fibre board production. In a large pooled study of European studies, exposure to formaldehyde was associated with increased risk of adenocarcinomas for both men and women in the high exposure groups (>1 ppm) [147]. The relative risks were 3.0 [CI 95% = 1.5-5.7] for men and 6.2 [CI 95% = 2.0- 19.7] for women. Because formaldehyde exposure is related to wood dust exposure the OR’s is adjusted for age, study, cumulative exposure to wood dust and leather dust. Some epidemiological studies indicate a risk for squamous cell carcinomas, but no risk for adenocarcinomas was reported. The literature regarding formaldehyde is inconsistent, perhaps because formaldehyde exposure is frequent in Western societies, and perhaps because there is combined exposure to wood dust and formaldehyde-releasing products. Overall, data indicate that an increased risk for nasal cancer can be associated with formaldehyde. Formaldehyde has also been evaluated by IARC in 1995[7] and was classified in group 2A (probably carcinogenic to humans), mainly based on animal studies.

It has also been documented that employees in the manufacturing of leather products and textile workers have increased frequencies of nasal cancer, all though there are inconsistencies in the evidence. Leather dust was initially suspected to be a nasal carcinogen after observation of a
cluster of sinonasal cancer among shoe makers in Northamptonshire in the UK [148]. Smaller case-control studies from US, Japan, the Netherlands, and France could not confirm the association between occupational exposure to leather dust and sinonasal cancer. In one of the biggest and most recent studies made in Italy [133,149], nasal cancer could be associated with employment in the leather industry with an odds ratio of 6.8 [CI 90% = 1.9-25]. In an analysis of pooled European case-control study an odds ratio of 1.92 [CI 90% = 1.10-3.35] was found for men occupational exposed to leather dust. For the adenocarcinoma histology type the odd ratio was 2.99 [CI 90% = 1.33-6.73] [135]. The difference in occurrence of nasal cancer between countries (studies) is suggested to be caused by different procedures in tanning [150]. An IARC evaluation concluded “that there was sufficient evidence for carcinogenicity associated with employment in the boot and shoe manufacturing and repair industries” [151].

Textile dust is also associated with nasal cancer in several studies, among both men and women. Odds ratios between 0.82 -17[133,152-154]and standardised incidence ratios between 115-559 [155,156]were found. However, none of these ratios were statistically significant probably due to the few cases in the studies, in a pooled analysis of 12 European case-control studies (see section below for description of the study) [147]. The female workers exposed to textile dust levels between 0.05 mg/m³ and 0.5 mg/m³ had a significant increase in the risk for adenocarcinomas (OR= 3.5 [CI 95% = 1.2-10.7]). For males, a non-statistically significant elevated risk for both adenocarcinomas and squamous cell carcinomas was found. An odds ratio of 1.6 [CI 95% = 0.6-4.2] was found for adenocarcinoma patients with a wood dust exposure <0.05 mg/m³. For squamous cell carcinomas an odds ratio of 1.3[CI 95% = 0.6-2.9] was found among patients with an exposure threshold > 0.5 mg/m³ [147]. Employment in textile manufacturing industry was found probably carcinogenic to humans (group 2A) by IARC in 1990 [157], mainly based on nasal cancer among weavers (possibly from exposures to dust from fibres and yarn) and other textile workers.

The pooled study for sinonasal cancer and occupational exposures mentioned above was published in 2002 [147]. In this publication, data from 12 case-control studies were pooled and occupational exposures was assessed with a job-exposure matrix. The pooled data set contained 195 adenocarcinoma cases (169 men and 26 women), 432 squamous cell carcinoma cases (330 men and 102 women), and 3136 controls (2349 men and 787 women). The study investigated the
risk of sinonasal cancer after exposure to the following chemical and dusts; formaldehyde, flour, coal, silica, textile, asbestos, four man-made vitreous fibres, mineral wool, continuous filaments (for insulating material like fibreglass), refractory ceramic fibres (helps to control heat flow in high-temperature, industrial situations), and micro fibres (not closely specified). There was an increased risk for adenocarcinomas after exposure to formaldehyde (see section about formaldehyde) and textile dust (see section about textile dust). In men, increased risk for adenocarcinomas was found after low concentrations of ceramic fibres (OR=13.6 [CI 95% = 3.5-53.7]), whereas in women, high exposure to silica (OR=17.0 [CI 95% = 2.6-112]) and continuous filaments (OR=6.6 [CI 95% = 1.6-27.2]) increased the risk for adenocarcinomas. Overall, for the investigated chemicals and dusts the odds ratios were above one but were not statistically significant. Looking at occupations besides the already mentioned, it has been suggested that farm workers [143,158], fishermen [141], electrical workers [141], and “bakers/pastry cooks/grain millers” [158] also have increased risk of nasal cancer.

**Wood dust and cancer at other localisations than the nose and paranasal sinuses**

Wood dust has also been suggested to be related to other cancer forms, although the associations are rather inconclusive. For instance a relation between lung cancer and exposure to wood dust could not be found [7]. In a very recent American case-control study by Barcenas et al. [159] on 1368 cancer patients and 1192 cancer free controls, exposure was assessed from self reported occupations and an evaluation was carried out by occupational hygienists. Employment in a wood dust related occupation (the most common was carpenters and related occupations by 68%) showed an increased OR ratio of 3.60 [CI95% = 1.51-8.56] even when adjusted for smoking etc. However, in a study of Estonian furniture workers the SIR ratio was 1.07 [CI 95% = 0.87-1.28] for cancer in the bronchi and lungs (ICD 9 code 162) [136]. No elevated SIR could be established in a Nordic survey between occupation and cancer. Here the standardized incidence ratio was 0.93 [CI95% = 0.90-0.95] for lung cancer (ICD 7th, code 162) [140]. Several single studies have found associations between wood dust and cancer at other sites. For instance an increased risk for gastric cardia adenocarcinomas was found among Swedish construction workers [160]. However, it is possible that these observations may be chance findings, which not will be confirmed in large cohort studies. The overall picture might also be influenced by publication bias.
HYPOTHESES AND AIMS

The background behind this thesis was that airway symptoms are found in workers exposed to wood dust. Both chronic inflammatory conditions and cancer in the nasal cavities have been associated in epidemiological case-control and cohort studies to wood dust exposure. The mechanistic background for these observations is very poorly investigated, so the aim of this study is to provide mechanistic knowledge to the effects seen after wood dust exposure. This can hopefully be used for the evaluation of wood dust in both relation to airway symptoms and cancer.

The following hypotheses were put forward.
Different species of wood dusts have different ability to induce DNA damage and inflammation.

Chronic inflammatory conditions in the sinonasal tract are causative for wood dust related cancer.

Specific mutagenic agents in wood dust are reflected in the spectrum of mutations. The mutation spectrum therefore differs between the tumours from wood dust exposed patients and non-exposed patients.

In order to address these hypotheses; an in vitro cell model system, in which human lung epithelial cells were exposed to dust from different species of wood dust, was used. The exposed cells were investigated for cytotoxicity, cytokine expression (marker for inflammation), and DNA strand breaks (marker for genotoxicity). In order to investigate if the tumours from patients with sinonasal cancer showed a specific pattern of mutations after wood dust exposure, patients were identified, place of treatment located, and the tumours collected. To ensure uniformity in the diagnosis all diagnosis were re-evaluated and the patient’s occupational history obtained from interview or registers. Finally, the ras genes were analysed for mutations in the hotspots codons 12, 13, and 61.
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<th>OVERVIEW OF STUDIES AND DESIGN STUDY:</th>
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<td>Investigation of the inflammatory potential of the most commonly used wood species in the wood industry</td>
<td><em>In vitro</em> exposure of the lung epithelial cell model A549 for time and dose effects of 7 wood dust species. Establishment of time and dose-response curves for interleukin 6 and interleukin 8 induction.</td>
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<tr>
<td>Investigation of the genotoxic potential of the most commonly used wood species in the wood industry</td>
<td>Establishment of time and dose-response curves for DNA damage measured as DNA strand breaks.</td>
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<td>Investigation of cancer patients for carcinogenic fingerprints of wood dust.</td>
<td><em>K-ras</em> and <em>H-ras</em> mutations analyses by restriction enzyme cleavage and sequencing of nasal tumours (ICD7 code 160) from Danish and Finnish cancer patients.</td>
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<td>Investigation of the association between occupational exposure to wood dust and sinonasal cancer in Denmark.</td>
<td>Matched case-control study with incidence density sampling, including all incident sinonasal cancers 1991 to 2002 obtained from the Danish Cancer Registry. Exposure was assessed by register data and/or interviews.</td>
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METHODS AND MATERIALS

Ethical considerations
This thesis is based on in vitro exposures of a cell line and molecular analysis of tumour material. Before the onset of the in vitro study, the possibility of doing animal in vivo exposures was considered. However, as knowledge on the underlying mechanisms behind the carcinogenic effects of wood dusts as well as what precisely are responsible for the non-malignant symptoms are limited, in vivo studies were abstained from. Furthermore, ethical guidelines states, that in vivo testing should be avoided if an equivocal result can be obtained by a different type of test, as is the case of the present study.

For the molecular epidemiology part of the project, we used paraffin embedded tissue blocks, stored at pathology departments in Denmark, Finland, and France. In Denmark, molecular analysis on tumour material (PET blocks) from patients, stored at the hospitals, demands no personal consent, as long as all personal data are being kept confidential within the research group. In other countries e.g. Finland, a personal consent is needed. The exposure assessment of the patients necessitated that we contacted the patients for information on occupational, exposure and history of smoking. For this an approval of the project was obtained from the ethical committee in Copenhagen (journal number KF 01-048/02). All personal data are kept confidential within the research group and stored according to the Danish Data Inspectorate.

The In Vitro Exposure

Choice of model
In vitro models with cell populations are widely used in toxicology. The in vitro models are attractive because of their simplicity, speed, and easiness of performance. There are two main principles of in vitro models; the use of primary cultures and secondary cultures (cell lines). Primary cell lines recovered from human or animal tissue and are usually not maintained for more than a few cell divisions. They are therefore inhomogeneous and under the influence of previous exposures to potential xenobiotics and the cells may respond individually. On the contrary, all cells in a cell line are derived from the same stem cell and therefore have the potential of a more uniform response. However, the specific characteristics of the cell type in
the tissue are often lost. *In vivo* models have the benefit of the interactions that occur in the whole body, such as uptake, distribution, and detoxification of the xenobiotics and interactions between different cell types. *In vivo* models are usually more costly and there are ethical considerations and legal restrictions. Therefore *in vitro* models are a valuable option. Since the knowledge of wood dust’s effects on DNA damage and inflammation is very limited, an *in vitro* model of the pulmonary epithelium was chosen.

The lung epithelial cell line A549 was used in this study. The cell line was chosen despite the fact that it derives from the lung, and wood dust have been associated mainly with nasal symptoms. However, in my search for nasal epithelium cell lines a very limited number of cell lines came up. One potential cell line the RPMI 2650 (CCL- 30) could have been an option. However, the cell line was until recently not well characterized for cytokine release [161].

The A549 cell line is a commonly used model of the pulmonary epithelium, especially in the toxicological literature [162]. The cell line was derived from lung adeno-carcinomatous tissue in a 58-year-old Caucasian male in 1972 [163,164]. The cell line is considered to be a type II epithelial-like cell line, and it exhibits characteristics like lamellar bodies, capability of absorptive/fluid-phase endocytosis and the cytochrome P450 system found in type II cells [162]. The A549 cell line also has the capacity for metabolic activation of volatile organic compounds and polycyclic aromatic hydrocarbons [165]. In our laboratories have A549 been used with success for the studying of IL-6 and IL-8 expression and DNA damage in relation to dust and diesel exposure [31,166,167] and it has also shown to respond to other xenobiotics e.g. cigarette smoke extracts [168].

**The doses**

Doses were chosen to cover a wide range of concentrations because of the sparse knowledge about genotoxic effects of wood dust. The doses in the study were based on our Finnish collaborators experiences with mouse macrophage RAW 264.7 cells during pilot studies. The doses were modified after the initial experiment to avoid cytotoxicity and resulted in the use of the following doses; 0, 10, 30, 100 and 300 µg/ml wood dusts. The dose 1000 µg/ml was tested but showed cytotoxic effects for some species. In addition to the cytotoxicity relevant
for the measurement of comets, the cells were at 1000 µg/ml visually fully covered by wood particles which could lead to anoxia of the cells. The 0 µg/ml was treated identically as the wood dust treated cells, but without dust, and functioned as negative control.

**Choice of time perspectives**

In a pilot study A549 cells were incubated with the different species of wood dust in all the selected concentration from 0 µg/ml to 1000 µg/ml for 3, 6, 24 and 48 hours. Cytotoxicity was measured as lactate dehydrogenase secretion to the media. As some wood dusts turned out to be cytotoxic at 24 hours (see manuscript I) incubation for 3 and 6 hours were chosen for the future exposures. For the detection of primary DNA damage by comet assay it is recommended that at least one short (3 or 6 hours) and one long (24 hours) exposure period should be tested [74]. Due to the cytotoxicity at 24 hours we did make a compromise and included both 3 and 6 hours of exposure instead of 24 hours. The time frame in relation to inflammation was based on experiments with diesel particles which showed elevated expression of IL-6 after 2, 5 and 24 hours [31].

**Cell culture**

The cell line was obtained from the American Type Culture Collection (Rockville, MD, US) and cultivated in Ham’s F12 with added 10% heat-inactivated foetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, all from Gibco BRL, Life Technologies, Denmark. The cells were cultured in a humidified atmosphere at 37°C and 5% CO₂.

**Wood dust exposure**

Two ml medium with 2*10⁵ cells was seeded in each well of 12-well plates (Nunclon™ Surface, Nunc, Kamstrup, Denmark). Fresh medium was added after 24 hours. After 48 hours wells were examined visually in a microscope and the cell density was estimated. Only dishes with a cell density between 75-90% confluences were used. The wood dusts were thoroughly suspended by vortexing in freshly prepared culture medium. The wood dust suspensions were added at the concentrations 0, 10, 30, 100, and 300 µg/ml. The cells were incubated for 3, 6, 24, and 48 hours. After incubation, the medium was collected and stored at -80 °C. The cells were washed twice with PBS and then lysed directly in the well with RLT lysisbuffer (Qiagen, Crawley, UK) for RNA purification. Alternatively, cells were treated with trypsin-EDTA and collected. The cell suspensions were kept on ice and the total cell number and viability were determined by hemocytometer counting and trypan blue dye exclusion. The remaining cells were centrifuged at 300 x g (4 °C) for 10 min and resuspended in culture medium with 10% DMSO followed by freezing at -1°C /min freezing rate in a 5100 Cryo freezing container (Nalge Europe Ltd.,Neereijse, Belgium) until storage at -80 °C.
Assessment of cytotoxicity by lactatedehydrogenase (LDH) activity

The LDH assay is based on LDH a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell-culture supernatant upon damage of the plasma membrane. The activity of lactate dehydrogenase (LDH) is determined using the cytotoxicity detection kit from Roche, Hvidovre, Denmark (cat no 11 644 793 001). The principle of the assay is the conversion of lactate into pyruvate by LDH, under the formation of NADH. The formed NADH is able to convert tetrazonium salt (pale yellow) into formazan salt (red) figure 14. The red colour intensity is read spectrophotometrically and compared to a maximum lactate dehydrogenase release (i.e. total lysis of the cells). The kit was used according to the manufacturer.

![Diagram of LDH assay](image)

Figure 14. Shows the principle in the cytotoxicity dectection kit. Illustration from[169]

All samples were run in duplicates and a standard deviation of 15% was allowed between the duplicates. For negative controls wells with pure culture medium, culture medium with wood dust and pure reaction mixture were included on each plate. For positive controls a sample with Triton-X lysed cells was used to normalize all the samples to. The positive control was included in each run and each wood dust was tested for interference with the measurements.

Assessment of inflammation

The cellular inflammatory response can be quantified at several levels. Quantification of genes transcription level is one important tool, but the activity can also be regulated post transcriptional, in which case quantification of the inflammatory protein levels is important. There exists a variety of techniques to quantify the expression of a gene. To summarize, some of the most commonly used techniques, Northern blotting, RNAse protection assays, in situ
hybridization and reverse transcription (RT) PCR can be mentioned. During the last decade new techniques like real time PCR and micro arrays have gained ground. For quantification of proteins, techniques like Western blotting, HPLC, and immunoassays like the ELISA are most commonly used.

Real-time RT-PCR has become a routine technique for quantification of gene expression, because of the large dynamic range, high sensitivity, and high specificity of the technique [170]. In this study the ABI PRISM® fluorescence sequence detection system (Applied Biosystems, Foster City, CA, US) was used, but other fabricates of detectors exists.

The principle in Taqman real-time PCR
The principal in real-time PCR is to detect PCR products as they accumulate after each PCR cycle. In RT-PCR, RNA is converted to cDNA by the use of reverse transcriptase. The cDNA is used as template for the PCR reactions. The PCR was performed with the ABI PRISM® fluorescence sequence detection system (Applied Biosystems, Foster City, CA, US), in which PCR products are detected as they are formed. Taqman real-time PCR is based on the use of an oligonucleotide probe containing one fluorescent moiety and a fluorescence quencher. When the probe is bound to the template during the PCR reaction the exonuclease activity of the AmpliTaq Gold DNA Polymerase hydrolyses the probe and the quencher is separated from the fluorochrome and a fluorescent signal can be detected (see figure 15 for an illustration of the principle).

Figure 15. The Taqman® probe which is gene specific contains a reporter dye in the 5' end and quencher in the 3' end. As long as the quencher is close to the reporter dye, little fluorescence is emitted. During the PCR cycle, the 5'-3' exonuclease activity of the AmpliTaq Gold DNA polymerase cleaves the probe and releases the reporter dye from the quencher. The emitted light is then monitored. As PCR cycles are performed more and more fluorescence is accumulated. From Applied Biosystems [171]
Two parameters are used to standardize the assay. In figure 16 an example on the fluorescence emitted is shown, with the most important terms added. The fluorescence signal is increased by two-fold with each PCR cycle. Despite of this, an increase in fluorescence will not be detected during the first rounds of PCR because low background fluorescence is emitted from the probe even in the quenched state and the increase needs to exceed this threshold. This background emission period is termed the baseline. As the PCR cycles progresses, fluorescence is accumulated exponentially. During these cycles the threshold cycle (Ct) is chosen.

The Ct value is proportional to the number of target copies present in the sample. To normalize the amount of cDNA added to the reaction, an endogenous control is used (with a second set of primers and probes). The ideal endogenous control is unaffected by the experimental treatment and are often a constitutively expressed gene, a so called household gene. The most commonly expressed and used genes are 18S rRNA > β-actin > GADPH > phosphoglycerokinase [172,173]. 18S rRNA is often found to be the best option for an endogenous control in RT real-time PCR [174]. However, the amount of the internal control should not be too high, as errors in the estimates occur when the background level can not be assessed probably. In the pilot experiments I determined that the amount of IL-8 mRNA was too low for using 18S rRNA as the endogenous control. I therefore chose β-actin as the endogenous control.

Figure 16. A schematic presentation of the key terms used in the analyzing of RT-PCR. The three graphs designated ‘sample in triple’ refer to a sample with template cDNA added run in triplet, measured for its IL-8 content. The purple graph designated ‘No template' refer to a sample with no template cDNA added.
Mesurement of IL-6 and IL-8 mRNA

Total RNA was isolated from frozen lysates of A549 cells with the QIAamp® RNA blood mini kit (QIAGEN, Maryland, US, cat no 52304), as recommended by manufacturer. To check purity and concentration the RNA were examined spectrophotometrically by measuring the $A_{260}/A_{280}$ ratio. To ensure that contamination with genomic DNA did not contribute as templates at the PCR reactions, the RNA was treated with DNase (QIAGEN, Maryland,US, cat no 79254) as described by manufacturer. The cDNA synthesis was performed using Taqman® reverse transcription reagents (Applied Biosystems, Foster City, CA, US) as recommended by manufacturer. RNA quality was not assessed directly, but a small scale cDNA synthesis was performed with 200 ng RNA in 10 µl. Then the β-actin mRNA level was determined (details see later section). If the expected yield was obtained, cDNA synthesis was carried out for all samples. If the expected yield was too low, a new DNAse treatment or replacing the reverse transcription reagents with new ones always helped.

Quantitative PCR was performed on ABI PRISM 7700 and 7300 sequence detectors (Applied Biosystems, Foster City, CA, US), using Universal Mastermix (Applied Biosystems, Foster City, CA, US). The probes and primers used were commercially available in form of pre-developed kit from Applied Biosystems; IL-6 (part.no. Hs99999032_m1), IL-8 (part.no. 4327042F) and β-aktin (part.no.4310881E). These kits are already optimized with respect to primer and probe concentrations. Pre-developed kits can potentially be run in the same tube, however in the case of IL-6, IL-8 and β-actin this was not possible due to interference in the fluorescence spectra (recognized by Applied Biosystems support division), the IL-6, IL-8 and β-actin were therefore quantified in separate tubes.

Quality control

For both the IL-6 and IL-8 assay, the independence of the amount of cDNA in the reaction was validated by making a standard curve. The target genes were normalized to β-actin by subtracting the threshold value of β-actin ($C_t$ endogenous gene) from the threshold value of the target gene ($C_t$ target gene) of interest, i.e. $C_t$ endogenous gene - $C_t$ target gene = $ΔC_t$. The relative expression was calculated by the comparative method $2^{-ΔCt}$ [175]. Using this method, the amplification efficiency has to be very close to 100% for both the target gene and the endogenous control gene. The assay was validated by making a 2-fold dilution of cDNA over a wide range. A plot like the one in figure 17 is made from the data and this makes up the standard curve. The slopes in the plot for the fam and vic values has to be close to -3.32 in order to be quantitative because of the exponential formation of PCR products. The slope of
ΔCₜ has to be very close to zero. As seen in figure 17, a print of the IL-8 vs. β-actin standard curve, the assay was stable over a dilution range of approximately 32.

![Graph showing the dilutions of cDNA.](image)

Figure 17. Plot showing the 2-fold dilutions of cDNA. The fam curve represents the values from IL-6 or IL-8. The vic curve represents the values from β-actin. Delta is the difference between the fam and vic values.

The samples were run in triplicates with an average standard variation of 18% for the IL-6 mRNA assay and 11% for the IL-8 mRNA assay. The standard derivation of repeated measurements of the same sample (positive control) in separate runs was 23%, indicating the day-to-day variation of the assay. Negative controls, one where RNA is not converted into cDNA and one with no template were included in all runs.

**The ELISA**

ELISA is the acronym for enzyme-linked immunosorbent assay. Several types of ELISAs exist; the general (indirect) ELISA, the sandwich ELISA and the competitive ELISA. The most commonly used form is the general ELISA, where antigens attached to the surface of a well captures the antibody examined and is finally detected by a second antibody coupled with a substrate-modifying enzyme. The higher the concentration of original antigen, the higher a signal can be detected.

**The principle of the sandwich ELISA**

The cytokine sandwich ELISA method is in principal performed by letting highly-purified cytokine antibodies (capture antibodies) being non-covalently adsorbed (coated) onto plastic microwell plates. After removing excess antibody by washing, the immobilized antibodies serve to specifically capture soluble cytokine proteins present in samples, which are applied to
the plate. After washing away unbound sample, the captured cytokine proteins are detected by biotin-conjugated anti-cytokine antibodies (detection antibodies) followed by an enzyme-labelled avidin binding. Following the addition of a chromogenic substrate, the absorbance of coloured product generated by the bound, enzyme-linked detection reagents is measured spectrophotometrically on an ELISA-plate reader at an appropriate optical density (OD) [176]. A schematic presentation of the IL-8 ELISA is shown in figure 18.

![Figure 18. Schematic presentation of the reaction steps in the OptEIA™ Human IL-8 Set from BD Biosciences, San Jose, CA, US.](image)

**Measurement of IL-8 by ELISA**

The ELISA was performed using the OptEIA™ Human IL-8 Set from BD Biosciences, San Jose, CA, US (cat no 555244) according to instructions of the manufacturer. In short, a 96-well micro plate was coated with 100 µl diluted capture antibody supplied in the kit over night at 4 °C. The next day the plate was washed and aspirated 3 times (PBS with 0.05% Tween-20). The plate was blocked with 200 µl assay dilution (PBS with 10% foetal bovine calf serum) for minimum one hour at room temperature. The plate was washed and aspirated once, before adding 100 µl sample, control, or standard (supplied in the kit) to each well. The plate was incubated for 2 hours before being washed and aspirated 5 times. 100 µl mixtures of diluted secondary antibody and avidin-HRP-enzyme, supplied in the kit, were added. The plate was incubated for one hour, washed, and aspirated 5 times. 100 µl substrate solutions (tetramethylbenzidine) was added and allowed to incubate for 15 minutes covered with aluminium foil before adding 50 µl stop solution (2N H₂SO₄). The substrates were mixed by tapping carefully on to the side of the plate. The absorbance was read spectra-photometrical...
after 10 to 30 minutes, on ELISA reading equipment (Elx808 from Bio-Tek Instruments, Winooski State, US).

**Quality control**
Controls obtained by the National Institute for Biological Standards and Control, United Kingdom (cat no. 89/520) were diluted into standards of 25, 100, and 400 pg/ml to ensure that the data was comparable over time. Data from all runs performed in the laboratory were registered and results when required to lie within two standard derivations of the mean. All samples were run in duplicates, but for each dose two independent samples were analysed in the same experiment. The duplicates were only allowed to vary 15% in standard derivation. Pure cell culture medium as well as culture medium with added wood dust was included in some of the runs, to ensure that they had no effect on the results.

**Assessment of genotoxicity**
Genotoxicity testing is performed to detect agents that induce damage to the genome. A combination of tests is generally applied to investigate the mechanisms behind DNA damage, gene, chromosome and genome mutations. In genotoxicity testing of fibers in vitro chromosome aberration test and micronucleus test demonstrated well the induction of structural chromosome aberrations [71]. As described in the section of genotoxicity in the background, the comet assay was found suitable to detect primary DNA damage induced by fibers [74]. The induction of oxidative damages such as oxidized purine bases can be measured by treatment with formamidopyrimidine DNA glycosylase (FPG) that removes the altered bases and the resulting alkaline-labile sites, which can be detected by the comet assay. However, this treatment was attempted established in our laboratory without success.

**The single cell gel electrophoresis assay (the comet assay).**
The technique is based on the assay introduced by Singh in 1988 [177]. However, the assay used in this thesis has been modified several times since then, one of these by McNamee [178]. The comet assay is based on the detection of single strand breaks in the DNA. These strand breaks occur either by exposure to a chemical agent, by intracellular DNA damaging agents, in the repair process of DNA, or they can be induced by enzymatic treatment in the assay to detect specific DNA adducts like 8-oxo-guanine residues. The principal of the assay is shown in figure 19. The cells are embedded in agarose and lysed, generating nucleus-like structures in the gel (referred to as nucleoids). Following alkaline electrophoresis, the DNA strands migrate toward the anode according to size. The extent of migration depends on the
number of strand breaks in the nucleoid. After staining the samples with fluorescence the migration is visualized in a fluorescence microscope. The nucleoids in damaged cells have the shape of a comet (see figure 19). The cells are then evaluated either on a scale by the observer or by a photo imaging system as either percentage tail DNA or tail length.

Figure 19. Schematic presentation of the comet assay. The cells are incubated with wood dust and brought to suspension. The cells are embedded into agarose on the GelBond film. The cells are lysed to remove cellular components and proteins. This leaves the DNA embedded in the agarose. Alkaline treatment unwinds the DNA and makes it single stranded. Alkali labile sites are broken. During electrophoresis the DNA will migrate according to the size of the fragments, depending of the frequency of strand breaks.

The standard method

The comet assay was basically performed as described by McNamee [178] but modified by Dybdahl et al [31]. The cell-agarose mixtures were cast on to a 100*85 mm GelBond film (Cambrex Bio Science, Rockland, ME) with a polyethylene moulding form (100*75*10 mm) with eight holes (d= 19.5 mm). The frozen cells were quickly thawed to 37°C in a water bath. Twenty five µl cell suspension were mixed with 225 µl of 0.75% low melting point agarose (Invitrogen, cat.no.15517-014). A 130 µl aliquot of this mixture was cast into a well and allowed to gel at 5 ºC for 10 min. Then the GelBond films were placed in cold lysis buffer (2.5 M NaCl, 10 mM Tris-base, 100 mM disodium-EDTA, 1% Na-sarcocinat, 10% DMSO, 1% Triton X-100, pH 8) for one hour before DNA was treated with alkaline solution (0.3 M NaOH, 1 mM EDTA-NA2, pH 13.2) for 40 minutes to allow the DNA to unwind. Electrophoresis was run for 20 min at 300 mA and 25V. The GelBond films were washed twice for 5 min in neutralizing buffer (0.4 M Tris, pH 7.5) and once in water before dehydration in 96% ethanol for 90 min. The samples were allowed to dry minimum overnight or until use. The dried gels were dehydrated and stained in a 1/20,000 stock dilution of SYBR
Gold (Invitrogen-Molecular Probes, Eugene, OR, US) in TE-buffer for 10 minutes before rinsed once with water. The samples were analysed on a Leica DM LB fluorescence microscope at 400x magnification, with a 450-490 nm emission filter and LP515 excitation filter.

The taillength in these experiments was measured despite that it is considered the least sensitive parameter. This was due to an overload of the head by fluorescence, because SYBR Gold binds to the GelBonds films. When the head in the imaging system is overloaded the percentage of tail DNA is no longer a quantitative measurement. Today Cambrex glass slides can be used instead of gel bond film eliminating this background fluorescence making it possible to measure the % of DNA in the tail or tail moment.

**Quality control**
The comet assay is a standard method in our laboratory and the standard method has been evaluated on H₂O₂ exposed A549 cells in our lab and in the development of the assay by McNamee [178]. A negative control from a batch of frozen unexposed cells and a positive control from a batch of 15µM H₂O₂ exposed cells for 30 minutes were included in each run. The negative controls had an average tail length ± S.D of 30.3 ±3.1µm (n=8) and the positive controls had an average of 64.3 ± 8.2. As some fluctuations occurred in the assay, all samples from a dilution series of a certain wood species were included in the same run and the tail length found were compared to the tail length from the unexposed cells in the same series. In our study 50 nuclei from each dose were evaluated, however in a very recent evaluation of the variance in the comet assay scoring of 200 nuclei per treatment was associated with the lowest residual variation [179]. We could therefore have reduced our variation if we have evaluated 200 nuclei instead of 50 cells.

**The molecular epidemiology study of ras mutations**

**Study design**
The study conducted in this thesis is partly a matched case-control study and partly a case-case study. The molecular part of this study is a case-case study, where we analysed tumour material from patients and collected exposure data from interviews and registers. In addition
to the case-case study, a matched case-control study was conducted in close collaboration with the Danish Cancer Society.

There are several study designs in analytical epidemiology. The case-case study used in this thesis was necessitated because of the use of tumour material. The case-case study is mainly used in molecular epidemiology and is a subtype of the case-control study. It is regarded the least powerful epidemiological study type, mainly due to size, especially if the illness (e.g. tumour type) investigated are rare. We also conducted a matched case-control study including interviews of three controls for each case with squamous cell carcinoma and five controls for each case with adenocarcinoma. The case-control study and the case-case study looks retrospective from effect to cause. The opposite is a prospective cohort study that is considered to look forward from cause to effect [180]. The case-control studies have both strengths and weaknesses compared to the cohort study. One large benefit is that a large longitudinal set up of the cohort, which takes time and large amount of money are avoided. This is especially true for cancer epidemiology with long latency time.

Our case-control study was matched with incidence density sampling, in this sampling method; controls, matched on sex and age, are chosen from the population of persons without a cancer diagnose at the time the cases were diagnosed. It is therefore possible for a person to be a control for several cases. It is also possible for a case to be chosen as a control of another case if this person first gets his cancer diagnose later in life. However, because of the rarity of sinonasal cancer, this is more a hypothetical scenario. This sampling method is used to avoid that controls are picked from an unnatural population of healthy persons.

The case-control study has several weaknesses, the major of which is including the differential reporting of exposure, also called recall bias. It is well known that patients tends to search for a cause of their disease (especially cancer patients) compared to healthy persons, and this can result in an overestimate of exposure of cases compared to controls. In studies of occupational cancers, the exposure bias can be reduced by adding information from registers of occupation, for example pension funds. These data are more objective, but usually less detailed than information from a personal interview.
The study cohort for the ras mutation identification

The study population was defined in collaboration with the Danish and Finnish national cancer registries. The study included initially all incident cases of adenocarcinomas and squamous cell carcinomas located of the sinonasal cavities (ICD7 code 160) in Denmark (from 1992 to 2002) and in Finland (from 1989 to 2002). In France, cancer registries do not cover the entire population, but there are several population-based cancer registries. The study was conducted in three areas, covered by the cancer registry from Isère, Somme, Doubs, in which sinonasal cancer cases diagnosed from 1990 onwards have been identified. In Finland the collection and purification of samples were carried out at the Finnish Institute of Occupational Health, Helsinki and included 116 DNA samples. In France the collection of samples was performed by the INSERM institute but only a limited number of DNA samples were received due to extensive problems with refusal by the pathology departments to participate and that a large proportion of the blocks have been treated with the Bouin fixative, which is not suitable for molecular analysis. Only 15 DNA samples were included from France.

<table>
<thead>
<tr>
<th>WHO code</th>
<th>Diagnosis</th>
<th>Comments</th>
<th>SNOMED 1991</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2.1</td>
<td>Sinonasal carcinoma</td>
<td>SNUC, refer to IHC</td>
<td>8121/3</td>
</tr>
<tr>
<td>1.2.1.1</td>
<td>Squamous cell carcinoma</td>
<td>keratinising vs nonkeratinising</td>
<td>8070/3</td>
</tr>
<tr>
<td>1.2.1.2</td>
<td>Cylindrical cell carcinoma</td>
<td></td>
<td>8121/3</td>
</tr>
<tr>
<td>1.2.2</td>
<td>Verrucous squamous cell carcinoma</td>
<td></td>
<td>8051/3</td>
</tr>
<tr>
<td>1.2.3</td>
<td>Spindle cell carcinoma</td>
<td>if recognizable carcinoma</td>
<td>8074/3</td>
</tr>
<tr>
<td>1.2.4</td>
<td>Adenocarcinoma</td>
<td>usually low grade NOS</td>
<td>8140/3</td>
</tr>
<tr>
<td>1.2.5</td>
<td>Papillary adenocarcinoma</td>
<td></td>
<td>8260/3</td>
</tr>
<tr>
<td>1.2.6</td>
<td>Intestinal-type adenocarcinoma</td>
<td></td>
<td>8144/3</td>
</tr>
<tr>
<td>1.2.18</td>
<td>Small cell carcinoma</td>
<td>rule out lung primary</td>
<td>8041/3</td>
</tr>
<tr>
<td>1.2.19</td>
<td>Lymphoepithelial carcinoma</td>
<td></td>
<td>8082/3</td>
</tr>
<tr>
<td>1.2.15</td>
<td>Adenosquamous carcinoma</td>
<td></td>
<td>8560/3</td>
</tr>
</tbody>
</table>

Table 7. A detailed list of histological diagnoses included in the molecular epidemiology study, with reference to the WHO classification of tumours (WHO, 1991). SNOMED, Systemic Nomenclature of Medicine

For the Danish part of the study cohort sinonasal cancers under the ICD9 code 160.0 and ICD7 code 160.2-160.9 were identified in the Danish Cancer Registry in collaboration with Johnni Hansen from the Danish Cancer Society. The patients included in the study all had their primary diagnosis given in the period 1991 to 2001. To retrieve the paraffin embedded tumour material (PET samples), a primary list of 466 patients was compiled (for details see
section about the case-control study). In collaboration with the pathologist assigned to this project, tumours with the diagnoses listed in table 7 were included in the study. After extensive considerations, the nasopharyngeal and vestibulum nasi cancers were excluded from the study.

**Retrieval and selection of blocks.**

In order to identify the location of PET samples, and retrieve them, I began by searching in the Danish hospital regions pathology databases. This was done at the university hospital departments of pathology (National Hospital of Copenhagen, Gentofte, Odense, Århus, and Ålborg hospitals). In these data bases I could identify the patients and the location of the relevant pathology reports from each patient. Sometimes there were several relevant reports for one patient. To retrieve the reports at the university hospitals I searched the archives myself. For the regional hospitals the reports were requested by letter. Upon arrival of the pathology reports, I was able to exclude some without any doubt, because I could ascertain that they did not fulfil the inclusion criteria, listed in table 7. The rest of the reports were evaluated by pathologists Torben Steiniche or Annemarie Antonsen. If a patient was selected suitable for the study based on the pathology reports, the pathologists selected one or more PET samples for review. The PET samples and the glasses with the original staining were requested by letters for the regional hospitals. At the five university hospitals I collected the blocks and the glasses myself. The glasses with the original staining were examined for amount of tumour and diagnosis and the pathologist selected a PET sample suitable for the molecular analysis. In very few cases where there was doubt of inclusion, it was decided initially to include these patients and await the final review. This was mainly the case for small fraction of squamous cell carcinomas due to their location on the border of the vestibulum. It was decided that if skin was identified in the sections the patient would be excluded. Of the original 466 patients, 174 patients were included in the molecular study (see figure 20 for the inclusion process). For 43 patients of the 466 patients no report could be retrieved. 11 of the patients had no information on the place of biopsy or treatment in the cancer registry reports (not even in the original paper version of the cancer report). The pathology reports were missing in the archives for 32 patients. For the remaining 423 patients one or more reports were retrieved. Of these patients, 215 patients were excluded on the basis of the pathology report. For 33 patients the reports were not accurate enough for exclusion. Blocks and original sections were therefore retrieved and reviewed by the pathologist before exclusion. One patient was excluded because the PET sample contained less than 10% tumour DNA. In the end 174 patients were included in the study.
**Standard protocol for sections**

PET samples were sectioned serially to prepare material for pathological review and molecular analysis: First one section hematoxylin-erosin stained and one section alcien-blue-PAS stained were cut. These were used for the revision of the diagnosis; two times five 10-μm sections were collected for DNA extraction. Finally a tissue section was cut for hematoxylin-erosin staining, to confirm that tumour was still present in all the sections.

**Revision of diagnoses**

Diagnostic procedures may change over time and between pathologists. To ensure uniform and correct diagnoses according to modern criteria, newly-made HE and PAS-alcien sections were evaluated independently by pathologist Michael Dictor and Torben Steiniche without knowledge of the original diagnosis. If there was disagreement between the given diagnoses, both reviewers re-examined in order to reach a consensus. In a handful of cases, additional immunostainings had to be performed before it was possible to set a correct diagnosis. The immunohistochemical stainings were performed in Finland by Pathologist Henrik Wolff. The percentage of tumour tissue in the blocks was estimated by revision of the first cut HE-section.

**DNA purification for molecular analysis.**

Genomic DNA was prepared from the five 10-μm sections. The sections were extracted twice with 1000 μl xylene (Merck) and twice with 1000 μl 96% ethanol for 10 min at 55 °C with centrifugation in between. After the last wash with ethanol the tissue was allowed to dry before adding 500 μl lysis buffer (50 mM Tris-HCl pH 8.5, 1.0 mM EDTA, 0.5% Tween-20)
and incubation for 2 hours (55 °C) under rotation. The tissue was treated under rotation with 50 µl to 100 µl 20 mg/ml proteinase K (Finnzymes, Espoo, Finland), depending on sample size, overnight or until the tissue was totally degraded. The samples were extracted twice with 500 µl phenol/chloroform/water mixture (Applied Biosystems, Foster City, CA, US cat. no 400765) and once with 500 µl chloroform (AMRESCO, Ohio, US). The DNA was precipitated for 2 hours at -20 °C with 27 µl 3M sodium acetate in ice-cold ethanol. The DNA was then washed once with 70% ethanol, and dried at 50 °C, before it was dissolved in RNase free water. The DNA concentration was determined spectrophotometrically at 260 and 280 nm and when it could be achieved, the concentration was adjusted to 100 µg DNA /ml.

**Identification of ras mutations**

Mutations in critical genes such as tumour suppressor genes or oncogenes, which are involved in cell growth and regulation, are important in relation to carcinogenesis. There exist a variety of techniques to identify mutations of specific genes like denaturing gradient gel electrophoresis (DGGE), direct sequencing, single-strand conformation polymorphism analysis (SSCP), PCR-restriction fragment length polymorphism analysis (RFLP), mismatch RFLP, oligonucleotide ligation assay and RNase mismatch protection assay, as well as combinations of these techniques [181].

**The principle of mismatch RFLP**

The mismatch RFLP technique is a further development of the RFLP technique. The principal in RFLP technique is to multiply the area of interest by PCR, and to digest the PCR fragments by restriction enzymes specific for the oligonucleotide sequence, where the mutation is expected to be. The enzyme will cut the PCR fragment if the template DNA contains the wild type (normal) sequence, but not if the template DNA has a mutation. The digested PCR fragments are visualised after gel electrophoresis (see figure 21). The assay is limited by the availability of a restriction enzyme with the right sequence specificity. To overcome this obstacle, artificial sequences with restriction enzyme digestion sites can be generated by mismatch PCR. This is achieved by replacing one or two bases overlapping the restriction site in the 3’ end of one of the primers. This expands the possibilities for making an RFLP assay by many times, but options are still not unlimited.
Figure 21. The figure shows the results of the codon 12 RFLP analyses. From the left, lane 1:100 base pair ladder, lane 2: Undigested PCR product (DK 211), lane 3: BstN1 digested PCR product (DK211- no mutation), lane 4-11: Samples undigested/BstN1 digested, lane 12: Undigested negative control (P48), lane 13: BstN1 digested negative control (P48), lane 14: Undigested positive control (A549) and lane 15: BstN1 digested positive control (A549).

Identification of mutations in K-ras gene codon 12, 13, and 61

In order to identify mutations in the K-ras gene codon 12, codon 13 and codon 61, three assays were developed. The assays were modified versions of the methods used by Saber et al [95] and Vachtenheim et al [182]. Separate PCR reactions were performed for the three assays. The reactions were performed with the primers listed in table 8 under the conditions listed in table 9, the amount of DNA varied between 0.5 µl to 4 µl since individual optimization was necessary.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Codon</th>
<th>Primers</th>
<th>Sequence 5’ to 3’</th>
<th>Restriction enzymes/restriction site</th>
<th>PCR product/cut fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12</td>
<td>mmkras12F</td>
<td>ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT</td>
<td>BstN1: 5’..CCTGG..3’</td>
<td>157 bp/ N: 114+29+14</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mmkras1213R</td>
<td>TCA AAG AAT GGT CCT GGA CC</td>
<td>3’..GGACC..5’</td>
<td>M:143+14</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>mmkras13F</td>
<td>ATA TAA ACT TGT GGT AGT CCC AGC TGG</td>
<td>Van 911: 5’..CCA(N)TGG..3’</td>
<td>152 bp/ N:126+26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mmkras1213R</td>
<td>TCA AAG AAT GGT CCT GGA CC</td>
<td>3’..GGT(N)ACC..5’</td>
<td>M:152</td>
</tr>
<tr>
<td>2</td>
<td>61</td>
<td>mmkras61BlcF</td>
<td>CTT GGA TAT TCT CGA CAC AGC TGA T</td>
<td>Bcl1: 5’..TGATCA..3’</td>
<td>179 bp/ N:155</td>
</tr>
<tr>
<td></td>
<td></td>
<td>mmkras61BlcR</td>
<td>AAC TAT AAT TAC TCC TTA ATG TCA GCT TA</td>
<td>3’..ACTAGT..5’</td>
<td>M: 179</td>
</tr>
</tbody>
</table>
The PCR reactions were performed on a PTC-100™ Programmable Thermal Controller (MJ Instrumentation, Inc., Ramsey, Minnesota, US). Cycling conditions for the mmkrascodon12 assay and the mmkrascodon13 assay were; DNA was denatured at 94 °C for 1 min, taken through 35 cycles at 94 °C for 30 sec, 55 °C for 45 sec, 72 °C for 45 sec and finally incubated at 72 °C for 10 min. For the mmkrascodon 61 assay the cycling conditions were: DNA was denatured at 94 °C for 2 min, taken through 12 cycles at 94 °C for 30 sec and 67 °C for 1 min with a decrement of -1°C for each cycle (67 °C to 56 °C). Then taken through 28 cycles at 94 °C for 40 sec, 56 °C for 1 min and finally incubated at 72 °C for 10 minutes.

Before restriction enzyme treatment the PCR products for the mmkrascodon13 assay was purified by GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK), as the Van91I enzyme was not able to cut due to the high salt concentrations of the PCR buffer.

The restriction enzyme treatment in the mmkrascodon12 was performed with 7µl PCR product, 1x NE Buffer 2, 100 µg/ml BSA and 0.7µl BstN1 I (New England Biolabs, Ipswich, MA, US) and overnight incubation at 60 °C. The restriction enzyme treatment in the mmkrascodon13 was performed with 7.6-12.7 µl PCR product, 1x Surecut buffer B, 1-6 µl miliQ and 0.4 µl Van 911 I (Boeringer Manheim-Roche, Hvidovre, Denmark) and 6 hr incubation at 37 °C. The restriction enzyme treatment in the mmkrascodon61 was performed with 5-10 µl PCR product, 5-10 µl miliQ, 1x NE Buffer 2, 0.4 µl Bcl I (New England Biolabs, Ipswich, MA, US) and 6 hr incubation at 50 °C. The cut and uncut PCR products were visualised on a 3% NuSieve® 3:1 agarose (Cambrex, San Francisco, CA, US) gel run in 1x TBE buffer.

Table 9. Lists the reaction conditions for the three RFLP assays.

<table>
<thead>
<tr>
<th>Assay</th>
<th>PCR conditions</th>
<th>KCl</th>
<th>Tris-HCl</th>
<th>MgCl₂</th>
<th>dNTP</th>
<th>Taq polymerase</th>
<th>Primers</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmKras codon 12</td>
<td>50 mM</td>
<td>20 nM</td>
<td>0.25 mM</td>
<td>0.2 mM</td>
<td>0.75 unit</td>
<td>0.5 µM</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>mmKras codon 13</td>
<td>50 mM</td>
<td>20 nM</td>
<td>0.25 mM</td>
<td>0.2 mM</td>
<td>0.75 unit</td>
<td>0.5 µM</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>mmKras codon 61</td>
<td>50 mM</td>
<td>10 nM</td>
<td>1.5 mM</td>
<td>0.2 mM</td>
<td>1.0 unit</td>
<td>1.0 µM</td>
<td>8.3</td>
<td></td>
</tr>
</tbody>
</table>

**Quality control**

For all PCR reactions a no template control was included. All PCR products was visualised on a 1.5% NuSieve® 3:1 agarose gel and only samples where a distinct band in the correct size was observed were used for restriction enzyme treatment. In two of the assays it was not possible to include a restriction control site so DNA from a healthy person was included in all
the runs instead. To ensure that we were able to distinguish between a mutant sequence and a normal sequence, we include positive controls as well. For the codon 12 assay the A459 cell line (GGT → AGT) was used [183]. For the codon 13 assay the MDA-MB231 cell line (GGC → GAT) was used [184]. For the codon 61 assay no cell line was available, so DNA from a cancer patient with a codon 61 mutation (CAA → CTA) was used (kind gift from Kirsti Husgafvel-Pursiainen, Finnish Institute of Occupational Health). Validation experiments with 2-fold dilutions of the positive controls down to 6.25 % mutated DNA, was performed, and the results depicted in figure 22.

Direct sequencing
During the last decade, direct sequencing has become a golden standard in screening for mutations in minor well defined area. Since the development of automated sequencing it has become much less time consuming and affordable.

**Principle in direct sequencing on an ABI 310 Genetic Analyser.**
Direct sequencing is based on PCR of the target area. Dideoxynucleotides marked with fluorescence (one colour for each of the four nucleotides) are in a second PCR reaction incorporated into the PCR products of the target area. Because dideoxynucleotides prohibit elongation of the PCR product, products differentiating in length will be formed (figure 23).
The fluorescence-labelled PCR products are analysed by the Genetic Analyser. In principal a capillary electrophoresis separates the PCR products by one base length, with the smallest PCR migrating fastest. In the end of the capillary a laser detector registers the fluorescence emitted from the marked probes. An electrophoretogram is produced electronically by the machine. A representative electrophoretogram is shown in figure 24.

Figure 23. Schematic presentation of the principle of direct sequencing on a Genetic Analyser.

Figure 24. Shows a print of the first page of a electrophoretogram from a run of the negative control (P48) with the reverse sequencing primer. Codon 12 and 13 are marked with green and should be read in reverse.
**Standard procedure**

In order to identify the mutations in *K-ras* and *H-ras* codon 12, codon 13, and codon 61 by direct sequencing, four assays were developed: The K-ras codon1213, K-ras codon61, H-ras codon1213 and H-ras codon61 assays. Four sets of PCR and sequencing primers listed in table 11 were developed. The PCR conditions for the four assays were 50 mM KCl, 10 mM Tris-HCl, 0.2 mM dNTP, 1 unit Taq polymerase and 1.0 μM forward and reverse primers. Only the MgCl₂ concentration and pH varied (listed in table 10). The PCR reactions were performed in 50 μl reactions, on a PTC-100™ Programmable Thermal Controller.

<table>
<thead>
<tr>
<th>Assay</th>
<th>Primers</th>
<th>Sequence 5’ to 3’</th>
<th>MgCl₂</th>
<th>pH</th>
<th>PCR fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-ras codon1213</td>
<td>Kras1213forward</td>
<td>AACCTTATGTGTGACATGTTC</td>
<td>1.5</td>
<td>9.0</td>
<td>220</td>
</tr>
<tr>
<td></td>
<td>Kras1213reverse</td>
<td>ATGGTCCTGACGACCAGTAAAT</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kras1213sequence</td>
<td>ATATAGTCACATTTTCATTATT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kras1213rev</td>
<td>TCCGCACCAGTAATATGCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K-ras codon61</td>
<td>Kras61forward</td>
<td>AAAGGTGCACTGTAATAATCC</td>
<td>1.5</td>
<td>9.0</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Kras61reverse</td>
<td>TGATTTAGTATTATTATGGGCAA</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kras61sekvens</td>
<td>TGGTTCTCCCTTCAGGATTTC</td>
<td></td>
<td></td>
<td>123</td>
</tr>
<tr>
<td>H-ras codon1213</td>
<td>Hras1213forward</td>
<td>GAGGAGCGATGACGGAATATCC</td>
<td>2.0</td>
<td>8.9</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Hras1213reverse</td>
<td>CTGCAGCCGACCCCTATCTCT</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hras1213sekvens</td>
<td>GCGCCAGGCTCACCTCTATA</td>
<td></td>
<td></td>
<td>132</td>
</tr>
<tr>
<td>H-ras codon61</td>
<td>Hras61forward</td>
<td>CTGTCTCCTGCTTCTCTTAGA</td>
<td>1.0</td>
<td>9.2</td>
<td>326</td>
</tr>
<tr>
<td></td>
<td>Hras61reverse</td>
<td>GTACTGGTGAGGTGCCTCAA</td>
<td>mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hras61sekvens</td>
<td>GAGGCTTGCTGTGTAACCT</td>
<td></td>
<td></td>
<td>278</td>
</tr>
</tbody>
</table>

For the K-ras codon1213 assay the DNA was denatured at 94 °C for 1 min and amplified in 12 cycles at 94 °C for 30 sec and 57 °C for 1 min with a decrement of -1 °C for each cycle (57 °C to 45 °C). Additional amplification was performed through 28 cycles at 94 °C for 40 sec, 45 °C for 1 min, and incubated at 72 °C for 10 minutes. For the K-ras codon61 assay the amplification was done at 95 °C for 2 min, then 40 cycles at 94 °C for 1 min, 48 °C for 30 sec, and 72 °C for 1 min. For the amplification of H-ras codon1213 cycling conditions were; 95 °C for 2 min then 35 cycles at 94 °C for 1 min, 61 °C for 30 sec, and 72 °C for 1 min. For the H-ras codon61 assay the amplification was done at 95 °C for 2 min then 35 cycles at 94 °C for 1
min, 57 °C for 30 sec, and 72 °C for 1 min. The PCR products (3.0 µl) were visualised by electrophoresis, on a 1.5% NuSieve® 3:1 agarose gel, run in 1xTBE buffer. Only PCR reactions where a distinct band of the right size appeared was purified using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, UK) according to the manufacturer.

To generate the sequencing templates the sequence primers in table 11 were used with matching annealing temperature (K-ras codon 1213, 45 °C; K-ras codon 61, 50 °C; H-ras codon 1213, 57 °C and H-ras codon 61, 55 °C) with the BigDye® Terminator Ready Reaction Kit (Applied Biosystems, part no 4303152) according to the manufacturer. The sequencing was performed on an ABI 310 Genetic Analyser, according to standard protocols.

Figure 25. Electrophograms from the validation with the cell line A549 which contains a total G → A mutation in the first base of K-ras codon 12. The sequencing is performed in reverse. The DNA from the A549 cell line was mixed with DNA from the negative control P48 before amplification. ½ A549 therefore represents a sample with 50% A549 cell line DNA and 50% P48 DNA.

Quality control
In these assays the same quality steps were used as in the RFLP assay with no template controls to ensure no contamination, visualisation of PCR fragments, positive and negative
controls included in the study, dilution assay to check for sensitivity, and finally confirmation of mutations by sequencing a second time of the opposite strand. Validation of the sensitivity was performed using the A549 cell line in dilutions to see if mutations could be recognized. In figure 25 the electrophograms for the dilutions are shown. It is possible to establish mutations by certain down to 25% mutated DNA. However, a peak is also found with 12.5% mutated DNA but is considered more uncertain due to the background spectrum.

The case-control study
The study was a matched case-control study with incidence density sampling among Danish sinonasal cancer patients. The study subjects were recruited from the Danish Cancer Register as all incident cases of sinonasal cancer (ICD7 code 160 and ICD9 code 160.0 and 160.2-160.9) registered with adenocarcinomas or squamous cell carcinomas histology between 1991 and 2001. This resulted in a total of 466 cancer patients. The criteria for participating in the interview were restricted to include adenocarcinoma patients born after year 1923 and squamous cell carcinomas patients born 1930 and onwards. For the patients where PET samples were retrieved interview were attempted for all cases, but the number was very limited due to technical reasons (see discussion). For each case of adenocarcinoma, five controls matched on birth year, sex, and vital status were selected and for each case of squamous cell carcinoma three control matched on birth year, sex and vital status were selected. Invitations to participate in the interview were sent out by mail and followed up by an interview. If the cases were deceased or refused to participate next-of-kin preferable the last spouse or a child was interviewed instead. The questions for the interview are enclosed in the appendix B. The interviews were carried out by trained interviewers at the Danish Cancer Society and me. All data was assembled in a data base held at the Danish Cancer Society. Electronically data cleaning and handling of the data base is processed by Johnni Hansen.

The strategy for the exposure matrix is based on the knowledge of exposure levels for wood dust in Denmark in the period 1990-1999[28]. Trades without wood dust measurements were assessed by using data from Finland, France and Germany as substitute. The estimates of historical exposures were done by extrapolation. The extrapolation constant was set on a 6% decrease per year since the year 1975. The extrapolation constant was estimated based on time trends in occupational exposures to air contaminants. The literature on which the decision was
based are two literature reviews [185,186] covering historical changes in total dust exposures of 119 plants in the period 1967 to 1996. A decreasing trend in concentration was found in 80 percent of the plants with a yearly downward trend between 4 percent and 14 percent. A third additional review [187] concludes that the yearly reductions were remarkably equal lying in the area of 6-7 percent across all industries. Analysis of exposure data bases provided a yearly decline in wood dust or confound exposures by 6 % [188]. Also exposure measurements by Schlünssen et al. [189] were used to decide the extrapolation constant on a 6 percent yearly decline. All jobs and trades given in the interview were classified according to International Standard Classification of Occupation 1968 [190] and Danish Industrial Classification of All Economic Activities 2003 [191]. If the trade codes did not agree with the information in the job descriptions the trade code were changed to a more suitable trade code. For instance a carpenter employed by an amusement park.

Our occupational exposure expert Vivi Schlünssen then estimated the GM for wood dust related job codes and trade codes. GM for trades is based on persons working close to the source with a constant exposure. From the job description, an interpretation of the extent and duration of wood dust exposure was performed by Vivi Schlünssen and a constant for exposure modification was assigned. Because all our GM measurements are based on exposure levels in Denmark from year 1990 to 1999, they are assigned year 1995. The cumulative exposure is calculated by the following formula for each job the patient held and added together:

\[
CE_x = W*EKSP(-LN(D)*X)/(-LN(D))*(EKSP(LN(D)*E)-EKSP(LN(D)*F))*T)*1
\]

- \(CE_x\) = cumulative exposure for job \(x\)
- \(D\) = disintegration constant (\(< 1975 = 1\) and \(1975 \leq 0.94\))
- \(W\) = Geometric mean in 1995
- \(X\) = year of estimated GM
- \(E\) = year of job start
- \(F\) = year of job end
- \(T\) = exposure modifier
A graphical illustration of the model for cumulative wood dust exposure is shown in figure 26. The cumulative exposure is represented by the hatched area under the curve.

Figure 26. Illustration of the cumulative wood dust exposure assessment. The hatched area represents the cumulative exposure. The year 1975 was a cut off for the 6% annual decline in wood dust exposure.

In the future when the data is cleaned and proofread, a statistical analysis will be performed using conditional logistic regression for matched case-control studies. Odds ratios adjusted for age, sex, smoking and the following occupational exposures; formaldehyde, leather dust, textile, and chromium. The odds ratios in relation to the cumulative exposure to wood dust will be calculated for all histologies, the adenocarcinomas, and the squamous cell carcinomas.
RESULTS

Exposure of cells to wood dust

The results from the in vitro exposures are described in the manuscript: Inflammatory response and genotoxicity of seven wood dusts in the human epithelial cell line A549, accepted for publication in Mutation Research (manuscript I). In addition to the submitted results the pictures in figure 27, shows examples on the cells exposed to wood dust from the different species.

Figure 27 Shows examples of A549 cells exposed to different species of wood dust. The species spruce, teak, birch and pine are shown after 6 hours of incubation.
As can be seen in the pictures the structure of the wood dusts varies between species. There was a tendency that the softwoods spruce and pine exhibited a more fibrous character than the hardwoods, which were of more particle character (round shaped). However, neither particle size distribution nor specific surface areas did differ between the wood dusts, table 11 and [72,192]. The content of secondary metabolites from the seven species according to literature findings are listed in table 4.

<p>| Table 11. The specific surface areas determined by liquid nitrogen adsorption |
|---------------------------------------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Wood species</th>
<th>Specific surface area (m²/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beech</td>
<td>3.24</td>
</tr>
<tr>
<td>Oak</td>
<td>2.92</td>
</tr>
<tr>
<td>Birch</td>
<td>2.89</td>
</tr>
<tr>
<td>Teak</td>
<td>3.07</td>
</tr>
<tr>
<td>Pine</td>
<td>3.26</td>
</tr>
<tr>
<td>Spruce</td>
<td>3.02</td>
</tr>
<tr>
<td>MDF</td>
<td>2.17</td>
</tr>
</tbody>
</table>

The result in the paper is in short: DNA strand breaks were increased 1.2 to 1.4-fold after 3 hours of incubation with beech, teak, pine and MDF dusts compared with the control experiments, without exposure to wood dusts. However, only exposure to MDF dust resulted in an elevation of DNA strand breaks after 6 hours. Increased expression of cellular IL-6 and IL-8 mRNA was induced by all of the wood dusts, and it increased both at 3 and 6 hours (figure 28 and table 13). Also the levels of IL-8 protein was elevated in the medium, and showed a pattern similar to that seen with the levels of IL-8 mRNA, except for oak dust in which case there was a reduction, which was not statistically significant. The slopes of the interleukin-mRNA dose-response curves varied up to 15-fold. By using the IL-8 mRNA expression, the wood dusts could be divided into three groups, with the teak dust being the most potent, MDF, birch, spruce and pine intermediate, and beech and oak being the least potent among the wood dusts studied. The induction of DNA strand breaks did not correlate well with the interleukin (IL-6 and IL-8) responses at mRNA or protein levels. In figure 29 the data are compiled and presented graphically.
Figure 28. Cellular effects in A549 cells, as a function of incubation to different concentrations of wood dust for 3 hours and 6 hours. Data are given as the mean and standard derivation. DNA strandbreaks are given as taillength relative to that of untreated cells, n=3. Cellular content of IL-6 and IL-8 mRNA relative to -actin, n=3). Excreted IL-8 protein in the culture medium normalised to untreated cells, n=4 (n=3 for beech and oak).
In table 12 the results are compiled according p-values for dose-effects curves made by linear regression. The arrows in the table indicate the level of significance for the dose-response effects for log-transformed interleukin mRNA data and IL-8 protein data analysed by linear regression. For the analysis on mRNA level only the concentrations between 0 and 100 µg/ml were used because the dose-response curves tended to level of at this concentration. For the DNA strand breaks linear regression performed on tail length relative to untreated cells. One arrow indicates a p-value with a cut of at 0.05, two arrows indicates a p-value with a cut of at 0.01 and three arrows indicate a p-value with a cut of at 0.001.

Table 12. Compilation of Interleukin 6 and 8 mRNA, Interleukin 8 protein and DNA strand breaks results.

<table>
<thead>
<tr>
<th></th>
<th>Beech</th>
<th>Oak</th>
<th>Birch</th>
<th>Teak</th>
<th>Pine</th>
<th>Spruce</th>
<th>MDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 mRNA</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>→</td>
<td>→</td>
<td>↑</td>
</tr>
<tr>
<td>IL-8 mRNA</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑</td>
<td>→</td>
</tr>
<tr>
<td>IL-8 protein</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
<td>→</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>DNA strand breaks</td>
<td>↑↑</td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
<td>↑↑</td>
<td>→</td>
<td>↑↑</td>
</tr>
<tr>
<td>6 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6 mRNA</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>IL-8 mRNA</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>IL-8 protein</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
<td>→</td>
<td>→</td>
<td>→</td>
</tr>
<tr>
<td>DNA strand breaks</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>→</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

The analyses of the tumour material for mutations in codon 12, 13 and 61 of the K-\textit{ras} and H-\textit{ras} genes.

The histology
For the epidemiological studies in the Wood Risk project a histological review of the cases was included to ensure a correct, uniform and modern classification of the histology types. The result from the pathological review of patients from all three countries, Denmark, Finland and France are listed in table 13. The number of patients included in the epidemiological

76
studies is 378. The samples can be classified into three main categories adenocarcinomas (127 patients), squamous cell carcinomas (229 patients) and other carcinomas (22 patients). In the epidemiological study the number of patients is higher than in the molecular study of *ras*. The reduction of cases is due to the Finnish rules of consent and the wide use of Bouin fixation among the French samples, which excludes a large number of patients from molecular analysis. Among the samples suitable for molecular analysis the samples distribution to the three main categories were; 86 adenocarcinoma, 188 squamous cell carcinoma and 16 other carcinoma.

Table 13. The diagnosis from the pathological review.

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Histology in the epidemiological studies</th>
<th>Histology in the <em>ras</em> molecular study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Danish</td>
<td>Finish</td>
</tr>
<tr>
<td>Carcinoma NOS</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Carcinoma NOS (low grade)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma NOS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma high grade</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Adenocarcinoma low grade</td>
<td>11</td>
<td>5</td>
</tr>
<tr>
<td>Adenocarcinoma intestinal type</td>
<td>23</td>
<td>7</td>
</tr>
<tr>
<td>Adenocarcinoma low grade (acinic like)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Adenocarcinoma papillary</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Squamous cell carcinoma</td>
<td>104</td>
<td>78</td>
</tr>
<tr>
<td>Squamous cell carcinoma (inverted papilloma)</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Squamous cell carcinoma- spindle cell</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Squamous cell carcinoma- dysplasia</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td>Undifferentiated carcinoma</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Verrucous carcinoma</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>174</td>
<td>119</td>
</tr>
</tbody>
</table>

For the Danish cases pension fund register data and telephone interview with the patient or next-of-kin and register data were used for assessing exposure of wood dust, smoking habits and other occupational exposures. For the Finnish patients the occupational exposure was
assessed by industrial hygienists and telephone interviews. In table 14 a description of the epidemiological data from the 174 Danish patients and 96 of the 100 Finnish patients included in the study are listed. For the last four Finnish patients no information was available.

Table 14. Epidemiological data from the Danish and Finnish study.

<table>
<thead>
<tr>
<th></th>
<th>DANISH PATIENTS</th>
<th>FINNISH PATIENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Men (n=111)</td>
<td>Women (n=63)</td>
</tr>
<tr>
<td>Average age at diagnose</td>
<td>66 67</td>
<td>63 69</td>
</tr>
<tr>
<td>Tobacco smoking</td>
<td>no information</td>
<td>53 37</td>
</tr>
<tr>
<td></td>
<td>smoking 49 20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>non smoker 9 6</td>
<td>8 10</td>
</tr>
<tr>
<td>Diagnose</td>
<td>adenocarcinoma</td>
<td>48 10</td>
</tr>
<tr>
<td></td>
<td>squamous cell carcinoma 58 51</td>
<td>5 5</td>
</tr>
<tr>
<td></td>
<td>other carcinoma 5 2</td>
<td>43 21</td>
</tr>
<tr>
<td>Wood dust</td>
<td>Likely exposed to wood dust 40 0</td>
<td>27 2</td>
</tr>
<tr>
<td></td>
<td>Likely not exposed to wood dust 71 63</td>
<td>30 23</td>
</tr>
<tr>
<td></td>
<td>Not assessable - -</td>
<td>9 5</td>
</tr>
</tbody>
</table>

The sensitivity of the assays used to identify ras mutations

To ensure that the sensitivity was high enough to identify all possible mutations, the combination of RFLP and direct sequencing were used. The combination provides a reliable but very time and work consuming process. Evaluating the sensitivity as little as 6.25% mutated DNA could be detected in the BstN1 RFLP assay (codon 12 position 1 and 2). The mutated DNA was isolated from the A549 cell line harbouring a homozygote K-ras mutation at the second position in codon 12. The sensitivity of the BstN1 RFLP assay has in some studies been shown to detect as little as 0.1 to 2 % mutated DNA [193,194]. Inactivation of tumour suppressor genes in principle requires functional loss of both alleles, while a single mutational event is sufficient for oncogene activation. K-ras mutations are therefore expected to occur as heterozygous mutations. With the sensitivity of the BstN1 RFLP assay, one should
be able to detect a mutation in samples containing 12.5% tumour tissue. The Van 91I assay and the Bcl1 RFLP assay were tested with heterozygous cell lines/samples. Using those we were able to detect 12.5% mutated DNA, which is equal 12.5% tumour tissue. The sensitivity test of direct sequencing revealed that mutations could be detected in samples containing 25% tumour tissue with a heterozygote mutation. A recent study shows that dideoxy sequencing is rarely sensitive below a 10% mutant allele frequency, which corresponds to 20% tumour sensitivity found in our study. Increasing the sensitivity of the direct sequencing is normally done by increasing the percentage of tumour tissue in the sample by micro dissection (laser or crude scraping). This is, however, very time consuming and demands skilled personnel not tissue harbouring a heterozygous mutation [195]. This is in accordance with the level of available in our study, the samples contains therefore both normal and cancerous tissue.

![Image of gel electrophoresis](image)

Figure 29. Shows the BstN1 digestions of the samples with mutations. Picture A. Lane 1: DK 15 undigested, Lane 2: DK15 digested (mutation), Lane 3: DK 20 undigested, Lane 4: DK 20 digested (no mutation). Picture B. Lane 1: DK 91 undigested, Lane 2: DK91 digested (mutation), Lane 3: DK 92 undigested, Lane 4: DK 92 digested (no mutation), Lane 5: DK 93 undigested, Lane 2: DK93 digested (mutation). Picture C. Lane 1: DK 86 undigested, Lane 2: DK 86 digested (mutation), Lane 3: DK 97 undigested, Lane 4: DK 97 digested (no mutation).

**K-ras mutation analysis; Danish samples**

Results from the K-ras analyses on Danish sinonasal cancer are described in manuscript II published in BMC Cancer 2008, 8:53 (doi:10.1186/1471-2407-8-53). The results were in short. K-ras was mutated in 13 percent of the adenocarcinomas (7 patients) and in 1 percent of squamous cell carcinoma patients (one patient). Of these eight mutations, five mutations were located in the codon 12. The remaining three could only be identified ambiguously, because sequencing was suboptimal. Among the five identified mutations, the most common was the GGT → GAT transition, which was present in tumour tissue from two wood dust exposed adenocarcinoma patients and one patient with unknown exposure. In figure 29 the
digestions of the K-ras codon 12 mutations are shown. Due to the quality of the scan not all the mutations found are shown. In addition to the submitted results all electrophoretograms for the samples with a mutation, are shown in figure 30.

Figure 30. Shows the electrophograms from the direct sequencing of the samples with mutations (DK15, DK91, DK93 and DK178) and two electrophograms from the ambiguous sample DK86. The red ring indicate the base change.
K-ras mutation analysis; Finnish and French samples

The mutational analysis of the Finnish and French sinonasal cancers for K-ras mutations was performed on 101 Finnish and 15 French tumours. Among these patients no mutations could be identified. The success rates for the sequencing are shown in the table 15. Some samples were not sequenced, because no PCR product could be generated. The Finnish samples performed well and I was able to sequence with a success rate of 74% to 100%, whereas the quality of the French DNA was lower, with a success rate between 31% and 81%. However, for one Finnish and French sample no PCR products could be amplified in any of the PCR assays used.

Table 15. Shows the success rate for the direct sequencing

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Denmark</td>
<td>141 (81%)</td>
<td>158 (91%)</td>
<td>152 (87%)</td>
<td>107 (61%)</td>
</tr>
<tr>
<td>Finland</td>
<td>98 (98%)</td>
<td>100 (100%)</td>
<td>99 (99%)</td>
<td>74 (74%)</td>
</tr>
<tr>
<td>France</td>
<td>9 (56%)</td>
<td>13 (81%)</td>
<td>9 (56%)</td>
<td>5 (31%)</td>
</tr>
<tr>
<td>Total</td>
<td>248 (86%)</td>
<td>271 (93%)</td>
<td>260 (90%)</td>
<td>186 (64%)</td>
</tr>
</tbody>
</table>

Due to the low sensitivity of the sequencing, RFLP was performed as well. For K-ras codon 12 (position 1+2) both the Finnish and French samples was tried amplified except for nine samples, where no DNA was left. On the remaining 107 samples, PCR was performed for 97 of these the quality of the amplification was good enough for digestion. None of the digestions indicated mutations. For the third position in codon 12 and first and second position of codon 13, successful PCR amplification was performed on 87 Finnish samples. The rest of the Finnish could not be amplified (8 samples) or no DNA remained (2 samples). Because of the quality of the French DNA, none of the samples with remaining DNA (6 samples) could be amplified. None of these digestions indicated mutation. Based on the low frequency of K-ras mutations among the Danish squamous cell carcinomas, RFLP analysis was limited to the codon 61 for adenocarcinomas, because of the limited time and resources.

H-ras mutation analysis by direct sequencing; Danish, Finnish and French samples

In addition to the K-ras mutations, direct sequencing of H-ras codon 12, 13 and 61 were performed. The success rates for tumours from all three countries are shown in table 15. For codon 12+13, successful sequencing was performed for 152 Danish samples (87%), 99
Finnish samples (99%) and 9 French samples (56%). The PCR amplification resulted in no product for the rest of the samples. For codon 61 of the H-ras gene 107 Danish samples (61%), 74 Finnish samples (74%) and 5 French samples were sequenced successfully, for the rest no PCR product could be amplified. For the H-ras codon 61 assay the PCR reaction did not run very well despite many attempts to optimize. The quality of the French samples was lower than the Finnish and Danish, probably due to difference in the preservation techniques of the tissue. Among the sequenced samples no mutations could be identified. However, since no second method for detection of mutations has been performed on these samples, the results for the H-ras gene should be considerate preliminary. The lack of mutations could be due to the high backgrounds in the sequences which are able to hide mutations in sample with low amount of tumour tissue.

**Case-control study of sinonasal cancer in Denmark**

In addition to the molecular analysis of the ras genes, a matched case-control study was performed in close collaboration Danish Cancer Society. The data from this study are in the process of being verified at the Danish Cancer Society. Exposure data are being assessed by Vivi Schlünssen on basis of the given job and trade codes. Exposure data are therefore not presented in this thesis.

Of the 466 study subjects retrieved from the Danish Cancer Registry, 222 complied with our criteria for participating in an interview among these 174 cases was successfully interviewed. Because this study was a matched case-control study with incidence density sampling a number of 443 controls was invited to participate in the interview and 295 interviews were successfully completed. In table 16 the distributions of interviews are listed.

<table>
<thead>
<tr>
<th>Table 16. Distribution of interview among invited participants.</th>
<th>Controls</th>
<th>Cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Successful completed interview</td>
<td>295</td>
<td>174</td>
</tr>
<tr>
<td>Refrained from participation</td>
<td>77</td>
<td>24</td>
</tr>
<tr>
<td>Could no be contacted despite of a minimum of 10 calls</td>
<td>34</td>
<td>10</td>
</tr>
<tr>
<td>No correct telephone number could be identified</td>
<td>24</td>
<td>10</td>
</tr>
<tr>
<td>Dead or emigrated during the period of interview</td>
<td>13</td>
<td>4</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>443</strong></td>
<td><strong>222</strong></td>
</tr>
</tbody>
</table>
DISCUSSION

Health effects associated with occupational exposure to wood dust are of both non-malignant and malignant character. In 1999, the European Commission launched the directive EU 99/38/EC stating that dust from hard wood are considered carcinogenic to humans and a 5 mg/m³ threshold limit value was set. However, little is known about the exposure levels and mechanisms behind the carcinogenic effects of wood dust.

In 2000, the Wood Risk project was launched (http://www.ttl.fi/NR/rdonlyres/98D74781-98B5-440A-8474-5C3160D5BEB8/1983/Woodrisk1.pdf). The purpose of the project was to provide information on exposure and the mechanisms behind the adverse health effects of wood dust. This thesis is a part of the Wood Risk project and has main focus on the carcinogenic effects of wood dust exposure. The objectives and aims of this thesis were to investigate the wood dust induced inflammation in relation to DNA damage, and the relationship between occupational exposure to wood dust and the recognition of specific mutational patterns in human tumours. The project was originally planned to include sinonasal cancer cases from the three countries Finland, France and Denmark, but unfortunately, our French collaborator was not able to retrieve a sufficient number of samples. The number of cases was also significantly reduced in both Finland and Denmark compared to the original listings from the cancer registries. The reduction was caused by very strict ethical rules of consent in Finland and the fact that a large fraction of the squamous cell carcinomas were located in the vestibulum nasi or the nasopharynx. Cancers at these sites are epidemiologically not thought to be associated with wood dust exposure and were consequently excluded.

In cancer epidemiology, a crucial factor is the correctness of the diagnosis, as misclassified cases dilute the true effects, since exposures not related to the specific cancer will be included. In countries with national cancer registries, the most common mistake is including metastasis as primary cancers [196]. Wood dust exposure has mainly been associated with adenocarcinomas [7,141]. It was therefore important to perform a review of the diagnosis in order to eliminate the potential misclassification which might have occurred at the regional hospitals because of the rarity of this tumour.
In our review of the pathology of the Danish samples, we found discrepancy in 14% of the cases. The most common misclassification (21% of the misclassifications) was assessing squamous cell carcinomas as adenocarcinomas. Other frequent misclassifications were squamous cell carcinomas classified as carcinomas, carcinomas NOS classified as adenocarcinomas, and malignant tumours NOS classified as squamous cell carcinomas. Only in a few cases, adenocarcinomas were not diagnosed correctly. If we had used the hospital based diagnosis we would have had an overestimation of adenocarcinomas, because of the misclassified squamous cell carcinomas as the adenocarcinomas. Using the hospital records would have diluted the wood dust related effects, since squamous cell carcinoma is not as strongly associated to wood dust exposure [192] as adenocarcinomas are [7,141].

The mutational spectrum of wood dust

The ras protooncogene family was selected for mutational analysis in the hotspot codons 12, 13, and 61. Cancer related genes are frequently classified as either tumour oncogenes or tumour suppressor genes. Among tumour oncogenes and suppressor genes the most frequently mutated genes are the p53 suppressor gene and the ras proto-oncogene family. A shift in the frequency or spectrum of mutations in these genes can be used for understanding chemical carcinogenesis. Different carcinogens are associated with a variety of mutation spectra in the p53 gene and the ras genes. As an example, ozone exposure produce only a marginal increase in the the cancer frequency in animal models, but an evaluation of K-ras mutations revealed two chemical-specific mutations (CAA→CTA in codon 61 and increased number of G→T transversions at codon 12), indicating that ozone causes both direct and indirect DNA damage [101]. In humans, G→T transversions at codon 249 in the p53 gene are observed in individuals who are heavily exposed to aflatoxin B1 or Hepatitis B virus [197].

Based on the previous findings of K-ras mutations in the smaller studies [95,192] the p53 and the ras genes were chosen to be examined for mutations. As this PhD project is a part of the WOOD RISK collaboration, it was decided, that I should do the ras gene analyses, while the Finnish group should perform the p53 mutational analyses. The aim was to screen both the K-ras and H-ras for mutations in the activating codons 12, 13, and 61. In the initial set up we had decided on using direct sequencing. This method is time and work consuming, but is regarded as the gold standard for the verification of mutation [198]. In order to increase the
sensitivity we decided to use an additional method not based on sequencing as well. The choice was set on RFLP, a commonly used assay in detecting point mutations. Screening codon 12 by RLFP revealed several potential mutations which then were confirmed by an additional sequencing of the non-transcribed DNA-strand. However, the mutations proved to be difficult to separate from the background in direct sequencing. This was mainly due to a high background spectrum which is often observed, using DNA extracted from PET samples. I therefore chose to combine the two methods and focus the efforts on the K-ras gene. Because of limited time resources I chose to use already existing RFLP assays. For the K-ras codon 13 only an assay without an internal restriction control site was available. It had, however, previously been used with success in our laboratory [95]. In order to avoid false positive mutations the presence of an internal control site is recommended.

The mutational analysis of the sinonasal cancers revealed only a limited numbers of mutations. All the mutations were among the Danish workers (manuscript II). Among the Danish samples, 13 percent of the adenocarcinomas and one percent of the squamous cell carcinomas were mutated in the K-ras gene. The frequency of K-ras mutations in sinonasal squamous cell carcinomas were determined for the first time, but the frequency for the adenocarcinomas are similar to the 14 percent reported previously by Saber et al [95] and the 13 percent reported by Yom et al [98]. Among the Finnish cases, no mutations were found. However, the mutational frequency varies from study to study. An Italian study [99] reported of a mutation frequency of 50 percent of the ras gene among 17 intestinal type adenocarcinomas. The difference in frequency between the studies could be due to difference in the occupational/environmental exposures. Unpublished data from the mutation analyses of the p53 gene from our patients shows a significant lower frequency of mutations among the Finnish patients than among the Danish and French patients (personal communication Reetta Homilia, Finnish Institute of Occupational Health, Helsinki). This supports that the differences in the prevalence of mutations might be related to differences in environmental/occupational exposures rather than detection methods. The smoking prevalence among the interviewed Finns (69 percent) was lower than among the interviewed Danes (82 percent). In contrast, the prevalence of wood dust exposure was higher among the Finnish cases (35 percent) compared to the Danish cases (22 percent), without taking into consideration the difference in exposure assessment. But other occupational/environmental
differences between the countries could influence the mutation rate as well, since wood dust was only non-significantly associated with having an adenocarcinomas by an odds ratio of 3.6, which was reduced when adjusting for smoking and formaldehyde. The environmental (mainly smoking) and occupational factors (e.g. formaldehyde, nickel, chromium and dusts), which were included in the interviews did not differ between the two countries in a way that could explain the difference in the K-ras mutations. It is more likely that a combination of several environmental/occupational factors is responsible for the difference in the K-ras mutation rate between the Finnish patients and the Danish patients.

As described in manuscript II, only four of the patients with mutations had been occupationally been exposed to wood dust, three working as furniture makers and one working as a sawmill truck driver. The remaining patients three had no record of occupational exposure to wood dust working with servicing of machines, trench-digging machine operator and on one patient no information about the occupational history could be obtained. With the limited number of mutations found in this study, no specific mutation spectrum relating to wood dust exposure could be identified.

In order to identify a wood dust induced fingerprint, all available mutations from sinonasal adenocarcinomas was included in a pooled analysis. The previous published mutations are listed in table 17.

<table>
<thead>
<tr>
<th>Table 17. K-ras codon 1213 mutations from sinonasal adenocarcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mutation frequency</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Saber et al [95]</td>
</tr>
<tr>
<td>Pérez et al [96]</td>
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<tr>
<td>Yom et al [98]*</td>
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<tr>
<td>Frattini et al [99]</td>
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</tbody>
</table>

* In this study no exposure data are linked to the patients and therefore not included in manuscript II. \(^\text{1}\)Two of the patients contained double mutations in both codon 12 and 13, the numbers in brackets are the second mutation.

The mutation data from the present study and those previously published, suggests that cancers harbouring K-ras mutations represent only a small fraction of the sinonasal adenocarcinomas. The mutations were primarily located at codon 12 (16/20), and were in 85
percent of the cases G→A transitions (17/20). The primary amino acid change was glycine to aspartic acid, causing the K-ras protein to be in a constitutive active state [82]. Despite the limited number of mutations in the K-ras gene, the high frequency of G→A transitions may suggest an environmental component involved for this subgroup of sinonasal cancers. In B6C3F1 mice K-ras codon 12 G→A transitions were predominant in chemically induced lung neoplasm’s [199]. Occupational exposure to wood dust could not alone explain the high frequency of G→A transitions, since the frequency of G→A transitions did not differ between the wood dust exposed patients and non-exposed (Fisher exact, P = 0.25). However, among our eight patients with K-ras mutations two had been occupationally exposed to diesel particles working with servicing of machines and as a trench-digging machine operator. These types of particles are also known to cause mutations [31]. Leather dust and other organic dust, also epidemiologically associated to sinonasal cancer was common the other K-ras mutation studies [95] [96] [98] [99]. It could therefore be hypothesized that these dusts cause the same type of mutation by a similar mechanism as wood dust. Also environmental chemical exposures like tobacco smoke including second hand smoking, known to cause G→A transitions [107,200,201,201], may also play a role in the carcinogenesis of this small subpopulation of sinonasal cancers.

Unpublished data from our partner in Finland show frequent TP53 mutations detected by CE-SSCP analysis. TP53 was mutated in 277 of the 358 (77.4 percent) cases (with 477 mutations detected altogether). The risk of a TP53 mutation increased with the duration and cumulative level of wood dust exposure. The risk of a mutation increased with the duration of exposure, with a statistically significant 8-fold elevated risk among those exposed to wood dust for more than 42 years. These results suggest that TP53 is frequently involved in the carcinogenesis of sinonasal cancer, which could be one of the explanatory factors for the limited role of K-ras.

Despite the limited role of K-ras mutation in the carcinogenesis of sinonasal cancer, our study of the occupational history revealed that exposure to wood dust was associated with an 21-fold increased risk of having an adenocarcinoma than a squamous cell carcinoma compared to unexposed [OR=21.0, CI= 8.0-55.0]. In the literature a pooled case-control study by Demers et al [4] found an increased risk for having an adenocarcinoma for males employed in wood-related occupations [OD=13.5, CI=9.0-20.0]. They also found no increased risk for squamous
cell carcinomas in relation to occupational exposure to wood dust. In a more recent Swedish cohort study a SIR of 13.7 was found for having an adenocarcinoma for workers exposed to wood dust. In a Norwegian case-control study [134], squamous cell carcinomas was associated to soft wood exposure. In a pooled analysis on eight case/control studies by Mannetje et al. [135] wood dust exposed adenocarcinomas (74.2 percent were five times more common than wood dust exposed squamous cell carcinomas (14 percent). Our results are overall in concordance with the already published data, showing an overrepresentation of wood dust exposure among adenocarcinoma patients. This association between exposure to wood dust and having an adenocarcinoma, together with the risk of TP53 mutation, and a correlation with the duration and cumulative exposure to wood dust [202], supports that wood dust is involved in the carcinogenesis of sinonasal cancer.

**Inflammation and cancer**

The mechanisms behind sinonasal cancer are poorly understood. However, there is substantial evidence for chronic inflammation and malignant development in general are causally linked [203]. An inflammatory response after wood dust exposure was seen in our *in vitro* model [185] as well as after *in vivo* installation in mice [204]. Infiltration of lymphocytes and neutrophils into the lungs as well as increased content of cytokines was observed after *in vivo* exposure to wood dust. An elevated release of ROS has been measured after both *in vivo* and *in vitro* exposure [205][204]. In addition to ROS leukocytes release reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, which too can generate mutagenic adducts [203]. Despite that most DNA-damaging effects of ROS and RNS are non-specific it is also known that some cellular responses are affected by specific ROS/RNS. K-ras mutations are often associated with ROS, whereas nitric oxide is mostly associated with the generation of TP53 mutations [203]. In human colon cancer a strong relationship between the presence of NOS2 and C to A transitions at CpG sites was established. Enhanced NOS2 expression is linked to the chronic inflammatory state in ulcerative colitis, hemochromatosis and Wilson disease [206]. A study of gallbladder cancers from patients with a very high frequency of chronic cholecystitis showed K-ras codon 12 mutations [207]. Since chronic bronchitis caused by chronic inflammation have been observed frequently in wood dust exposed workers [117-119], we hypothesised that a special mutational spectrum could be found in sinonasal cancers. However, we were not able to detect a mutational pattern in our few K-ras mutations,
so this hypothesis has not been proven. Despite the missing link of a mutational spectrum in K-ras, several of our results from the WOOD RISK project still indicate that chronic inflammation could play a role in the carcinogenesis of wood dust induced sinonasal cancer. We had a high frequency of TP53 mutations and some K-ras mutations, in the sinonasal cancers. We saw a release of ROS in animals models [205] [204]. Elevated expression of the COX-2 enzyme, which has been implicated in carcinogenesis, in human sinonasal adenocarcinomas (squamous cell carcinomas, had a some what lower expression) were associated with exposure to wood dust and a non-smoking status [202].

Effects of different wood dusts
Before the onset of the WOOD RISK programme the literature was very limited on inflammatory effects of wood dust, we therefore measured the expression and level of IL-6 and IL-8. The results from the study indicated that wood dust can cause an inflammatory response in epithelial cells and the size of the response depends on the wood species. The results from this study are in accordance with previously published studies [72,192].

The results suggest that the dusts of teak, MDF, and birch are more inflammatory than dusts, from pine, spruce, beech, and oak. Interestingly, these materials are not usually been pointed out as harmful to the health of exposed workers. The inflammatory potency seems to be intermediate for the typical softwoods spruce and pine, whereas the woods that have received most attention for being linked to cancer in wood workers, beech and oak, were less inflammatory. Other studies with controlled exposures of wood dusts in relation to their inflammatory potential have been limited to a few in studies on mouse macrophage RAW 264.7 cells [72,192]and alveolar macrophages from Sprague-Dawley rats[205]and a single study on humans[208]. The results from these studies are, however, in accordance with our findings. In a recent study by Maatta et al. [204], repeated intranasal installations with 50 µg of birch or oak dust in 50µl PBS were given to BALB/c mice. The doses were administrated twice a week for three weeks. Both species of wood dusts caused lung inflammation which was accompanied by the induction of the pro-inflammatory cytokines. In the study, oak dust seemed to have a marginally higher inflammatory potential than birch dust, without this being statistically significant. In an additional study, oak dust was tested in non-allergic and allergic mice by a similar exposure protocol. The study demonstrated a differential effect of wood
dust exposure between allergic and non-allergic mice. However, both types of mice had increased influx of macrophages and neutrophils as well as increased production of pro-inflammatory cytokines [209]. In the human inhalation study, 11 healthy volunteers were exposed to airborne concentrations of approximately 5 mg/m³ pine wood dust for 1 hour. Cells of vital importance (eosinophils, mast cells, and lymphocytes) for the development of an asthmatic inflammatory reaction were elevated in numbers in the recovered bronchoalveolar lavage fluid [210].

The limited numbers of studies published with a controlled experimental setup with wood dust all identify an inflammatory reaction after exposure to wood dust. The potency of the species of wood did vary between the studies depending on the duration of wood dust exposure and model used. *In vivo results* are influenced by the interaction between cell types and clearance from the nose and lungs. This might explain the small inconsistencies between the results from the *in vitro* and the *in vivo* exposures. However, although there is a need for more data, there seems to be reasonable coherence between the *in vitro* and the *in vivo* data and the epidemiological evidence with regard to non-carcinogenic effects in the respiratory tracts, where practically all types of wood are able to cause pulmonary symptoms [211].

There are only a limited number of studies, focusing on the DNA damaging effect of wood dusts. In this thesis DNA strand breaks were observed primarily after 3 hours of exposure to the wood dusts, after 6 hours of exposure the increase in strand breaks was no longer present. The DNA damaging potential depended on the species of the wood. Birch, beech, teak, pine, and MDF are the wood species which caused a significant increase in DNA strand breaks.

In some of the few *in vitro* studies performed on wood extractives in the shape of dried condensates or ethanol and methanol extracts were used to investigate the genotoxicity. In an *in vitro* study of human embryonic lung cells (MRC-5) chromosome aberrations were found after exposure extracts from beech and oak, but not after pine [212]. The cytotoxicity and genotoxicity for Southern yellow pine, Eastern white pine, Douglas-fir, and Red oak were examined using the condensates from wood chip drying in a closed laboratory extraction – drying facility at 121°C. Genotoxic and cytotoxic effects were observed for the fir and pine
wood species at concentrations of 0.1 to 1 µl/ml medium of the condensates. However, no effect was seen after exposure to the oak dust [213-217].

Studies of more epidemiological character have also been conducted. These studies measured biomarkers for cancer risk on exposed workers. In a Polish study, workers from a wooden furniture plant had increased amounts of DNA single strand breaks in peripheral lymphocytes. The plant mainly used pine, beech and oak tree in their production [218]. White blood cells taken from polish workers at another wooden furniture manufacture confirmed a increased level of DNA damage [219]. In a Turkish study of workers in a wood working shop increased rates of micronuclei could be detected in exfoliated buccal mucosa cells from both non-smokers and smokers [220]. The wood working shop used a mixture of soft and hardwood, but no formaldehyde or solvents. Finally, a Finnish study found chromosome aberrations in peripheral lymphocytes of workers employed in plywood production processing pine, spruce, and birch [221]. All in all, the studies conducted so far show a genotoxic potential for exposure to wood dust, but the knowledge of the underlying mechanisms is still sparse.

When the wood dusts was ranked by the magnitude of effects in the present study (manuscript I), the DNA damaging potency and the cytokine expression levels did not seem to correlate. After three hours, the DNA damage was greatest for birch and beech, whereas only birch was among the most potent inducer of interleukins. On the other hand, the most potent inducers of interleukins were not very DNA damaging. DNA damage in form of DNA strand breaks were in the present study (manuscript I) detected before the pro-inflammatory cytokines had reached their maximum. These primary DNA damages might be linked to secondary metabolites of the woods, since some of these have genotoxic [30] and cytotoxic [33] potentials. However, these metabolites vary in concentration not only among wood species but also among individual trees depending on the trees conditions for growth.

We concluded in manuscript I that the DNA damage was not a result of the inflammatory response observed after wood dust exposure. However, our experimental setup was not suited for detecting secondary genotoxicity as a result of ROS release from immigrating neutrophils. In vivo an increase in the same pro-inflammatory cytokines would trigger an immigration of neutrophils into the affected area for example the lungs. The ROS released
from the neutrophils are able to cause DNA damage and thereby inducing mutations. DNA
damage and mutations have been observed after various exposures to particles, some of the
effects observed are attributed to the chemical compounds adsorbed to the core of the particle
and some to the primary carbon particle it self. In recent studies diesel particles were found to
induce inflammation, DNA damage and mutations both \textit{in vitro} and \textit{in vivo} [31,222]. The
inert particle carbon black (a particle with very small amounts of polycyclic aromatic
hydrocarbons adsorbed to the surface) increased both DNA strand breaks and mutations after
repeated exposures \textit{in vitro} [223]. This suggests that not only adsorbed chemicals influence
the processes towards carcinogenicity, but other factors like inflammation could play a role.
The carcinogenic effect of wood dust are could probably to be a combination of both direct
chemically induced genotoxicity and inflammation induced release of ROS causing DNA
damage, but more research are need for a conclusive statement.
CONCLUSION AND PERSPECTIVES.

The molecular mechanisms behind the carcinogenic effect of wood dust, especially in relation to wood dusts ability to induce DNA damage and inflammation, were the major themes in this thesis. This project was a part of the EU funded WOOD RISK project, aimed to assess the exposure to wood dust and investigate the biological mechanisms behind wood dust related diseases.

The WOOD RISK project did result in an exposure assessment, where it was estimated that about 3.6 million European workers were exposed to wood dust. The exposure level to hardwood dust was usually below 5 mg/m³, (the OEL of the EU), but approximately 0.5 million workers may be exposed to a dust levels (any type of wood dust) exceeding 5 mg/m³. These finding suggests that wood dust related health effects affect a large population of workers.

Despite that epidemiological studies consistently relate wood dust exposure to illnesses like chronic bronchitis, asthma and nasal cancer, knowledge about the underlying biological mechanisms are almost non-existing. In this study, we show for the first time that pure wood dust is able to cause primary DNA damage, independent of inflammation. This conclusion is based on the finding that exposure to wood dust resulted in DNA strand-breaks for four out of seven wood dusts examined, before inflammation had reached its maximum. However, secondary genotoxicity due to the release of ROS from immigrating neutrophils can not be ruled out, since this was not investigated in our model. This aspect would however be very interesting in the light of our results and our Finnish partners’ results with wood dusts ability to cause inflammation in animals [73].

In our study, an inflammatory response was identified for the first time after exposure to pure wood dust in a human cell line. We were able to compare the potential of several wood species and found contrary to what was expected, that hardwoods were not more inflammatory than softwoods. Our Finnish collaborator in the WOOD RISK project confirmed the patterns of inflammatory responses in both a murine macrophage cell line (RAW 264.7) [72,192] and after in vivo installation in mice [73]. Based on the inflammatory
potential of wood dust and findings like high levels of COX-2 in sinonasal cancers [224] and a higher frequency of chronic bronchitis in workers occupationally exposed to wood dust [7], makes it possible that chronic inflammation also are an important player in wood dust related sinonasal cancer. However further research is needed for a conclusion.

The carcinogenesis of sinonasal cancer has been linked to wood dust exposure in epidemiological studies, however knowledge about molecular mechanisms are almost non-existing. Previous small studies showed that K-ras mutations occur in sinonasal cancers. In an attempt to identify a mutational fingerprint after wood dust exposure, we therefore examined all incident cases of sinonasal cancer in Denmark 1991-2001 for K-ras mutations. We found that for the patients in this study, exposure to wood dust was associated with a 21-fold increased risk of having an adenocarcinoma than a squamous cell carcinoma compared to unexposed [OR=21.0, CI= 8.0-55.0]. Mutational activation of K-ras was restricted to a small subgroup of the Danish adenocarcinomas (13%), and very limited among squamous cell carcinomas (1%). Wood dust exposure among the patients with activating K-ras mutations was frequent (50 percent) in paper II. Collectively, taking into account our present results and all published K-ras mutations in sinonasal cancers, the GGTGLY →GAT ASP transition was the most common mutation. A large proportion of the mutation positive cases had been exposed to wood dust occupationally, although no statistically significant correlation was found. However, since many of the mutation positive cases were smokers, exposure to cigarette smoke, including involuntary smoking may also be involved. Contrary to mutations in the K-ras gene, mutations in the TP53 are associated with exposure to wood dust. Overall, the data presented in this thesis suggests only a limited role for K-ras mutations in development of sinonasal cancer.

Overall, wood dust is a very common occupational exposure. All tested wood species so far causes inflammation, but varies in their potential. The present in vitro experiments suggests that wood dust possess a genotoxic potential independent of inflammation. But wood dust induces inflammation both in vitro and in vivo, and is epidemiologically associated with illnesses relating to chronic inflammation. In the carcinogenesis of sinonasal cancer chronic inflammation might also be a possible key player as well.
In order to fully understand the biological mechanisms behind the effects of wood dust, further studies are needed. It is continuously problematic that so far no one has been able to induce sinonasal cancer in animal models as a result of wood dust exposure. This could be due to large anatomical difference between animals and humans. Because sinonasal cancers epidemiologically are strongly associated to wood dust, continuous research in the mechanisms behind the carcinogenesis is important.

One approach in continuation of the recent research could be to characterize and purify the bioactive components from wood dust and examine their effects in relation to inflammation and genotoxicity, e.g. in the nasal cell line RPMI 2650 (CCL-30) since it has now been characterized in relation to inflammation or in an in vivo inhalation model. The mutagenesis of wood dust under controlled conditions could also be very interesting to investigate. The MML cell line which derives from the genetic modified MutaMouse™ is a potential assay. Using this cell line one would be able to investigate whether the increased numbers of DNA strand breaks seen in manuscript I lead to mutations. To establish the role of inflammation more accurately it would be interesting to look for oxidative damages like the adduct 8-oxo-guanin or oxidation of proteins. Based on the inflammatory response found in mice, genotoxicity and mutagenicity would be interesting to investigate in vivo as well, including the use of the MutaMouse™ and/or BigBlue™ rats. Since inhalation is the natural way of exposure, an inhalation model provides a more realistic route of exposure and opens up to investigations of the sinonasal cavities for inflammation, genotoxicity, and mutagenicity.

It is still problematic that it has not been possible to induce sinonasal cancer in animals after wood dust exposure. Seen in that light it would be interesting to examine molecular biomarkers relating to inflammation, genotoxicity and mutagenesis in humans, but ethical considerations have to be taken into account at the design of such studies. Since we now have a unique collection of PET samples from well characterized sinonasal cancers, in regard to histology and exposures, it could be interesting to further use it for the investigation of the mechanism of carcinogenesis. A new technology present at the department of pathology in Vejle, enables us to punch out microscopic samples from the PET samples and transfer them to one PET sample block containing up to 100 samples. Immunohistochemical stainings for a
large number proteins linked to inflammation, DNA damage and carcinogenesis, could be performed fast and at a reasonable cost.

During the WOOD RISK project, we found a piece of the puzzle. However, there is still a long way to go before the mechanisms behind wood dust related diseases especially, the carcinogenesis is fully understood. Meanwhile many workers all around the world are daily exposed to wood dust.
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