General introduction
LUNG CANCER

Epidemiology and etiology
For several decades, lung cancer is the most common cancer worldwide. In 2012, global lung cancer incidence was estimated at 1.8 million new cases (13% of all cancer cases), being the most commonly diagnosed cancer in men and the third most common cancer in women following breast and colorectal cancer. The estimated death rate of 1.6 million cases (20% of all cancer deaths) is the same year that lung cancer is also the leading cause of cancer-related death in the world. In the Netherlands, 11,967 lung cancer diagnoses and 10,322 lung cancer-related deaths were recorded in 2012.

Since the 1980s, the burden of lung cancer among males is declining in most developed countries, including the Netherlands. In contrast, incidence continues to rise in many developing countries among females and in Asia and Africa, particularly in males. Since tobacco smoke exposure accounts for 80-90% of all lung cancers, disease rates gradually follow smoking prevalence among the population with a lag time of about 30 years. Therefore, the observed trends largely reflect spatio-temporal changes in tobacco consumption with female peak rates lagging behind male peak rates for several decades, and more recent peak rates in Asian and African regions lagging behind those of Western regions.

Besides smoking, main risk factors for lung cancer include second-hand smoke, long-term exposure to occupational agents, including asbestos, arsenic, chromium, nickel and radon gas, and in-/outdoor air pollutants. A contributory role of certain viral agents, such as high-risk human papillomavirus (hrHPV), to the process of lung carcinogenesis has been implicated as well, however, this etiological link remains controversial.

Staging, prognosis and therapeutics
The prognosis of lung cancer patients is primarily based on stage of disease at time of initial diagnosis. The stage of disease can be determined using the 7th edition of the Tumor-Node-Metastases (TNM) classification for lung cancer. The TNM classification is based on evaluation of the primary tumor size and spreading of the tumor into adjacent tissues (T descriptor), regional lymph node involvement (N descriptor) and the presence of distant metastases (M descriptor). The TNM descriptors are grouped together in several stages, ranging from stage 0 (carcinoma in situ) and stage IA (tumor ≤ 3 cm without lymph node involvement and/or distant metastasis) to stage IV (tumor with distant metastasis).

For lung cancer (Table 1), the 5-year survival rates are progressively declining from ~50% in case of localized disease (Stage I-IIA disease), to ~20% for locally advanced disease (Stage IIB-III) and <5% for advanced disease (Stage IV). Importantly, the prognosis of in situ lesions (Stage 0) is excellent (> 90%). Since the disease causes no or only non-specific symptoms during its early stages, the majority of lung cancer patients are diagnosed with (locally) advanced disease after symptoms have become manifest, resulting in an overall poor prognosis of the disease with a 5-year survival not exceeding 17%.

Complete resection represents the mainstay treatment for patients diagnosed with early-stage lung cancer (Stages I-II). The standard treatment regimen for patients who present with more advanced, non-resectable disease (Stage III-IV) consists of a combination of chemo- and/or radiation therapy. Recently, a number of chemotherapeutic agents have been introduced that target specific genetic aberrations in advanced-stage lung tumors, for instance in EGFR and ALK genes.

### Classification of lung cancer

In general, lung cancers are classified according to their histological appearance into one of two main categories: small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC). SCLC, which accounts for ~15% of all lung cancer diagnoses, originates from neuro-endocrine cells that reside in the epithelial linings of the main and lobar bronchi and is typically localized in the peribronchial tissue compartment. NSCLC (85% of all lung cancer cases) represents a heterogeneous group of histological subtypes. The three major NSCLC subtypes include adenocarcinoma (AdCa), squamous cell carcinoma (SqCC) and large cell carcinoma (LCC). Of these, AdCas originate from bronchiolar or alveolar cells (peripheral lung). SqCCs were traditionally thought to originate from the bronchial epithelial cells (central lung). However, recent reports indicate that an increasing percentage of SqCCs are found in the peripheral compartment of the lungs. The origin of the large cell carcinomas can be found either within the central or peripheral compartment of the lungs.

<table>
<thead>
<tr>
<th>Stage</th>
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<th>N</th>
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<td>Tis</td>
<td>N0</td>
<td>M0</td>
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<td>M0</td>
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<td>N1</td>
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<td>IV</td>
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Classification of pre-invasive lesions

Besides cancer categorization, the current histological classification system for lung cancer - as introduced by the World Health Organization (WHO) and the International Association for the Study on Lung Cancer (IASLC) - also recognizes distinct histopathological entities defined as pre-invasive lesions for lung cancer.\[32,35\]

SqCCs are believed to arise through a sequence of histologically defined precursor stages (Figure 1). Among the earliest microscopically visible changes within the bronchial epithelium, hyperplasia (H; increased proliferation of cells) and squamous metaplasia (SQM; cellular transition from non-squamous to squamous morphology) are generally considered as reversible events that reflect morphological manifestations of repair responses to airway tissue damage caused by exposure to exogenous factors, e.g. tobacco smoke. However, occasional progression to SqCC has been noted for these lesions. The sequence of precursor changes is continued by squamous dysplasia and carcinoma in situ (CIS), lesions that are recognized as true cancer precursor lesions. Squamous dysplasia can be categorized as mild (miD), moderate (moD) or severe (SD), depending largely on the degree of cytonuclear atypia in the multilayered squamous epithelium. Whereas miD is characterized by mildly atypical cells that are confined to the lower one-third of the epithelium, SD is recognized by minimal cell maturation and a high degree of cellular atypia that has spread into the upper third of the epithelium without reaching the luminal surface. In case of CIS, cells show extreme cytological abnormalities and cellular involvement has extended throughout the full thickness of the epithelium, but has not infiltrated the basement membrane.

The development of peripheral AdCa is less well-defined, but AdCa is thought to be preceded by atypical adenomatous hyperplasia (AAH; focal proliferation of slightly atypical cells lining alveoli and/or bronchioles) and adenocarcinoma in situ (AIS; small tumor ≤ 3 cm) with an exclusive lepidic, non-invasive growth pattern (Figure 1).

**FIGURE 1 Schematic overview of pre-invasive lesions for lung cancer.** Histological changes during the development of squamous cell carcinoma (upper panel) and adenocarcinoma (lower panel) are shown as recognized by the WHO classification of lung tumors. Figure was adapted from Fagot et al. \[33,35,43\].

### Prevention Strategies

**Primary prevention**

Since most lung cancers are directly attributable to inhalation of tobacco smoke, decreasing the (second-hand) exposure to these substances is clearly the most effective primary prevention strategy to reduce the burden of lung cancer. In 2003, the WHO Framework Convention on Tobacco Control (FCTC) was developed to cope with the dramatic spread of tobacco consumption worldwide. The WHO treaty included measures that restrict use of tobacco products in indoor public places, to raise public awareness of the harmful effects of smoking, and to reduce tobacco demands (tobacco taxation, introduction of smoking cessation programs) and tobacco sales (restricted sales to minors, limited advertising, warnings on packages). These measures are now generally accepted as an internationally standardized set of guidelines for tobacco control policy in many countries, including the Netherlands. Nevertheless, in spite of these measures, 25% of all Dutch people (≥ 15 years of age) nowadays still use tobacco on a regular basis. Together with the fact that 30% of the current Dutch population consists of former smokers, a substantial number of future lung cancers are still to be expected from the (ex-)smoking population due to their elevated lifetime risk. Therefore, additional (secondary) preventive strategies, such as screening for early lung cancer/early detection, are needed in order to control disease rates.

**Screening and early detection**

The inverse relationship between patient prognosis and disease stage at time of diagnosis implicates the urgent need for early lung cancer detection strategies. Implementation of early detection techniques in screening of individuals at risk is expected to result in identification of earlier stage (pre-)invasive lesions that are still eligible for curative treatment, and therefore are potentially of benefit in reducing lung cancer mortality rates. Several early detection strategies have been investigated, but thus far none has proven to be effective for mass screening purposes. In previous studies, the use of chest radiography (CXR) - either alone or in combination with sputum cytology - has been proposed as a means of non-invasive screening. Although several randomized controlled trials (RCTs) in the 1970s and 1980s showed slight improvements in terms of disease stage distribution, resectability and short-term survival in screened vs. control groups, this approach was not able to demonstrate reduced lung cancer mortality. Due to shortcomings in study number and design in these early RCTs, a large-scale and methodologically rigorous trial has been set up more recently to definitively investigate CXR screening. This trial further en-forced previous findings by showing its failure of efficacy in screening.

During recent decades, new technological advancements such as low-dose spiral computed tomography (LDCT) and autofluorescence bronchoscopy (AFB; further discussed in the next paragraph) have resulted in renewed interest in early lung cancer detection strategies. LDCT allows for more sensitive detection of small peripheral lung cancers as compared to chest radiography. Several observational studies have shown the superior sensitivity of LDCT, with higher lung cancer detection rates and detection of disease at earlier, resectable stages.
then, multiple large-scale RCTs have been initiated to assess the efficacy of LDCT in lung cancer screening\(^{65-70}\). The first results have now been released from the US National Lung Screening Trial (NLST), which showed a significant reduction (20%) in lung cancer-specific mortality in LDCT-screened vs. CXR-screened participants\(^{71}\). Nevertheless, high numbers of false-positive findings, potential overdiagnosis bias and possible harm to screening participants due to excessive radiation exposure are problems faced in NLST. Confirmation of efficacy of LDCT screening awaits results from ongoing European trials, including the world’s second-largest Dutch-Belgian Lung Cancer Screening Trial (NELSON)\(^{72}\), which are expected to be released in the upcoming years.

### Autofluorescence bronchoscopy

LDCT imaging technology is unable to detect early (pre-)invasive lesions in the central airways. The introduction of white light flexible fiberoptic bronchoscopy (WLB) has enabled visual inspection of the central airways. However, regardless of the technological improvements that have led to state-of-the-art videobronchoscopy systems, conventional white light imaging for detecting pre-invasive and early invasive endobronchial lesions has only low sensitivity for detecting pre-invasive lesions (pooled sensitivity of ~65%)\(^{73,74}\). Autofluorescence bronchoscopy (AFB) is a more advanced endoscopic technique that is able to visualize subtle differences in fluorescence and absorption properties between normal and abnormal (i.e., squamous metaplastic and dysplastic) bronchial epithelium by utilizing an alternative light source (e.g. helium-cadmium laser or xenon lamp). Illumination of the bronchial mucosa at a wavelength of 395–445 nm (blue light) will cause autofluorescence at a wavelength of 460–700 nm (green to brownish red), depending on the degree of abnormality of illuminated tissues. Whereas normal areas will appear green, abnormal areas will appear brownish red owing to changes in the composition of the endobronchial epithelium (e.g. increased thickness of the bronchial epithelium, changes in cellular density and changes in the molecular contents of the extracellular matrix)\(^{73-75}\). Thus, autofluorescence imaging facilitates the detection of early (pre-)invasive lesions (pooled sensitivity of AFB is ~90%), which would otherwise remain undetected during WLB procedure (Figure 2)\(^{76,77}\). However, the increase in sensitivity of AFB comes at the expense of a loss in specificity as compared with WLB (pooled specificity of AFB versus WLB is ~60% vs. ~80%)\(^{75}\), which is mainly due to the fact that AFB has difficulties in differentiating early pre-invasive lesions from airway inflammatory changes\(^{78}\). This results in unnecessary biopsies and prolonged procedural time, which negatively impacts the cost-effectiveness of this technique. Recent technological developments, such as narrow band imaging\(^{79}\) and optical coherence tomography\(^{80,81}\), may further enhance the diagnostic specificity of AFB and WLB for detecting pre-invasive lesions without compromising the sensitivity.

**FIGURE 2 Imaging of a pre-invasive endobronchial lesion.** Appearance of a pre-invasive lesion (indicated by white arrow) under a) white light imaging and b) autofluorescence imaging. Figure was adapted from\(^{85}\).

### PRE-INVASIVE ENDOBRONCHIAL LESIONS

#### Prevalence of pre-invasive endobronchial lesions

The relative ease of tissue procurement from the central airways has enabled several studies investigating SqCC precursor lesions. Auerbach et al were the first to describe these epithelial changes in autopsy studies and showed that pre-invasive endobronchial lesions were more frequently found in smokers than in non-smokers and that the frequency of epithelial changes increased along with increasing smoking intensity\(^{82,83}\). More recently performed bronchoscopy studies demonstrated a prevalence of 1-5% for CIS lesions, 25-60% for dysplasias and 30-50% for lower-grade lesions amongst groups of heavy smokers (≥ 20 pack-years; smoking at least 20 cigarettes/day for twenty years)\(^{84,85}\). Lesion severity was found to be related to the extent of tobacco exposure with more high-grade pre-invasive lesions (SD and CIS) observed in individuals with highest smoking intensities and a gradual decrease of higher-grade lesions seen in individuals following smoking cessation\(^{86-88}\).

#### Natural history of pre-invasive endobronchial lesions

The stepwise progression model of squamous cell lung carcinogenesis is mainly based on cytopathological findings in serially collected sputum samples\(^{89,90}\) and histopathological findings in cross-sections of the tracheobronchial tree\(^{91,92}\). However, sputum cells and cross-sections (can) originate from different parts of the tracheobronchial tree and therefore do not necessarily reflect progression of a single pre-invasive lesion. In an attempt to further clarify the natural history of pre-invasive endobronchial lesions, several longitudinal studies using serial AFB with repeated biopsies from the same site(s) have been performed. However, comparison of
in patients with exclusively low-grade lesions and risks of 33% (one year) and 75% (five years) in patients bearing high-grade lesions. In both studies the majority of detected cancers developed at a remote site rather than at the site of the preexistent precursor lesion. This is consistent with the concept of field cancerization, in which the entire airway epithelium is at risk of developing malignancy. The abovementioned findings and the fact that the majority of detected cancers in these studies were early-stage lung cancers (Stage I-II) suggest that combined surveillance (AFB + CT) may serve as an interesting approach to facilitate early detection of lung cancer in individuals harboring pre-invasive endobronchial lesions.

**Molecular alterations**

As described above, classical histomorphological characteristics fail to make an accurate distinction between lesions that do progress to an invasive carcinoma and those that will regress or remain indolent. The risk and rate of lesion progression, as well as the underlying mechanisms of progression or regression, are incompletely understood. Better understanding of the biology of SqCC development may help to identify pre-invasive lesions that ultimately will become malignant; thereby allowing more tailored preventive measures.

The development of cancer in general is associated with accumulation of genetic and epigenetic changes that lead to aberrant functioning of genes and/or gene products involved in cell cycle regulation, apoptosis, angiogenesis, and tissue invasion\(^{96}\). Whereas genetic changes alter the DNA sequence itself, epigenetic changes alter the accessibility of the DNA for transcription factors by modifying either bases within the DNA sequence or the histone proteins, around which the DNA is wrapped. Activation of genes that promote tumorigenesis (oncogenes) and inactivation of genes that inhibit tumorigenesis (tumor suppressor genes) can be achieved through several genetic mechanisms, including mutation, numerical and structural chromosomal aberrations (e.g. chromosomal gain and/or loss) and loss of heterozygosity (LOH), and epigenetic mechanisms such as DNA hypermethylation of gene promoter regions.

During recent years, several studies have been performed with the aim to characterize the (epi)genetic events in pre-invasive endobronchial lesions that are associated with lungSqCC development. Alterations described include TP53 mutations, hTERT overexpression, p16\(^{96,98}\) hypermethylation and LOH on chromosomes 3p, 9p and 17p\(^{100,102}\). However, most of these studies involved cross-sectional sample series, thereby not taking into account single lesion outcome\(^{100,102}\). Since only a small fraction of squamous metaplastic and dysplastic lesions eventually progress to carcinoma (in situ), longitudinal studies are necessary to shed light on the molecular events that truly drive development of these lesions to an invasive phenotype. Thus far, only few studies have investigated molecular changes in pre-invasive lesions with known clinical outcome and these have largely been restricted by low-resolution analyses or limited sample sets\(^{97,111,112}\). As a consequence, at the start of this project no molecular marker suitable for risk assessment of AFB-visualized lesions was known.
AIM AND OUTLINE OF THIS THESIS

Secondary prevention, i.e., detecting lung cancer and its precursor lesions with invasive potential at an earlier, curable stage, holds promise in reducing mortality from lung cancer. With the advent of sensitive techniques to identify pre-invasive endobronchial squamous lesions (e.g. AFB) and the presence of curative treatment modalities for early-stage disease, this approach is nowadays feasible. However, since current detection and diagnostic tools lack specificity in recognizing potentially malignant lesions among the many transient lesions found, extensive follow-up examinations and/or overtreatment of individuals presenting with AFB-visualized lesions form a major drawback for efficacy of secondary prevention. We hypothesize that molecular biomarkers allow more accurate classification of pre-invasive endobronchial squamous lesions and better identification of lesions that ultimately will progress to invasive cancer. In light of the above, we asked the following research questions. The studies that were performed to answer these questions form the basis of this thesis.

What is the risk of lung cancer in individuals with pre-invasive endobronchial lesions?
Chapter 2 describes the long-term clinical outcome of a cohort of individuals harboring pre-invasive endobronchial lesions. All study participants underwent combined surveillance with repeated AFB and LDCT to assess their risk of lung cancer, both in the endobronchial and parenchymal lung compartments. Furthermore, we sought to identify factors associated with higher risk. All individuals described in chapters 3, 4 and 5 were part of the cohort described in chapter 2.

Are molecular biomarkers, i.e. specific DNA copy number alterations, able to identify AFB-visualized squamous metaplastic lesions with malignant potential?
In Chapter 3 we describe the identification of a novel biomarker panel comprising of specific DNA copy number alterations at 3p26.3-p11.1 (loss), 3q26.2-q29 (gain) and 6p25.3-24.3 (loss) that has the potential to serve as a molecular classifier to predict endobronchial cancer risk in individuals presenting with AFB-visualized squamous metaplastic lesions.

Is the pre-defined molecular classifier based on specific DNA copy number alterations able to serve as risk predictor for endobronchial cancer in an independent series of squamous metaplastic and dysplastic lesions?
Chapter 4 addresses the independent validation of the molecular classifier defined in Chapter 3, to assess an individual’s risk for subsequent endobronchial cancer among subjects who present with endobronchial squamous metaplastic and dysplastic lesions. In addition, we evaluated the diagnostic accuracy of a simplified single gene assay.

Can free-circulating plasma DNA quantification be used as a non-invasive means to identify individuals harboring pre-invasive endobronchial lesions?
Detection of molecular biomarkers in non-invasively collected biomaterials, such as blood or sputum, might serve as a prerequisite for AFB surveillance by identifying individuals at highest risk of lung cancer. Chapter 5 evaluates whether quantification of free-circulating plasma DNA could be used to identify individuals harboring pre-invasive endobronchial lesions.

Is there a causal contribution of hrHPV infection to lung cancer in the Dutch population?
Chapter 6 addresses the role of human papillomavirus infections in lung cancer from a Dutch study population. Since a causal contribution of HPV infection to the development of lung cancer is still a matter of debate, a comprehensive study was performed to investigate a potential etiological link between HPV and lung cancer.

To conclude, Chapter 7 provides a summary of all study findings described in this thesis and puts the data into perspective by presenting a general discussion.
REFERENCES


CHAPTER 1  GENERAL INTRODUCTION


CHAPTER 1


