IMPROVING LUNG CANCER SURVIVAL *Time to move on*

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Improving lung cancer survival;
Time to move on

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Ingenuas didicisse fideliter artes emollit mores,
nec sinit esse feros.

Ovidius, *Epistulae ex Ponto 2.9.47*

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General introduction and outline of the thesis

Chapter 1

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GENERAL INTRODUCTION

1.1 Lung cancer

In 1761, lung cancer was first described as a distinct disease based on autopsies by Giovanni Morgagni.[1] In 1810, Gaspard Laurent Bayle described lung cancer in more detail in his book entitled *Recherches sur la phthisie pulmonaire*.[2] At that time it was an extremely rare disease; in 1878, malignant lung tumors included only one percent of all cancers discovered during autopsies at the Institute of Pathology of the University of Dresden in Germany. Nowadays lung cancer is the major cause of cancer deaths worldwide.[3] There are two major groups of lung cancer: non-small-cell lung cancer (NSCLC) and small-cell lung cancer, accounting for approximately 85% and 15% of lung cancer cases, respectively.[3] NSCLC can be divided into four histological subtypes: squamous cell carcinoma, adenocarcinoma, large cell lung carcinoma and undifferentiated NSCLC. Squamous cell carcinoma mostly develops from bronchial epithelial cells in the central airway, while most tumors that are not related to smoking, like adenocarcinoma, develop from basal bronchial cells and type II pneumocytes and arise in the more peripheral parts of the lung.[4]

Although the subdivision of NSCLC has no direct treatment consequences in limited disease, in advanced disease treatment choices depend on these histological differences.[5]

The 5-year survival of lung cancer is 73% for localized NSCLC and only 13% for metastasized disease.[6] One of the reasons for this extremely poor survival is that most lung cancer cases are diagnosed at an advanced stage due to the relative lack of clinical symptoms during early stages. Metastatic NSCLC is currently an incurable disease for which standard chemotherapy provides only minor improvement in overall survival. Less than 30% of unselected patients with advanced-stage NSCLC have a clinical response to platinum-based chemotherapy, which is in general considered to be the most effective first line treatment at this stage of the disease.[7]

1.2 Prognostic factors of lung cancer

1.2.1 Stage of disease
Stage of lung cancer is an important prognostic factor. The 5-year survival rates significantly differ between the different subgroups (Table 1). The stage of lung cancer is determined based on the characteristics of the 7th revised tumor, node and metastases (TNM) criteria, established by the International Associations for the Study of Lung Cancer (IASLC) (Table 2). These criteria have defined four major stages of lung cancer. The TNM classification can be subdivided in the clinical TNM stage (cTNM) and the pathological TNM stage (pTNM). The cTNM is based on the results of a chest X-ray,

Table 1 Survival of non-small cell lung cancer according to stage of the disease[70-72]

Lung cancer stage	Median survival (months)	5-year survival (%)
IA	59	73
IB	48	58
IIA	30	46
IIB	24	36
IIIA	14	24
IIIB	9	9
IV	4	13

Table 2 Lung cancer staging according to the TNM descriptor and subgroups[71,73]

T/M	Detailed T/M	N0	N1	N2	N3
T1 ($\leq$ 2 cm)	T1a	IA	IIA	IIIA	IIIB
T1 (> 2-3 cm)	T1b	IA	IIA	IIIA	IIIB
T2 ($\geq$ 3-$\leq$ 5 cm)	T2a	IB	IIA	IIIA	IIIB
T2 (> 5-7 cm)	T2b	IIA	IIB	IIIA	IIIB
T2 (> 7 cm)	T3	IIB	IIIA	IIIA	IIIB
T3 invasion		IIB	IIIA	IIIA	IIIB
T4 (same lobe nodules)		IIB	IIIA	IIIA	IIIB
T4 (extension)	T4	IIIA	IIIA	IIIB	IIIB
M1 (ipsilateral lung)		IIIA	IIIA	IIIB	IIIB
T4 (pleural effusion)	M1a	IV	IV	IV	IV
M1 (contralateral lung)		IV	IV	IV	IV
M1 (distant)	M1b	IV	IV	IV	IV

computer tomography (CT) scan of the chest and upper abdomen, a fluorodeoxyglu-cose- positron emission tomography (FDG-PET) scan, and/or a magnetic resonance imaging (MRI) of the brain, while the pTNM is based on the pathological results of a surgical resection. Because the latter is most accurate, there can be differences between cTNM and pTNM. Treatment options are determined based on the stage of the disease.

1.2.2 World Healthy Organisation (WHO) performance status
WHO performance status is next to the stage of lung cancer the most pivotal inde-pendent prognostic factor.[8] It runs from 0 to 5, with 0 denoting perfect health and 5 death (Table 3). The 5-year survival rates are significantly different between the five categories.[9,10] From this it can be concluded that patients will benefit from a diagnosis of lung cancer in an asymptomatic stage.

Table 3 WHO performance score[9]

WHO-score	Description
0	Asymptomatic (Fully active, able to carry on all activities without restriction)
1	Symptomatic but completely ambulatory (Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature)
2	Symptomatic, <50% in bed during the day (Ambulatory and capable of all self care but unable to perform any work activities)
3	Symptomatic, >50% in bed, but not bedbound (Capable of only limited self-care)
4	Bedbound (Completely disabled. Not able to perform any self-care)
5	Death

1.3 Pathogenesis of lung cancer

1.3.1 Risk factors

The most important risk factor for NSCLC is smoking, which account for approximately 85% of all lung cancer cases.[11] In 1929, the German physician Fritz Lickint already recognized the link between smoking and lung cancer, which led to an aggressive anti-smoking campaign. However, the first major epidemiological study about the link between lung cancer and smoking was published by sir Richard Doll and sir A. Bradford Hill in 1956.[12] Smoking causes all types of lung cancer but is most strongly correlated with small-cell lung cancer and squamous-cell lung cancer. Adenocarcinoma is the most common type in never smokers.[13]

Environmental factors and genetic susceptibility interact to influence carcinogenesis. Lung tissue injury, from tobacco smoke or other environmental factors, such as asbestos, initially occurs in the form of genetic and epigenetic changes, like mutations, loss of heterozygosity and promotor methylation. This can lead to changes in the lung tissue, such as inflammation. These changes can persist long term and can eventually lead to aberrant pathway activation and cellular function, like dysregulated proliferation and apoptosis. This can cause premalignant changes, including dysplasia and clonal patches and can eventually lead to angiogenesis, invasion and early-stage cancer.[14,15]

1.3.2 Genetic risk factors

Epidemiologic studies show an association between family history and increased risk of lung cancer. Consequently, multiple studies have been performed on inherited predisposition to lung cancer including study of polymorphisms associated with lung cancer risk and familial linkage studies. This provides evidence of host susceptibility. Several independent genome-wide association studies (GWAS) identified single nucleotide polymorphism (SNP) variations at 15q24-q25.1 that were associated with an increased risk of both nicotine dependence and developing lung cancer.[16-18] The

region of the SNP includes genes encoding nicotinic acetylcholine receptor (nAChR) subunits (CHRNA5, CHRNA3, and CHRNB4). More recently, meta-analyses have provided further evidence that variation at 15q25.1, 5p15.33, and 6p21.33 influences lung cancer risk.[19] It has not yet been elucidated whether there is a mechanistic association with these nAChR polymorphisms and nicotine addiction, carcinogenic derivatives of nicotine exposure, or the effect of nicotine acting on nAChRs known to be expressed in lung epithelial cells.[11]

Lung cancer susceptibility also increases when there is a reduced capacity to repair DNA, especially in combination with tobacco smoke. This reduced capacity can be due to germ-line alterations in nucleotide excision repair genes, such as ERCC1.[20] Next to this there are also inherited cancer syndromes caused by rare germ-line mutations in the p53 suppressor gene[21], the epidermal growth factor receptor (EGFR) gene[22] and the retinoblastoma gene.[23]

1.3.3 Immune system

In the process of carcinogenesis, the tumor-microenvironment and the immune system play an important role. Tumor cells can elicit a specific immune response by the host through the expression of tumor-associated antigens (TAA). TAA emerge by mutations leading to synthesis and overexpression of abnormal proteins. The immune system will recognise these TAA and can thereby discriminate between malignant cells and other cells. During the early stage of tumor development there are different immunological cell types from the innate and adaptive immune system involved in the recognition and destruction of tumors. The innate immune system, including macrophages, neutrophils, natural killer (NK) cells, NKT cells, gamma-delta T cells and certain cytokines (IL-12), plays a role in the early lines of defence, while the adaptive immune system plays a more specific role against certain tumor antigens. To generate this adaptive immune response, TAA need to be presented to the cells of the adaptive immune system. Antigen presenting cells (APC), such as dendritic cells (DC) and macrophages, can achieve this. They play a pivotal role in the presentation of antigens to cells of the adaptive immune system and can thereby lead to activation and differentiation of lymphocytes. APC are cells that originate from bone marrow precursor cells. They appear in peripheral tissues where they detect and take up foreign substances, including TAA, which are released from dying tumor cells. DC will migrate to regional draining lymphoid organs after they captured antigens. The antigens are processed and presented by major histocompatibility complex (MHC) molecules of the APC. This will lead to antigen-specific activation of lymphocytes. T lymphocytes mediate the cellular immunity, while B lymphocytes will mediate the humoral immunity, as they are able to produce antibodies. T lymphocytes can be divided into at least two major subsets: T helper cells (Th cells) and cytotoxic T cells

(CTL). Th cells can stimulate the proliferation and differentiation of B and T cells, and CTL are able to kill cells that express foreign antigens, including tumor cells.

Nevertheless, the role of the immune system in carcinogenesis is two-folded; it can either suppress tumor expansion by attacking cancer cells or it can activate tumor progression by establishing conditions within the tumor microenvironment that smooth the progress of tumor development.[24] So, there is a complex interaction between tumor cells and the immune system with both pro-tumor and anti-tumor functions.[24]

Leukocyte infiltration is an important characteristic of lung cancer and the main components of these infiltrates include T and B lymphocytes, tumor-associated macrophages (TAM), myeloid-derived suppressor cells (MDSC), regulatory T-cells (Treg), mast cells and natural killer (NK) cells.

Infiltration of mature DC (mDC), cytotoxic CD8 T-cells, M1 type TAM, CD4 T-cells, natural killer (NK) cells, neutrophils and possibly Th17 cells will lead to tumor regression. While, immature DC (iDC), regulatory T cells (Tregs), myeloid-derived suppressor cells (MDSC) and M2 type TAM, will stimulate progression of the tumor.[25-29]

1.3.4 Tumor immune escape

It has been described that during tumor development tumor cells undergo multiple changes to escape the immune surveillance, by either evading the induction of an immune response or the inhibition of anti-tumor responses via a variety of mechanisms.[30] The mechanisms that can be used are related to the tumor cells whereas others act via the interaction with the immune system. One way in which tumor cells can avoid recognition by CTL is by downregulation of the MHC class I molecules on their cell membrane, as this will lead to a decreased TAA presentation to T cells. Tumor cells can also down-regulate the expression of TAA, so there will be no recognition of foreign antigens by APC. In addition, the TAA can be presented by APC to lymphocytes in a tolerogenic form, which will lead to specific immunologic tolerance. Next to this, antigents on tumor cells can be hidden from the immune system by glycocalyx molecules, such as sialic acid-containing mucopolysaccharides. Tumor cells can also induce immunologic tolerance through the expression of programmed death ligand-1 (PDL-1), which can inhibit T cells through interaction with the negative co-stimulatory receptor PD-1. Another tumor escape mechanism is the production of soluble immunosuppressive mediators, including prostaglandin (PGE), interleukin (IL)-10, IL-23, vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-β. These factors can directly inhibit immune responses, by the suppression of APC and T cells. [30]

Many of the tumor-derived factors listed above are suppressive and in this way create an immunesuppressive environment or they can indirectly induce immune

suppressor cells, such as Treg, M2 type TAM or MDSC. The physiological role of these cell types is to prevent autoimmunity.[31] However, cancer cells take full advantage of these cells.

1.4 Early detection of lung cancer

1.4.1 CT-screening

Lung cancer screening aims to detect lung cancer at an early disease stage to improve survival of this devastating disease, as the chance of curative treatment is higher in limited disease. After several trials showed that chest radiography was unsuccessful, the low-dose multi-detector CT scan has been investigated to reduce lung cancer mortality. The advantages of these CT scans are improved spatial resolution, the capacity to reconstruct multiple series from a single data acquisition and higher scan speed. Several large screening studies showed that high numbers of lung cancer could be detected in high-risk patients with CT scan compared to chest radiography and that most of these lesions were detected in an early and thus resectable stage.[32,33] The National Lung Screening Trial (NLST) recently showed in a selected population that there is a 20% reduction in lung cancer specific mortality in the screened arm, compared to the non-screened arm.[34]

When comparing survival rates, it is important to realize that there are several biases, such as lead-time bias, length time bias and overdiagnosis that could play an important role. Lead-time bias occurs when testing increases the perceived survival time without affecting the course of the disease. Length time bias occurs because screening is more likely to detect slow growing tumors than fast growing tumors. This can give the impression that screening prolongs survival. However, this is simply because slow growing tumors are less aggressive and have a higher chance of being detected, but the prolonged survival of the detected tumors is not due to screening.

Overdiagnosis is the diagnosis of a disease that will never have caused symptoms or death during a patient's life. This may unnecessary lead to harm, due to treatment and psychological stress.

The clinical applicability of lung cancer CT screening is at present criticized, due to important factors that need to be addressed first. This is described in chapter 5.[35,36]

1.4.2 Blood biomarkers

Another screening method that could lead to early detection of lung cancer is detecting biomarkers of in peripheral blood. A large number of biomarkers have been studied, for example the detection of transcriptomics (micro RNA and messenger RNA). Micro RNA is a class of small non-coding RNA gene products that can regulate certain gene expression. It has been shown that the micro RNA patterns between

lung cancer patients and healthy controls are significantly different. Therefore these patterns can be used for risk stratification and prediction models. Messenger RNA can be used for detecting circulating tumor cells. Recent studies show promising results in tracing lung cancer.[37-39]

Next to messenger RNA, lung cancer might also be detected in the blood based on circulating tumor DNA. It has been described that persons with circulating DNA levels of > 20 ng/ml have a high chance of having lung cancer, however more studies are needed to make the cut-off values more accurate.[40]

Another blood biomarker that might be useful is the analysis of promoter hyper-methylation in plasma. It has been shown that lung cancer patients have elevated levels of methylated genes in their plasma. Hypermethylation is associated with silencing of promoter regions of growth controlling genes and is found in cancer cells.[41]

Currently, blood biomarkers cannot yet be used in daily clinical practice, as most studies have not been validated independently and there are still contrary results between the different studies. Integration of biomarkers and clinical parameters in a model may improve results; therefore more research in this field is needed.

1.4.3 Exhaled breath measurements

The detection of lung cancer in exhaled breath measurements is another interesting screening method, especially because it is non-invasive. This method is originally based on cancer detection with sniffing dogs[42], but better standardisation is expected with the 'electronic nose'. The detection is based on the presence of volatile organic compounds (VOC) in lung cancer patients.[43] The exhaled breath reflects the meta-bolic activity in the body, resulting in different VOC profiles between lung cancer patients and healthy controls. Recently, studies showed that the exhaled breath could moderately distinguish lung cancer patients from control subjects.[44,45] The accuracy improved when clinical risk factors were taken into account.[46]

1.4.4 Sputum cytology

Sputum analysis represents a promising tool for early lung cancer detection.[47] The exfoliative cytology is used to identify the early stage of cancer and to prevent tu-mor mortality.[48] It is thought that lung cancer can be detected based on genetic or epigenetic changes in exfoliated cells. Two randomized studies, the Memorial Sloan-Kettering Lung Study and the Johns Hopkins Lung Project,[49] were identically designed to evaluate the benefits of sputum cytology to annual chest radiography. No decrease in lung cancer mortality rates were seen in the more intensely screened arm of either study. However, both studies showed a modest benefit among the heaviest smokers and a moderate reduction in deaths due to squamous cell and large cell lung cancer.[49]

Another trial, the Mayo Lung Project, provides strong evidence that combining sputum cytology and chest X-ray could lead to a higher detection rate.[48,50] However, these results did not show a mortality benefit from screening: lung cancer-specific mortality was 4.4 and 3.9 per 1000 person-years in the screened and control arms, respectively, (two-sided P for difference = 0.09).[51]

It has been described that conventional sputum cytology is an adequate method of establishing the diagnosis of lung cancer, with a pooled sensitivity rate of 0.66. In addition, the sensitivity of sputum cytology increased to 76% by the use of fluorescence in situ hybridization to the conventional sputum cytology.[52] So, the addition of different techniques to analyze the cytological specimen may improve diagnostic values. However, these results should be replicated in larger cohorts before they can be used in clinical practice.

1.5 Lung cancer treatment

15.1 General
Currently, lung cancer treatment largely depends on the stage of the cancer. In NSCLC patients with stage I and II surgical resection is the treatment of choice and adjuvant chemotherapy is optimal in patients with stage II. Radiotherapy remains an important treatment for patients that are medically inoperable or refuse surgery.[53] Combination chemoradiotherapy, especially delivered concurrently, is the preferred treatment for lung cancer patients with stage IIIA and IIIB. Also surgery may be indicated for carefully selected patients with T4N0-1M0.[53] The treatment of stage IV NSCLC is palliative with platinum-based doublets as the standard of care in patients with good performance score. There is scientific evidence that the addition of bevacizumab, an antiangiogenic agent, to carboplatin/paclitaxel in patients with stage IV disease improves survival. However, this only works in patients with non-squamous NSCLC.[53]

1.5.2 Chemotherapy
Lung cancer patients have a high chance of tumor recurrence. Even early stage NSCLC patients with complete surgical resection can have undetectable metastases at diagnosis.[54,55] Therefore, several studies have addressed the role of adjuvant chemotherapy.[7,56] The effect of cisplatin-based chemotherapy was shown in a large meta-analysis of stage II patients. They found that cisplatin-based chemotherapy leads to a 27% mortality reduction. [57,58]

Chemotherapy in patients with stage IV NSCLC and a good performance score shows an improved survival and it palliates disease-related symptoms. The role of chemotherapy in patients with a poor performance score is less convincing and the optimal approach has not yet been determined.[59]

1.5.3 Targeted therapy

Recently, new treatment options have become available for NSCLC, which can lead to increased survival. These therapies consist of targeted agents. Targeted agents block cancer cell growth by interfering with specific targeted molecules needed for carcinogenesis and tumor growth, rather than by interfering with all rapidly dividing cells, like chemotherapy does.[60]

One target for these agents is the epidermal growth factor receptor (EGFR, also called HER1 or erbB-1) tyrosine kinases (TK). EGFR exists as a monomer on the cell surface, and it must dimerize to activate the TK. A significant group of lung cancer patients have mutations in the EGFR.[61] These mutations mostly lead to over expression of the EGFR in NSCLC.[61] The EGFR regulates important processes in the carcinogenesis of cells, like proliferation, apoptosis, angiogenesis, and invasion.[62] EGFR treatment involves EGFR TK inhibitors, such as erlotinib or gefitinib, or monoclonal antibodies against EGFR, such as cetuximab.[62] Erlotinib and gefitinib demonstrated clinical activity in patients who had been previously treated with cytotoxic chemotherapy. Further study defined clinical and molecular parameters that have enabled the identification of those who are most likely to benefit from such therapy. Objective response rates of 55 to 90 percent were observed in phase II studies with both erlotinib and gefitinib when patients were selected based upon molecular criteria.[61]

Another target for which recently an agent has become available is the translocation of the echinoderm microtubule-associated protein-like 4 (EML4) and anaplastic lymphoma kinase (ALK).[4] A subgroup of NSCLC tumors contain an inversion in chromosome 2 that juxtaposes the 5′ end of the EML4 gene with the 3′ end of the ALK gene, resulting in the novel fusion oncogene EML4-ALK.[63] Tumors that contain the EML4-ALK fusion oncogene or its variants are associated with clinical features quite similar to the patients with EGFR mutations, including never or light smoking history, younger age, and adenocarcinoma. ALK gene arrangements are largely mutually exclusive with EGFR or KRAS mutations.[64] In the near future, selection of patients with EML4-ALK fusion oncogene will be important, since crizotinib, an ALK targeted inhibitor, has shown very promising results in a phase I and III trials.[65]

1.5.4 Treatment resistance

In most lung cancer cases, specific mutations of the tumor are unknown. In the patients with identified tumor mutations, there is the possibility that these mutations will modify during treatment because of the genetic instability of the tumor cells.[66] These epigenetic changes could force drug resistance[67] and because treatment could encourage these changes[68], the patients will eventually need to switch to the basic treatments as well. Tumors can harbour groups of cells with the same genetic mutations (clones). If the clones dwell in different tumors and reveal differential sensitivity

to a treatment, only a mixed treatment response will be observed and the treatment must be switched. If two different clones dwell in the same tumor, it is dependent on the differential sensitivity of the clones how the response to treatment will be.[66,69] Even though, chemotherapeutic agents can be effective in the treatment of lung cancer, most patients will eventually relapse,[54] because of an initial intrinsic resistance or resistance after initial response to treatment.[66,69]

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Chapter 2

Aims and outline of the thesis

AIMS OF THE THESIS

In the previous chapter, general aspects of lung cancer were described, including the limited treatment options and the low survival rates. During the past decades, numerous efforts have been made to decrease the death rate among lung cancer patients. Nonetheless, the improvement in long-term survival was limited and for most lung cancer patients it is still a devastating disease. Recent reports show a decrease in lung cancer mortality by screening programs. In addition, modulation of the patient's immune system by immunotherapy either as monotherapy or combined with conventional cancer treatments might offer the prospect of tailoring treatments much more precisely and might lead to a better response to treatment and overall survival of NSCLC patients. Although recently the addition of targeted agents has increased survival for advanced disease, it also became clear that tumors develop resistance against these agents, either mutation driven or non-mutation driven. Since only small improvements in survival can be expected in advanced disease with the use of conventional therapies, more research should focus on lung cancer screening programs and patient tailored immunotherapy with or without conventional therapies. This thesis evaluates the role of the immune system in lung cancer patients. In addition, the role of CT screening to detect early stage lung cancer patients is examined. The aims of this thesis are to evaluate the role of new strategies that can improve lung cancer survival.

OUTLINE OF THE THESIS

Part I: General introduction and outline of the thesis

Chapter 1 gives a general overview of lung cancer and the aims and the outline of the thesis are discussed in **chapter 2**. In order to evaluate new strategies to improve the survival of lung cancer we describe the role of lung cancer screening and immunotherapy in **chapter 3**. We describe that survival of lung cancer can be strongly improved by controlling two prognostic factors: stage and treatment.

Part II: Evaluation of the role of lung cancer screening

In **chapter 4** the inclusion criteria of lung cancer screening are discussed regarding the generalizability of the findings from large screenings trials to the total population of lung cancer patients, to investigate the clinical value. **Chapter 5** evaluates the applicability of lung cancer screening in more detail. The advantages and limitations of screening and the implications of lung cancer survival are here discussed.

Part III: The contribution of the immune system in lung cancer

Chapter 6 provides an overview of the relevant immunological cell types in lung cancer and their complex and dynamic roles within an established tumor microenvironment. In **chapter 7** we determine the role of specific immune system in lung cancer patients. Therefore, we analyzed an important population of immune suppressive cells, myeloid derived suppressor cells (MDSC). In this chapter we describe a new receptor on MDSC, named ILT3, which plays a role in suppression of T cell responses. In **Chapter 8** we describe the composition of the immune system of lung cancer patients, therefore we determined the immunological baseline characteristics of 185 advanced stage non-small cell lung cancer (NSCLC) patients. In addition, we describe the characterization and optimal assessment of MDSC to examine their presence and function in the peripheral blood of advanced stage NSCLC patients. In **chapter 9** we investigate the role of cytotoxic T lymphocytes (CTLs) and MDSC in the peripheral blood of NSCLC patients with different prognostic factors. We assessed the relation of the immune cell composition between patients with different stages of the disease and the WHO performance categories. **Chapter 10** describes the relationship between inflammation and lung cancer. We investigate whether a history of pulmonary tuberculosis is an independent risk factor for lung cancer survival in Caucasian patients.

Part IV: General discussion and summary

In **chapter 11** the study results are discussed and the interpretations as well as the future directives for further research are given.

Chapter 3

Improving lung cancer survival;
time to move on

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ABSTRACT

Background

During the past decades, numerous efforts have been made to decrease the death rate among lung cancer patients. Nonetheless, the improvement in long-term survival has been limited and lung cancer is still a devastating disease.

Discussion

With this article we would like to point out that survival of lung cancer could be strongly improved by controlling two pivotal prognostic factors: stage and treatment. This is corresponding with recent reports that show a decrease in lung cancer mortality by screening programs. In addition, modulation of the patient's immune system by immunotherapy either as monotherapy or combined with conventional cancer treatments offers the prospect of tailoring treatments much more precisely and has also been shown to lead to a better response to treatment and overall survival of non-small cell lung cancer patients.

Summary

Since only small improvements in survival can be expected in advanced disease with the use of conventional therapies, more research should be focused on lung cancer screening programs and patient tailored immunotherapy with or without conventional therapies. If these approaches are clinically combined in a standard multidisciplinary policy we might be able to advance the survival of patients with lung cancer.

KEYWORDS

- Lung cancer
- Survival
- Lung cancer screening
- Immunotherapy

BACKGROUND

Lung cancer is the leading cause of cancer-related death worldwide. Approximately 85% of all cases of lung cancer are non–small cell lung cancer (NSCLC). The 5-year survival of this aggressive disease is only 16%.[1] One of the reasons for this extremely poor survival is that most lung cancer cases are diagnosed at an advanced stage due to the relative lack of clinical symptoms during early stages. Metastatic NSCLC is currently an incurable disease for which standard chemotherapy provides only minor improvement in overall survival. In addition, less than 30% of patients with advanced-stage NSCLC have a response to platinum-based chemotherapy, the most commonly used first line treatment at this stage of the disease.[2]

During the last decades, advances in diagnostic and therapeutic approaches of this devastating disease have been made, however, long-term survival rates have hardly changed in the past 50 years.[3] Therefore, new approaches are required.

DISCUSSION

Survival of lung cancer could be strongly improved by controlling two pivotal prognostic factors: stage and treatment. Early stages of lung cancer have a better prognosis; thus early diagnosis of lung cancer by screening programs is one way that leads to a reduction in lung cancer mortality. However, given the high chance of tumor recurrence, even alleged early stage NSCLC patients with adequate surgical resection can have undetectable metastases at diagnosis.[4,5] It is known that adjuvant chemotherapy can reduce these metastases; nevertheless, in 24% of the patients metastasis occurs after adjuvant chemotherapy.[4] Therefore, besides lung cancer screening programs, an additional approach next to the conventional therapy must be developed to tackle lung cancer. In recent years it has been established that the immune system plays an important role in carcinogenesis and makes an essential contribution to the antitumor effects of traditional therapies. Modulation of the patient's immune system by immunotherapy either as monotherapy or combined with conventional cancer treatments offers the prospect of tailoring treatments much more precisely and could lead to a better response to treatment and overall survival of NSCLC patients.

Taken together, when early diagnosis by screening programs and patient-tailored immunotherapy are combined in a standard multidisciplinary policy for NSCLC treatment, we might be able to advance the survival of patients with early stage lung cancer. We will discuss both topics and their role in improving lung cancer survival below.

Lung cancer screening

Multiple randomized trials have investigated the effectiveness of lung cancer screen-ing and it is shown that lung cancer can be identified at an early stage with detection rates varying between 40-66%.[6,7] The survival rates of lung cancer patients diagnosed in screening programs are very high; 5- and even 10-year survival rates close to 90% can be achieved.[8,9] The largest lung cancer screening trial[10] recently showed that screening of high risk persons is very effective in reducing the mortality from lung cancer. Persons with more than 30 pack-years (PY) and aged between 55 and 74 years at time of randomization were included in this study. They found a relative mortality reduction of 20% when this high-risk group is screened with a low-dose computer tomography (CT) scan compared to chest radiography.[10] However, this is probably an underestimate, as the mortality reduction was measured at the time of closure of the trial. The introduction of low-dose multi-detector CT has led to important advantages, such as advanced scan speed, better spatial resolution, and the capacity to reconstruct multiple series from a single data acquisition. Before public policy recommendations are crafted, there are major concerns in lung cancer screening such as the effects of false positive findings, lead-time bias, the impact of overdiagnosis, and the generalizability of the results.[11]

Another important aspect that should be considered in generalizing the results of screening studies are the therapeutic options for patients with a positive screening, as lung cancer treatment is an important prognostic factor. In developed countries, lung cancer patients are treated with surgery, chemotherapy, and radiotherapy. In recent years, peri-operative mortality has decreased by the introduction of video assisted thoracoscopy (VATS) and better peri-operative management.[12] Early stage patients who are not eligible for surgery are frequently treated with radiotherapy with curative intent. Novel radiotherapy techniques, such as stereotactic ablative radiotherapy, show local control rates of 90% or more for stage I NSCLC.[13] Adjuvant chemo-therapeutic regimens have been shown to increase survival especially in resected patients with stage II and IIIA disease.[14] These regimens are expensive and therefore the results of the published screening trials can only be applied to the selected group of individuals in countries with well-developed health care systems with a quality comparable to the US.

Adjuvant immunotherapy

Treatment of lung cancer is currently based on the patient's clinical signs and symptoms, tumor stage and subtype, medical history, and data from imaging and laboratory evaluation. Until now, most cancer research is focused on therapies based on tumor characteristics to improve the prognosis of NSCLC, as cancer has long been considered as a cell-autonomous genetic disease. However, the sobering outcome

of current NSCLC therapy has shifted the attention to combining adjuvant treatment approaches.

Recent experimental findings and clinical observations have led to cancer-related immune inflammation being acknowledged as a new hallmark of cancer.[15-17] Evidence that the immune system of the host can influence cancer incidence, cancer growth, response to therapy, and the prognosis of the disease, is growing.[18] Therefore it was thought that conventional therapy combined with immunotherapy based on a pretreatment profile of the immune system of the host could be a valuable tool to increase the survival of early stage NSCLC.[19]

Cancer immunotherapy attempts to activate the host's immune system to recognize and destroy the residual lung cancer cells that conventional therapy misses. Immunotherapy can be divided into two main types: passive and active immunotherapy.[20,21] The most common form of passive immunotherapy is monoclonal antibody therapy.[20] It makes use of antibodies that have been produced in vitro and can bind to specific cell surface proteins that can influence tumor growth.[22] However, there will only be a response of the immune system during the time the antibody is present in the body. Ipilimumab (anti-CTLA-4), bevacizumab (anti-VEGF), and anti programmed death (anti-PD-1) or anti-PD ligand 1 (Anti-PD-L1) are examples of passive immunotherapy that could be useful in NSCLC.[23-26]

Ipilimumab blocks the negative cytotoxic T-lymphocyte antigen (CTLA)-4 that enhances T-cell responses to tumor cells, leading to effective immune responses. For NSCLC, ipilimumab is now in phase II development [24], but it is already approved by the US Food and Drug Administration (FDA) for the treatment of unresectable or metastatic melanoma.[24,27,28] Studies show that the two- and three-year survival rates in ipilimumab-containing treatment arms in metastatic melanoma patients are almost twice as high as in the non-ipilimumab-containing treatment arm.

Bevacizumab is an antibody that neutralizes the vascular endothelial growth factor (VEGF) ligand. As a result, it will inhibit angiogenesis.[29] Moreover, research has shown that adding bevacizumab to chemotherapy is associated with afferent vascular dilatation and efferent vascular constriction of tumor vessels that may help concentrate chemotherapy at the tumor site. Bevacizumab combined with taxane-platinum chemotherapy is the first approved antiangiogenic agent for cancer therapy that showed increase of progression-free survival and overall survival in first-line treatment of stage IV NSCLC.[29-30] Recently, data have been published on the immunogenic effect of VEGF. VEGF seems to be involved in a number of mechanisms negatively influencing the immune system; it makes dendritic cells more tolerogenic, and induces myeloid derived suppressor cells. Adding bevacizumab prevents immunotolerance and could thereby contribute to a better survival of lung cancer.[31,32]

Two other recently described antibodies that could play important roles in passive immunotherapy are anti-PD-1 and anti-PD-L1.[25,26] PD-1 is a co-inhibitory receptor on activated T-cells that plays an important role in immunosuppression. PD-L1, the ligand of PD-1, is expressed on cancer cells and is involved in negative regulation of immune responses, as they increase apoptosis of T-cells and inhibit CD4 and CD8 T-cell activation.[25,26] Inhibition of the interaction between PD-1 and PD-L1 can improve T-cell responses and mediate antitumor activity. Recent studies show that in NSCLC the objective response rates to anti-PD-1 and anti-PD-L1 are 18% and 10% respectively.[25,26] Blockage of both receptors induced durable tumor regression and prolonged stabilization of the disease. These findings confirm that the pathway between PD1 and PD-L1 could play an important role in therapeutic intervention and that it causes an increase in survival of lung cancer patients.

Active immunotherapy tries to persuade and boost immune effector cells *in vivo* against tumor cells through the administration of immune mediators capable of activating the humoral (antibodies) and cellular (T cells) immune system.[33] Therefore the duration of this broad response persists for a long time, because of the immunologic memory and it is less prone to antigen mutational responses.[33] Currently, multiple trials are investigating the effectiveness of different lung cancer vaccines.[33-36] In 2001, one of the first synthetic lung cancer vaccines showed that 16 out of 65 patients had an immune response after vaccination, and the median survival time was more than doubled (30.6 months, instead of 13.3 months in controls).[37] After that, other tumor-antigens vaccines, such as Wilms tumor antigen-1 and IDM-2101 were tested and showed immunological responses and prolonged survival in patients with lung cancer.[21,36] Next to synthetic vaccines there are trials that test dendritic cell (DC) vaccines.[34,38,39] In DC vaccines, tumor associated antigens are used to load immature autologous DCs. These DCs are injected into patients to stimulate antigen-specific immune responses in lung cancer patients. Different studies have shown biological activity of DC vaccines and phase I and II trials report that a group of lung cancer patients had therapeutic benefit.[34,39,40] Nevertheless, until now, reports about clinical applicability are anecdotal.

Other examples of active immunotherapy in lung cancer are natural killer (NK) cell transfer and adoptive T cell transfer.[41,42]

As described above, recent literature provides evidence for many potentially useful immunotherapy combinations. However, these therapies show drastic antitumor responses in only small subsets of patients. Currently, there is lack of predictive biomarkers to rationally choose combinations of immunotherapy for individual patients that benefit from these therapies. Therefore, it is necessary to further elucidate the mechanisms that are responsible for clinical benefit in small groups of patients and identify relevant pre-treatment biomarkers that distinguish responders from non-

responders. This patient-tailored treatment approach is able to redress the balance towards efficacious antitumor responses that can improve the overall survival for more patients.

Taken together, passive and active immunotherapy might have an important adjuvant role in early stage NSCLC by consolidating responses to conventional therapy and thereby leading to increased lung cancer survival rates. However, further research in this field is warranted to improve these therapies and to define subsets of responders.

SUMMARY

During the past decades, numerous efforts have been made to decrease the death rate among lung cancer patients. Nonetheless, the improvement in long-term survival has been limited and lung cancer is still a devastating disease.[3]

Since only small improvements in survival can be expected in advanced disease with the use of conventional therapies, more research should be focused on early stage lung cancer. Combining lung cancer screening programs and patient tailored immunotherapy with or without conventional therapies should be further explored. If these approaches are clinically combined in a standard multidisciplinary policy we might be able to advance the survival of patients with lung cancer.

COMPETING INTEREST

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTION SECTION

All authors were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Part II

Evaluating the role of
lung cancer screening

Chapter 4

Generalizing lung cancer screening results

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To the Editor:

The results of the National Lung Screening Trial (NLST) (ClinicalTrials.gov number, NCT00047385) (Aug. 4 issue)[1] showed that screening of high-risk persons is very effective in reducing mortality from lung cancer. Persons with a history of cigarette smoking of at least 30 pack-years who were between 55 and 74 years of age at the time of randomization were included in this study.

All inhabitants of the Rotterdam suburb of Ommoord who were 55 years of age or older were eligible to participate in the Rotterdam Study,[2] an ongoing population-based prospective cohort study in Rotterdam, the Netherlands. A total of 7983 participants (78% of persons who were invited to participate) were enrolled between 1990 and 1993 in the first cohort. Between 1990 and 2009, there were 208 deaths due to incident lung cancer in this cohort.

When we compare the relative reduction in mortality from lung cancer in the NLST with mortality from lung cancer in the Rotterdam Study, we find different results because of the strict inclusion criteria used in the NLST.

In our cohort, only 62 cases of lung cancer (29.8% of the total number of cases of lung cancer) occurred in subjects who met the criteria of the NLST (in our study, persons with unknown smoking history were included in the >30-pack-year group). In our study, a total of 12.5% of the patients with lung cancer between 55 and 74 years of age were never smokers or had a smoking history of less than 30 pack-years at baseline, and 57.7% of the patients with lung cancer were older than 74 years. Consequently, 70.2% of cases in the Rotterdam cohort would not have been included in the NLST.

A relative reduction in mortality from lung cancer of 20%, as shown in the NLST would correlate with a reduction in mortality from lung cancer of 6% in the Rotterdam Study population (i.e., 0.2×62=12.4 of the 208 persons would not have died from lung cancer).

Screening for lung cancer with the inclusion criteria in the NLST reduced mortality from lung cancer as compared with the standard of care, but because only a minority of patients with lung cancer meet these criteria, clinicians should be cautious in generalizing findings to all patients with lung cancer. A reduction in mortality may be different in countries with other demographics and health care systems.

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Chapter 5

Generalizability of results from the National Lung Screening Trial

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ABSTRACT

Lung cancer is the major cause of cancer-related death worldwide, with a 5-year survival of only 16 %. Most lung cancer cases are diagnosed at an advanced incurable stage. As earlier stages have a better prognosis, the key to reducing mortality could be early diagnosis of the disease. At present, low-dose computed tomographic (CT) screening has shown promising data. Lung cancer death rates were reduced by 20 % when CT screening is compared to chest radiography in a high-risk group. There are many advantages of CT screening in lung cancer, however there are also some important issues that should be taken into account. Therefore, the applicability of the results to clinical practice is not clear yet. In this Commentary we discuss different aspects that play important roles in the balance between harms and benefits of screening, including overdiagnosis, availability of treatment options worldwide, ethical considerations, costs, and prolonged life expectancy. We conclude that clinicians should be cautious in generalizing findings to the total population of smokers and take into account that the use of lung cancer screening in clinical practice may have limitations.

Lung cancer is the major cause of cancer-related death in both men and women worldwide. The 5-year survival of this aggressive disease is only 16%.[1] One of the reasons for this extremely poor survival is that most lung cancer cases are diagnosed at an advanced stage due to the relative lack of symptoms during early stages. Early stages of lung cancer have a better prognosis. Therefore, early diagnosis of lung cancer could lead to reduction of lung cancer mortality. Recently, the National Lung Screening Trial (NLST)[2] showed that lung cancer death rates can be reduced by 20% when a high-risk group is screened with a low-dose computer tomography (CT) scan compared to chest radiography. The participants of this study comprised non-symptomatic persons aged 55-74 years, who had smoked at least 30 pack-years (PY) in their lifetime. Participants received a baseline CT and two annual screening CTs and were then followed for a median of 6.5 years. The study demonstrated a shift in stage at cancer diagnosis as in the screening arm more cases of early stage lung cancer and fewer cases of advanced disease were detected. In conclusion, screening of high-risk individuals led to a better opportunity for curative treatment. The International Association for the Study of Lung Cancer (IASLC) recently held a CT Screening Workshop after which the Strategic CT Screening Advisory Committee was set up.[3] This committee is currently engaging professional societies and organizations who are stakeholders in lung cancer CT screening implementation across the globe, to focus on delivering guidelines and recommendations in six specific areas: (i) identification of high-risk individuals for lung cancer CT screening programs; (ii) develop radiological guidelines for use in developing national screening programs; (iii) develop guidelines for the clinical work-up of "indeterminate nodules" resulting from CT screening programmers; (iv) guidelines for pathology reporting of nodules from lung cancer CT screening programs; (v) recommendations for surgical and therapeutic interventions of suspicious nodules identified through lung cancer CT screening programs; and (vi) integration of smoking cessation practices into future national lung cancer CT screening programs.[3]

There are many potential advantages of CT screening. Low-dose CT is not only a valuable tool to detect lung cancer at earlier stages; it can also be used to monitor other causes of morbidity and mortality in heavy smokers, such as ischemic heart disease and chronic obstructive lung disease (COPD). Early detection of coronary-artery calcification could be used to identify patients who are likely to experience ischemic heart disease. In addition, early detection of COPD could signify patients with a rapid progression of their COPD, leading to an intensive treatment. Screening for other causes of morbidity and mortality in heavy smokers might therefore increase the cost-effectiveness of screening programs, especially if positioned in multidisciplinary programs that provide smoking cessation programs.[4] CT scans could also play an important role in research to understand the aetiology and pathophysiology of COPD.

However, major concerns in lung cancer screening are the management of false positive findings and the impact of overdiagnosis. Up to 20% of screening tests lead to false positive findings. This exposes participants unnecessarily to potentially harmful diagnostic evaluations and psychological discomfort. Overdiagnosis occurs when a screening test detects a lung cancer that would otherwise have remained unnoticed, either because the disease remains stable, or grows so slowly that the patient dies before the disease is diagnosed due to other causes. It has been estimated that between 10% to as much as 25% of screen detected lung cancers may be overdiagnosed cases.[5] Overdiagnosis in lung cancer screening is a problem, because it is difficult to predict which early-stage cancers will ultimately progress and will be the cause of death of the patient.

There are different types of lung cancer, all with a different growth and developmental rate, but at present it is not possible to predefine these subgroups in different populations. Due to the recent developments in molecular medicine in lung cancer increasing knowledge is developing on this subdivision of lung cancer. It is now generally accepted that lung cancer consists of a group of cancers with different driving mutations. For instance, there are special characteristics, like EGFR mutations, showing a different susceptibility to therapeutic approaches apart from the fact that it is also a positive prognostic factor. This heterogeneity of lung cancer entities can change screening efficacy. How much this heterogeneity changes on average is dependent on the incidence of the different types of cancer and for instance EGFR copy number in tumour cells. The distribution between the different lung cancer types varies between distinct populations.

Another important issue that has not been clarified following the results of the NLST is related to the generalizability of its results to the general population, as most trials use very strict inclusion criteria and thereby select only a subset of the individuals. At a population level, the observed mortality reduction in the NSLT may vary depending on certain factors In case of the NLST the 20% lung cancer mortality reduction and the relative risks of harm and other side-effects was measured on a relative scale, and therefore it is difficult to extrapolate the effects in lung cancer mortality from the NLST to population with a lower and higher incidence. However, from a health-care point of view absolute numbers are more indicative. At a population level, the observed mortality reduction in the NSLT may vary depending the characteristics of the screened population. For instance, in case of a more genetically susceptible population, the incidence of lung cancer will be higher, even if same smoking criteria are applied. Then, the anticipated 20% relative reduction in mortality with screening will lead to a higher absolute number of people who will be saved from lung cancer deaths. Conversely, the impact of screening will be decreased in countries with a population with lower susceptibility for lung cancer.   Of course, screening for

lung cancer is not considered to be a modifier of the incidence risk, though the effectiveness of screening may be different between populations and subgroups within each population.

Furthermore, extrapolating the NSLT results requires to consider the diagnostic accuracy of the screening test. The NLST made use of multidetector scanners with a minimum of four channels. Nowadays the available technology even allows measuring the volume of the tumour nodules. However, expensive high-quality CT scans are only available in developed countries. In addition, the assessment and interpretation of the scans is dependent on the quality of the radiologist. There is a large difference in quality of education of medical doctors worldwide.

Nevertheless, the most important aspect that should be considered in generalizing the results of screening studies are the therapeutic options for patients with a positive screening, as lung cancer treatment is an important prognostic factor. In the NLST, patients were treated with surgery, chemotherapy, and radiotherapy. In recent years, peri-operative mortality has decreased by the introduction of Video Assisted Thoracoscopy (VATS) and better peri-operative management. Early stage patients who are not eligible for surgery are frequently treated with radiotherapy with curative intent. Novel radiotherapy techniques, such as stereotactic ablative radiotherapy do increase survival. Adjuvant chemotherapeutic regimens have been shown to increase survival especially in resected patients with stage II and IIIA disease. These regimens are expensive and therefore the results of the NLST can only be applied to countries with health care systems with a quality comparable to the US.

An additional pivotal public health policy concern in lung cancer screening is whether it is ethical to limit screening programs to individuals with >30 PYs of smoking, while approximately one-third of the population of lung cancer patients has smoked less than this threshold.[6] Screening programs may therefore unintentionally give the reassurance that there is no need to stop smoking, leading to continuation of smoking.

Also the cost effectiveness of low dose CT screening must be considered. The use of three annual CT screenings, the follow-up of the participants and the additional clinical procedures in response to positive screening is very expensive: $725,000 to prevent one death from lung cancer.[7] Several research groups modelled the cost-effectiveness analyses of CT screening with varying conclusions.[8-9] Wisnivesky et al.[8] conclude that the cost-effectiveness ratio of a baseline CT scan is within the range of clinical practice and health policy acceptability, while Mahadevia et al.[9] state that lung cancer screening is unlikely to be highly cost-effective without substantial reductions in mortality, high rates of adherence, lower rates of overdiagnosis, and lower costs per screening test. This contradiction should be investigated in more

detail. Existing risk prediction models are approximately 70% accurate in identifying higher risk individuals who may benefit most from screening.

We have previously shown that in a large prospective population-based cohort study, the Rotterdam Study, only 62 lung cancer cases (30%) fulfilled the NSLT inclusion criteria.[10] Consequently, when we applied NLST's relative reduction in lung cancer mortality to the Rotterdam Study, we find that the relative reduction in mortality of 20%, would lead to an absolute reduction in lung cancer mortality in the Rotterdam Study population of 6.0% . This means that 12 persons out of the total 208 lung cancer deaths found in the Rotterdam Study would have been saved from mortality by lung cancer. This difference is mainly due to applying the age restriction, as 58% of incident lung cancer cases in the Rotterdam Study were above 74 years. In addition, recent literature shows that the highest lung cancer incidence rates were among persons aged 70-79 years.[11]

In the last decade there has been a consistent increase in life expectancy, of which two-third is caused by declines in mortality among those aged 65 years and older.[12] This is partly due to improvements in health care delivery, particularly for the elderly.[12] Recent evidence suggests that after infectious diseases have largely been eliminated as a cause of death, medical care, i.e. a more active approach towards the treatment of seriously ill elderly patients, plays the central role for further increases in life expectancy.[13] Nowadays, there is an increased number of possibilities for curative therapeutic interventions in the elderly with lung cancer such as minimal invasive surgery and advanced radiotherapy. Taking into account that persons between 70-79 years have the highest lung cancer risk, life-expectancy in the elderly is increasing and curative therapeutic options are available, screening may reduce lung cancer mortality, also in this population. A possibility is adjusting the age limits in screening trials from 74 years to at least 79 years. The effects of this on cost-effectiveness have to be determined similar to lung cancer screening in the present studies, taking into account prevention of other causes of mortality such as ischemic heart disease.

In conclusion, early detection of lung tumours by lung cancer screening programs has led to more curative therapeutic options for patients and to a mortality reduction of 20%. There can be no doubt about the importance of such screening programs. However, results from the trial may only apply to individuals with access to high quality lung cancer care. In addition, screening trials for lung cancer only include a minority of lung cancer patients as most patients do not comply with these criteria. Therefore, clinicians should be cautious in generalizing findings to the total population of smokers and take into account that the use of lung cancer screening in clinical practice may have limitations in reducing lung cancer mortality.

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Part III

The contribution of the immune system in lung cancer

Chapter 6

Patient-tailored modulation of
the immune system may
revolutionize future lung
cancer treatment

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ABSTRACT

Cancer research has devoted most of its energy over the past decades on unraveling the control mechanisms within tumor cells that govern its behavior. From this we know that the onset of cancer is the result of cumulative genetic mutations and epigenetic alterations in tumor cells leading to an unregulated cell cycle, unlimited replicative potential and the possibility for tissue invasion and metastasis. Until recently it was often thought that tumors are more or less undetected or tolerated by the patient's immune system causing the neoplastic cells to divide and spread without resistance. However, it is without any doubt that the tumor environment contains a wide variety of recruited host immune cells. These tumor infiltrating immune cells influence anti-tumor responses in opposing ways and emerges as a critical regulator of tumor growth. Here we provide a summary of the relevant immunological cell types and their complex and dynamic roles within an established tumor microenvironment. For this, we focus on both the systemic compartment as well as the local presence within the tumor microenvironment of late-stage non-small cell lung cancer (NSCLC), admitting that this multifaceted cellular composition will be different from earlier stages of the disease, between NSCLC patients. Understanding the paradoxical role that the immune system plays in cancer and increasing options for their modulation may alter the odds in favor of a more effective anti-tumor immune response. We predict that the future standard of care of lung cancer will involve patient-tailor-made combination therapies that associate (traditional) chemotherapeutic drugs and biologicals with immune modulating agents and in this way complement the therapeutic armamentarium for this disease.

KEYWORDS

REVIEW

Current NSCLC treatment

Treatment of lung cancer is currently based on the patient's clinical signs and symptoms, tumor stage and subtype, medical and family history, and data from imaging and laboratory evaluation. Most conventional cancer therapies, such as radiotherapy and chemotherapy are restricted by adverse effects on normal tissue. Currently NSCLC therapy is moving towards personalized medicine where the genetic profile of each patient's tumor is identified and specific therapies that inhibit the key targets of the oncogenic activation are targeted. In approximately 60% of all NSCLC cases, specific mutations can be identified, of which ± 20% can be targeted with specific drugs at this moment (e.g. erlotinib, gefitinib, crizotinib). However, most patients receiving conventional cancer treatments or targeted drugs will experience a relapse of tumor growth at a certain time. This sobering outcome demonstrates the necessity of innovative approaches in NSCLC treatment.

Recently, experimental findings and clinical observations have led to cancer-related immune inflammation being acknowledged as an additional hallmark of cancer [1,2]. There is currently overwhelming evidence that several immunological cell types of the host influence cancer incidence, cancer growth, response to therapy and thereby the prognosis of the disease. However, the immune system plays a paradoxical role by either preventing cancer growth or in sculpting tumor escape and stimulates its development. A better understanding of the interaction between cancer cells and host immune cells within the tumor environment is of importance for further progress in cancer treatment. This is an extremely difficult task because of the complicated cancer-host immune interactions. The field that studies these interactions, termed cancer immunology, is rapidly progressing. It provides insights into the contribution of the immune system in processes such as tumor invasiveness, metastasis, and angiogenesis and may predict the response to treatment. Most importantly, it also provides opportunities for improved anti-cancer therapies. Modulation of the patient's immune system combined with anti-tumor treatments offers the prospect of tailoring treatments much more precisely and better efficacy for patients with advanced lung cancer.

Immune cells involved in tumorogenesis

The individual immune related tumor infiltrating cell types are discussed below (Figure 1).
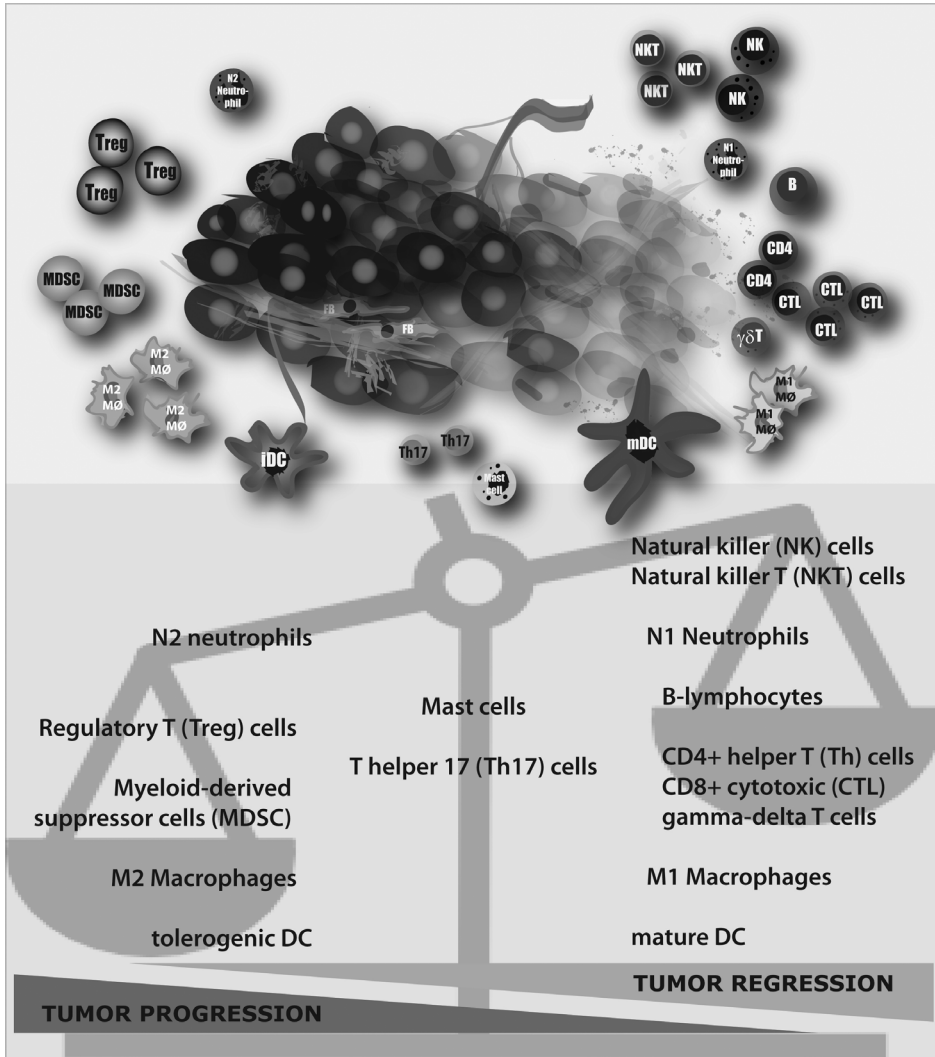
Figure 1: The tumor microenvironment is a heterogeneous and complex system of tumor cells (black) and 'normal' stromal cells, including endothelial cells and their precursors, pericytes, smooth-muscle cells, and fibroblasts of various phenotypes, located within the connective tissue or extra-cellular matrix (e.g. collagen). Leukocyte infiltration is an important characteristic of cancer and the main components of these infiltrates include natural killer (T) cells, neutrophils, B- and T-lymphocyte subsets, myeloid derived suppressor cells, macrophages and dendritic cells [3-7]. Based on their functions, these cells can be divided into cells with a potentially positive impact on the antitumor response (right) and cells with a detrimental effect (left). From mast cells and T helper 17 cells it is yet ambiguous what kind of effect these cells have within the micro-environment. The net effect of the interactions between these various cell types and their secreted products within the environment of an established tumor participates in determining anti-tumor immunity, angiogenesis, metastasis, overall cancer cell survival and proliferation.

Natural killer (T) cells

Natural killer (NK) cells (expressing the surface markers CD16 and CD56, but not CD3) are lymphocytes that play an important role in the rejection of tumors without previous sensitization and without restriction by the major histocompatibility complex (MHC) [8,9]. NK cells eradicate tumors through multiple killing pathways, including direct tumor cell killing. They also secrete cytokines and chemokines like Interleukin (IL) IL-10, Tumor Necrosis Factor (TNF)-α, and the principal NK-derived cytokine Interferon (IFN)-γ, which can coordinate the innate and adaptive immune responses to tumor cells and may lead to apoptosis of the attacked cells.

A large cohort study showed that an increase in NK cells in tumor tissue is a strong independent prognostic factor for the survival of lung cancer patients [10]. This is confirmed in mouse models, showing that stimulation of NK cell function protected against NSCLC metastasis [11,12], while depletion enhanced lung cancer metastasis [13]. However, it was recently shown that although the frequencies of NK cells in blood do not differ from healthy controls, stimulated blood NK cells from NSCLC patients with advanced disease had a reduced granzyme B and perforin A expression, lower production of IFN-γ, and decreased cytotoxic function indicating that these cells are functionally impaired in comparison with healthy controls [14,15]. Adoptive transfer of allogeneic, *in vitro* activated and expanded NK cells from haploidentical donors was proven potentially clinically effective in NSCLC [16].

Natural killer T (NKT) cells (CD16+, CD56+, CD3+) are a subset of NK cells that have been found in the peripheral blood, tumor tissue and pleural effusions of lung cancer patients in decreased numbers and with reduced functions [17,18]. It has been shown that NKT cells in cancer patients produce a decreased amount of IFN-γ and are therefore less effective than NKT cells in healthy controls [19,20]. They are currently exploited for cancer treatment by harnessing these cells with CD1d agonist ligands [21,22], or by adoptive transfer of NKT cells activated *in vitro* [23].

Mast cells

Accumulation of mast cells is common in angiogenesis-dependent conditions, like cancer, as mast cells are a major provider of proangiogenic molecules vascular endothelial growth factor (VEGF), IL-8, transforming growth factor (TGF)-β [24]. The density of mast cells in NSCLC tumors is correlated with microvessel density [25] and mast cells / histamine has a direct growth promoting effect on NSCLC cell lines *in vitro* [26]. However, the role of mast cells in the prognosis in NSCLC remains controversial [25,27–29]. Tumor-infiltrating mast cells can directly influence proliferation and invasion of tumors, by histamine, IL-8 and VEGF while the production of TNF-α and heparin can suppress tumor growth [26,30]. It has been shown that in NSCLC mast cell counts were noted to increase as tumor stage increased while another study

did not show this correlation [24,29]. Mast cells also play a central role in the control of innate and adaptive immunity by interacting with B and T cells (in particular Treg) and dendritic cells. The controversy of mast cells in cancer seems to be related to the type, microenvironment and stage of cancer and their role may depend on the tumor environment [29,31,32]. Therapeutic intervention by targeting mast cells, although technically possible [33], is too early without more knowledge on the paradoxical role of these cells in individual cases.

Neutrophils
Neutrophils play a major role in cancer biology. They make up a significant portion of the infiltrating immune cells in the tumor and the absolute neutrophils count and the neutrophils to lymphocyte ratio in blood are independent prognostic factors for survival of NSCLC [34–36]. Neutrophils are attracted to the tumor under the influence of specific chemokines, cytokines and cell adhesion molecules. Tumor-associated neutrophils (TAN) have polarized functions and can be divided into the N1 and N2 phenotype in a context-dependent manner [37,38]. The N1 phenotype inhibits tumor growth by potentiating T cell responses while the N2 phenotype promotes tumor growth [3]. The antitumor activities of N1 neutrophils include expression of immune activating cytokines (TNF-α, IL-12, GM-CSF, and VEGF), T cell attracting chemokines (CCL3, CXCL9, CXCL10), lower expression of arginase, and a better capacity of killing tumor cells *in vitro*. N2 neutrophils support tumor growth by producing angiogenic factors and matrix-degrading enzymes, support the acquisition of a metastatic phenotype, and suppress the anti-tumor immune response by inducible nitric oxide synthase and arginase expression. Neutrophils also influence adaptive immunity by interacting with T cells [39], B-cells [40], and DC [41]. In resectable NSCLC patients, intratumoral neutrophils were elevated in 50% of the patients and this was associated with a high cumulative incidence of relapse [42]. Recently, Fridlender *et al.* showed that TGF-β acquired the polarized N2 tumor promoting phenotype of neutrophils in a murine lung cancer model, and blocking of TGF-β shifted towards N1 tumor rejecting neutrophils with acquisition of anti-tumor activity *in vitro* and *in vivo* [43]. Blockade of TGF-β in humans might be a potential utility to prevent polarization towards the protumorigenic N2 phenotype and thereby may result in retarding tumor growth.

B-lymphocytes
B-cells may affect the prognosis of patients with lung cancer, as patients with stage I NSCLC contain more intratumoral germinal centers with B-lymphocytes than patients with stages II to IV [44]. These tertiary (T-BALT) structures provide some evidence of an adaptive immune response that could limit tumor progression in some patients. For instance, the production of antibodies by B-cells can activate tumor cell kill-

ing by NK cells and other inflammatory cells [45]. Auto-antibodies against tumor antigens are commonly found in patients with lung cancer [46–48] and can inhibit micrometastasis [49]. Recently, it has been shown in mice that antibodies produced by B cells interact with and activate Fcγ receptors on macrophages and in this way orchestrate antitumor activity [50] or tumor-associated macrophages (TAM)-mediated enhancement of carcinogenesis [51]. Thus, the role of B cells seems depending on the context.

CD4+ and CD8+ lymphocytes
CD4+ cells and CD8+ cells represent the strong effectors of the adaptive immune response against cancer [52]. There is controversy on the impact of T cells and their localization on the prognosis of lung cancer [53–59]. This may be caused by the presence of a special subset of T cells, the regulatory T cells, and myeloid-derived suppressor cells which are discussed below. Also tumor-derived factors can exhaust T lymphocytes or induce their apoptosis [60]. Recently it has been shown that cytotoxic T lymphocytes (CTL) within the tumor (the tumor-infiltrating lymphocytes [TIL]) are of beneficial prognostic influence in resected NSCLC patients in both adenocarcinoma [61] and squamous cell carcinoma [62]. Tumor-specific CD8+ effector T-cells are normally present at a low frequency in cancer patients, but can be expanded up to 50% of the total circulating CD8+ T-cells by dendritic cell vaccination or adoptive T-cell transfer therapy [63–65]. To enhance existing anti-tumor responses, recombinant CD40 ligand or CD40 activating antibodies are investigated as substitute for CD4+ T cell help [66]. Blocking T cell inhibitory molecules such as cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene-3 (LAG-3), T cell immunoglobulin mucin-3 (TIM-3), and programmed death-1 (PD-1) are currently investigated in NSCLC to improve T cell homing and effector functions [67,68]. Successes of these experimental therapies in small subsets of patients demonstrate that CTL can be directed against the tumor but mechanisms to induce CTL or overcome the inactivation of T cell function seems necessary to enable more patients from these treatments.

Regulatory T cells
Regulatory T cells (Treg), characterized by CD4+, CD25+, Foxp3+, and CD127-, are T lymphocytes that are generated in the thymus (natural Treg) or induced in the periphery (induced Treg) when triggered by suboptimal antigen stimulation and stimulation with TGF-β and IL-10 [69]. Treg are further characterized by the expression of glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), and cytotoxic T-lymphocyte-associated antigen 4 (CTLA4).

In cancer patients, Treg confer growth and metastatic advantages by inhibiting anti-tumor immunity. They have this pro-tumoral effect by promoting tolerance via

direct suppressive functions on activated T-cells or via the secretion of immunosuppressive cytokines such as IL-10 and TGF-β [70,71]. Treg are present in tumor tissue [72,73] and increased in peripheral blood of NSCLC patients compared to healthy controls [74,75]. This increase in Treg was found to promote tumor growth and was correlated with lymph node metastasis [56,73,76,77] and poor prognosis [73,78]. Many factors can increase Treg in NSCLC tumors, among them are thymic stromal lymphopoietin (TSLP) [79] and intratumoral cyclooxygenase-2 (COX-2) expression [80]. Treg are considered the most powerful inhibitors of antitumor immunity [81]. As a result, there is substantial interest for overcoming this barrier to enhance the efficacy of cancer immunotherapy. Strategies include I). Treg depletion by chemical or radiation lymphoablation or using monoclonal antibodies or ligand-directed toxins (daclizumab, basiliximab, denileukin diftitox [Ontak™], RFT5-SMPT-dgA, and LMB-2) or with metronomic cyclophosphamide. II). Suppression of their function (ipilimumab, tremelimumad [anti-CTLA4], DTA-1 [anti-GITR], denosumab [anti-RankL], modulation of Toll-like receptor, OX40 stimulation or inhibiting ATP hydrolysis using ectonucleotidase inhibitors). III). Inhibition of tumoral homing by blocking the selective recruitment and retention of Treg at tumor sites, e.g. CCL22, CXCR4, CD103, and CCR2. IV). Exploitation of T-cell plasticity by modulating IL-6, TGF-β, and PGE2 expression, e.g. the COX-2 inhibitor celecoxib [82]. Till now, a strategy that specifically target only Treg and no effector T cells is lacking and procedures that depletes or modulates all Treg should be avoided to minimize the risk of autoimmune manifestations. However, studies modulating Treg in patients are providing some early encouraging results supporting the concept that Treg inhibitory strategies have clinical potential, particularly in those therapies that simultaneously stimulate antitumor immune effector cells

Gamma delta T cells
Human γδ-T cells constitute 2-10% of T cells in blood and exhibit natural cytolytic activity in an MHC-unrestricted manner for microbial pathogens and tumor cells. A special TCR on γδ-T cells recognizes small nonpeptide antigens with a phosphate residue and isopentenylpyrophosphate (IPP) that accumulate in tumor cells [83]. Because γδ-T cells recognize target cells in a unrestricted manner, they may exert antitumor effects even on tumor cells with reduced or absent expression of HLA and/ or tumor antigens or by provision of an early source of IFN-γ [83,84]. Phase I clinical trials of *in vivo* activation of γδ-T cells with zoledronic acid plus IL-2 or adoptive transfer of *in vitro* expanded γδ-T cells are being conducted at present for lung cancer [85–87].

Th17 cells

Th17 cells are a subpopulation of CD4^{+} T helper cells that are characterized by the production of interleukin-17 (IL-17, also known as IL-17A). IL17 plays an important role in the host defenses against bacterial and fungal infections by the activation, recruitment, and migration of neutrophils [88,89]. *In vitro* experiments have shown that IL-1β, IL-6, and IL23 promote Th17 generation and differentiation from naïve CD4^{+} T cells [90]. Among the other cytokines secreted by Th17 cells are IL-17 F, IL-21, IL-22, and TNF-α. The role of Th17 cells in cancer is poorly understood. Th17 cells accumulate in malignant pleural effusion from patients with lung cancer [90]. Also higher levels of IL-17A were detected in serum and in tumor lesions of lung adenocarcinoma patients, indicating a potential role of these cells in cancer [91]. It has been shown that Th17 cells encouraged tumor growth by inducing tumor vascularization or enhancing inflammation, but other studies revealed also opposite roles for Th17 cells. Recent data indicate that IL-17 may play a role in the metastasis of lung cancer by promoting lymphangiogenesis and is therefore an independent prognostic factor in both overall and disease-free survival in NSCLC [92]. However, there is a distinct role for Th17 and Th17-stimulated cytotoxic T-cells in the induction of preventive and therapeutic antitumor immunity in mice by the promoted recruitment of several inflammatory leukocytes, like DC, CD4^{+} and CD8^{+} cells [93]. So, it is controversial whether Th17 cells in cancer are beneficial or antagonistic; this may be dependent on the tumor immunogenicity, the stage of disease, and the impact of inflammation and angiogenesis on tumor pathogenesis [94].

Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature myeloid cells and myeloid progenitor cells. MDSC inhibit T cells activation [95,96] in a nonspecific or antigen-specific manner, alter the peptide presenting ability of MHC class I molecules on tumor cells [97], influence B-cells [98], block NK cell cytotoxicity [99–101], inhibit dendritic cell differentiation [102], and expand Treg [103,104] signifying their crucial contribution in constituting a tumor suppressive environment. Furthermore, there is compelling evidence that MDSC, by secreting MMP9 and TGF-β1, are also involved in angiogenesis, vasculogenesis, and metastatic spread [105].

  MDSC suppress the immune system by the production of reactive oxygen species (ROS), nitric oxide (NO), peroxynitrite and secretion of the cytokines IL-10 and TGF-β [106]. Upregulated arginase-I activity by MDSC depletes the essential amino acid L-arginine, contributing to the induction of T cell tolerance by the down regulation of the CD3ζ chain expression of the T cell receptor [107–110]. However, the mecha-

nisms that are used to suppress the immune responses are highly dependent on the context of the microenvironment [111].

An increased subpopulation of MDSC in the peripheral blood of NSCLC patients was detected that decreased in those patients that responded to chemotherapy and patient undergoing surgery [112]. Because MDSC play an important role in mediating immunosuppression, they represent a significant hurdle to successful immune therapy in NSCLC. Therefore, targeting MDSC *in vivo* with drugs like 5-fluorouracil (5FU), gemcitabine or VEGF / c-kit blockers (e.g. sunitinib, imatinib, dasatinib) to elicit more potent anticancer effects is an exciting development [113–115]. Treatment of mice with all-trans retinoic acid (ATRA), along with NKT help, convert the poorly immunogenic MDSC into fully efficient APC and in this way reinforced anti-tumor immune responses [116]. Other MDSC suppressing or differentiation-inducing agents recently reported are 5-aza-2'-deoxycytidine, curcumin, IL-10, anti-IL4R aptamer, and vitamin D3 [117–120]. Agents that decrease arginase activity, ROS and/or iNOS expression by MDSC include Nor-NOHA, 1-NMMA, cyclooxygenase 2 inhibitors (celecoxib [121]), phosphodiesterase 5 inhibitors (sildenafil, tadalafil [122]) or reactive oxygen species inhibitors (nitroaspirin [123]). These agents promise to be a fruitful avenue of investigation in the coming years to overcome immune suppression associated by MDSC in advanced tumors [113,114].

Tumor–associated macrophages

Macrophages are part of the innate immune system and play important roles in the first line of defense against foreign pathogens. They can be divided into M1 macrophages (classical activation) and M2 macrophages (alternative activation). M1 macrophages attract and activate cells of the adaptive immune system and have anti-tumor and tissue destructive activity, while the M2 phenotype has been linked to tumor-promoting activities by subversion of adaptive immunity, promoting tumor angiogenesis and supporting cancer cell survival, proliferation, invasion and tumor dissemination. Macrophages in tumors are usually referred to as tumor-associated macrophages (TAM) and their presence can be substantial (10 to 65% of the tumor stroma). In the beginning, the TAM mainly consist of M1-like macrophages however, when the tumor starts to invade and vascularize, there is a skewing towards the M2 phenotype [124,125]. This takes place especially at those regions in the tumor that are hypoxic [126].

It has been reported by several groups that there is an association between the number of tumor islet macrophages and NSCLC survival [58,127–132]. Moreover, when looking at the different phenotypes of TAM (M1 and M2), it is shown that high numbers of M1 macrophages infiltrating the tumor are correlated with improved

survival [130,133]. On the other hand, the presence of M2-like macrophages is associated with poor clinical outcome [130,133].

Several strategies are currently investigated that influence M2 macrophages at multiple levels. For example, blockade of factors and cytokines secreted by tumor or immune cells to limit the induction of M2 macrophages are investigated [134–136], however this results in loss of typical M2 markers but not their function [137]. It has been shown that inhibiting IκB kinase (IKK) reprograms the M2 phenotype to the M1 subset [138,139]. Also CD40 therapy seems to skew tumor-infiltrating macrophages towards the M1 phenotype [140]. Influencing the attraction, the polarization or the activation of M2 macrophages may improve survival when combined with standard or other immunotherapeutic regimens.

Dendritic cells
Dendritic cells (DC) are widely acknowledged as the central surveillance cell type and play an important role in the activation of lymphocyte subsets to control or eliminate human tumors. Upon encountering tumor cells or tumor-associated antigens, DC engulf this material and begin migrating via lymphatic vessels to regional lymphoid organs. The density immature DC (Langerhans cell and interstitial DC) and mature DC, present in the tumor microenvironment is highly predictive of disease-specific survival in early-stage NSCLC patients [141] and the presence of DC in resected NSCLC material is a good prognostic factor [10,142]. Interaction between the DC and tumor cells results in the release of antitumour cytokines [143,144]. This suggests that DC within the tumor microenvironment of early-stage NSCLC are capable in initiating adaptive immune responses in situ [145–147].

In the peripheral blood and regional lymph nodes of lung cancer patients, the number and function of mature DC is dramatically reduced [148,149], partly due to abnormal differentiation of myeloid cells (e.g. MDSC) [150]. Tumor cells, stromal cells like fibroblasts, and tumor-infiltrating immune cells and/or their secreted products, like VEGF, M-CSF, IL-6, IL-10, and TGF-β are also responsible for systemic and local DC defects [151–154]. Affected DC are impaired in their ability to phagocytose antigen and to stimulate T cells, leading to a defective induction of anti-tumor responses.

NSCLC-derived DC produce high amounts of the immunosuppressive cytokines IL-10 and TGF-β [155]. It has been shown that the T cell co-inhibitory molecule B7-H3 and programmed death receptor-ligand-1 (PD-L1) are upregulated on tumor residing DC and these molecules conveys mainly suppressive signals by inhibiting cytokine production and T cell proliferation [156,157].

Tumor-induced modulation is one of the main factors responsible for tumor immune escape and correction of DC function might be a requirement to develop more effective immunotherapeutic strategies against cancer. This might include targeting of

those factors with neutralizing antibodies (e.g. anti-VEGF, anti-IL-6) to revert some of the inhibitory effects on DC. Another interesting finding is that culturing monocytes from cancer patients *ex vivo*, to circumvent the suppressive activity of the tumor milieu, generates DC with a capacity to stimulate allogeneic T cells [158,159]. [160] This finding is important for active DC-based immunotherapeutic approaches, where DC are generated *ex vivo* from monocytes and after arming with tumor-associated antigens, reinjected into the patient with the intension to restore proper presentation of tumor associated antigens (TAA) and T cell activation [161–163]. This concept is currently tested for NSCLC in therapeutic reality with encouraging results on the immune response, safety and tolerability, despite the small sample sizes of the trials [161–163].

Immunogenic cell death biomarkers

Lung cancer is a complex disease with limited treatment options, mainly caused by the close relationship between neoplastic cells and healthy cells. To develop a more effective treatment for lung cancer, we have to focus on the complex interactions that tumor cells have with the local stromal compartment and the involved immune cells, and all of their secreted factors. There is growing evidence that the efficacy of many traditional therapeutic treatments depends on their ability to induce proper immunogenic tumor cell death. This specific release of signals upon tumor cell death may lead to immune activation, and in particular anti-tumor immunity, that contribute to the therapeutic outcome for patients [164,165].

There are different candidate immune biomarkers that can predict the efficacy of specific NSCLC anticancer therapies [166,167]. In NSCLC, nucleosomes have already been proven useful for the early estimation of response to chemotherapy [168–170]. Presence of mature dendritic cells and CD4+ or CD8+ lymphocytes in NSCLC tumors are independent prognostic factors for overall survival, as described above [55,59,171,172]. In addition, other potentially pivotal markers for lung cancer are p53-specific autoantibodies and pyridoxal kinase (PDXK), the enzyme that generates the bioactive form of vitamin B6 [173]. Also a group of immunogenic cell death biomarkers called damage-associated molecular pattern (DAMP) molecules, can serve as prognostic markers for response to therapy and prognosis in cancer patients [174]. DAMPs, such as surface-exposed calreticulin (ecto-CRT) and the high-mobility group box 1 protein (HMGB1); are released in the blood circulation by late apoptotic and necrotic cells upon oxidative and endoplasmic reticulum (ER) stress. In peripheral blood, they bind to specific immune cells and trigger protective T cell responses and promote phagocytosis. One of the main functions of HMGB1 is the binding to specific receptors on dendritic cells and other antigen presenting cells, such as receptors for advanced glycation endproducts (RAGE) and toll-like receptors

4 (TLR4). It has been described that the release of DAMP during cell death is essential for the sustained therapy response after chemotherapy and the efficiency of HMGB1 was found to be increased when bacterial lipopolysaccharide (LPS), DNA or nucleosomes were bound to it. Knockdown of HMGB1 was observed to be associated with reduced anticancer immune response and poor therapy outcome. In contrary, overexpression of HMGB1 and its receptor RAGE is pivotal for the metastasizing of the tumor cells as it promotes neoangiogenesis [175]. Markers of immunogenic cell death are becoming a valuable tool in clinical practice for diagnosis and prediction of response to NSCLC therapy and prognosis [167].

Next to DAMP, there are other approaches using RNA- and DNA-based immune modifiers to augment cancer therapy efficacy by stimulating the immune system. Bacterial DNA is immunostimulatory and can be replaced using synthetic oligo-deoxynucleotides (ODN), for instance CpG oligodeoxynucleotides. CpG ODN are synthetic DNA sequences containing unmethylated cytosine-guanine motifs with potent immune modulatory effects via TLR 9 on DC and B cells [176]. They can induce cytokines, activate NK cells, and elicit T cell responses that lead to strong antitumor effects. It has been shown that CpG ODN downregulates regulatory T cells and TGF-β in peripheral blood of NSCLC patients [177].

Overall, analysis of new and conventional therapeutic strategies should not only be focused on the direct cytotoxic effects of tumor cells but also on the initiation of proper immune responses. Simultaneous modulation of the immune system by immune therapeutic approaches can then induce synergistic anticancer efficacy [178]. Overall, the composition of the immunological cells and cell death markers in the host is, next to the mutation analysis and histological features of the tumor, likely to determine the response to specific chemotherapeutic agents and the prognosis of the patients.

CONCLUSION

In this review, we have shown that the immune system plays a dual role in cancer development and progression and determines the response to treatment in NSCLC. These complex interactions between diverse immune cell types and tumor cells that can actively favor tumor rejection as well as tumor progression, depends on the tumor type, stage and the types of immune cells that are involved. The data presented here reinforce the importance of full understanding of the intricacy of the cellular interactions within the tumor microenvironment. There is a rapid progress in the field of the cancer immunology and the development of novel cancer immunotherapy approaches. Therefore, tumor immunology will probably be used more commonly in

clinical practice in the future, as increasing evidence indicates that the effectiveness of several chemotherapies depends on the active contribution of the different immune effectors. Selecting conventional chemotherapeutic agents that induce proper immunogenic tumor death can synergize with immune response modifiers to revolutionize cancer treatment [179]. Understanding the local and systemic immune mechanisms will lead to new potential therapeutic targets.

We predict that the future standard of care of lung cancer will involve patient tailored combination therapies that associate molecules that target specific genetic mutations or chemotherapeutic drugs with immune modulating agents, driven by the increasing understanding of the immune system in the cancer cell's environment. The future for cancer treatment is bright if we are able to: I). Find a chemotherapeutic drug that induces immunogenic cell death in tumor cells while leaving the normal cells and stimulating immune cells intact. II). Explore ways to efficiently activate the good-natured immune system, for instance, the adoptive transfer of *in vitro* expanded activated T-cells or NK-cells, and III). Modulate the tumor environment to reduce local and systemic immune suppressive components while limiting potential side-effects for the patient; e.g. by the depletion of Treg by denileukin diftitox or polarizing the M2 macrophage towards the M1 subtype. The treatment has to be tuned to the cellular make-up of each patient individually, based on their own both tumoral and immunological characteristics, rather than by the anatomic location of the tumor in the body or by the tumor histology or genetic make-up. This individualized, multi-targeted approach will be able to redress the balance towards efficacious antitumor responses that can improve the overall survival for more patients.

ABBREVIATIONS

APC, Antigen presenting cell(s); CTL, Cytotoxic T lymphocyte(s); CTLA-4, Cytotoxic T lymphocyte-associated antigen 4; DC, Dendritic cell(s); MDSC, Myeloid-derived suppressor cell(s); NK(T), Natural killer (T) cell(s); TAM, Tumor-associated macrophage(s); TIL, Tumor infiltration lymphocyte(s); Treg, Regulatory T cell(s)

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

MH contributed to literature research, data-analysis, interpretation of findings and drafting of the manuscript. JA contributed to study design, literature research, data-analysis, interpretation of findings and critical editing of the manuscript. RC contributed to literature research, data-analysis, interpretarion of findings and drafting of the manuscript. HG contributed to drafting of the manuscript. HH contributed to drafting of the manuscript. JH contributed to study design, literature research, data-analysis, interpretation of findings and critical editing of the manuscript. All authors read and approved of the final manuscript.

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Chapter 7

Elevated ILT3 expression on
myeloid-derived suppressor cells
in peripheral blood of patients with
non-small cell lung cancer;
Sub-analysis of the NVALT12 study

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Chapter 8

Arginase-1 mRNA expression correlates
with myeloid-derived suppressor cell
levels in peripheral blood of patients
with non-small cell lung cancer

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ABSTRACT

Myeloid-derived suppressor cells (MDSC) are a heterogeneous population of immature and progenitor myeloid cells with immunosuppressive activity that are increased in cancer patients. Until now, the characterization of MDSC in humans was very challenging. The aim of this study was to determine the characterization and optimal assessment of MDSC and to investigate their presence and function in blood of advanced-stage NSCLC patients. We determined MDSC and lymphocyte populations in peripheral blood mononuclear cells (PBMC) samples of 185 treatment-naïve NSCLC patients and 20 healthy controls (HC). NSCLC patients had an increased population of PMN-MDSC compared to HC ($p<0.0001$). Frequencies of CD4$^+$ and CD8$^+$ T-cells were significantly decreased in NSCLC patients ($p<0.0001$ and $p=0.05$). We found that PMN-MDSC were able to suppress T-cell proliferation *in vitro*. qRT-PCR showed that arginase-1 (Arg-1) mRNA is mainly expressed by MDSC and that the level of Arg-1 in PBMC correlates with the frequency of MDSC in PBMC (Spearman's rho: 0.797). There were significant differences in MDSC and lymphocyte populations between NSCLC patients and HC. We found that MDSC frequencies are stable up to six hours at room temperature after blood was drawn and that cryopreservation leads to a strong decrease of MDSC in PBMC. We show that Arg-1 mRNA expression is a valuable method to determine the levels of MDSC in peripheral blood of cancer patients. This method is therefore a useful alternative for the complex flowcytometric analysis in large multicenter patient studies.

INTRODUCTION

The modulating effect of tumors on the immune system is challenging.[1] Recent studies have revealed that a specific population of cells called myeloid-derived suppressor cells (MDSC) plays a pivotal role in tumor-associated immune suppression.[2,3]

MDSC are a heterogeneous population of immature myeloid cells and myeloid progenitor cells that can accumulate at the tumor site, in the lymphoid organs and in peripheral blood.[4,5,6] Under pathological conditions, such as cancer, the bone marrow is stimulated by chemokines and cytokines like interleukin (IL)-1β, IL-6, IL-10 and IL-6, vascular endothelial growth factor (VEGF), and colony stimulating factors (GM-CSF, G-CSF and M-CSF). This can lead to early release of MDSC from the bone marrow.[7]

Two major subpopulations of MDSC are defined based on the difference in expression of CD14: polymorphnuclear (PMN) MDSC and monocytic (M) MDSC. PMN-MDSC are characterized as $CD16^{low}$,$CD11b^+$,$CD14^-$,$HLA-DR^-$,$CD15^+$,$CD33^+$ cells, while M-MDSC are characterized by $CD16^{low}$,$CD11b^+$,$CD14^+$,$HLA-DR^-$,$CD15^+$,$CD33^+$.[7,8,11] In addition, PMN-MDSC and M-MDSC differ in function and quantity; M-MDSC use different suppressive mechanisms and are present in much smaller amounts in the peripheral blood of non-small cell lung cancer (NSCLC) patients than PMN-MDSC.[9]

There are several mechanisms by which MDSC suppress anti-tumor responses[6,10-12] and these are highly dependent on the context of their microenvironment.[6,10-12] MDSC can block anti-tumor responses by secreting cytokines, like IFN-γ, TGF-β, and IL-10 and by the production of reactive oxygen species (ROS), inducible NO synthase (iNOS), nitric oxide (NO), and arginase-1 (Arg-1).[6,10-12] The presence of these factors suppresses features of immune responses, like T cell- and natural killer (NK) cell-proliferation.[13] In addition, MDSC can enhance immune suppression via the induction of regulatory T cells (Treg).[3,5,10,11]

In this study we have investigated the presence, phenotypic characteristics and functionality of MDSC in blood of stage IV NSCLC patients. In addition, the differences between healthy controls and NSCLC patients in MDSC subpopulations and the other pivotal immunological subpopulations, $CD4^+$ T cells, $CD8^+$ T cells, and $CD19^+$ B cells were determined. Furthermore, the applicability of Arg-1 mRNA expression as an alternative method for flowcytometry to determine the levels of MDSC in the PBMC fraction of these patients was analyzed.

MATERIALS AND METHODS

Study population

All patients were participants in the NVALT12-study (trial number NCT01171170) a randomized phase II multicentre study on the effect of a nitroglycerin patch or placebo in patients with stage IV non-squamous NSCLC treated with Carboplatin Paclitaxel and bevacizumab. Blood samples were collected at baseline from 185 patients who were not applicable for treatment with curative intent. The stages are in accordance with the American Joint Committee on Cancer (AJCC). All patients were treated in one of the following participating hospitals, Amphia Hospital, Breda; Dutch Cancer Institution, Amsterdam; VU Medical Center, Amsterdam; Haga Hospital, The Hague; UMCG, Groningen; UMC, Maastricht; Isala Clinics, Zwolle; Jeroen Bosch Hospital, Den Bosch; Martini Hospital, Groningen; Sint Antonius Hospital, Nieuwegein; Deventer Hospital, Deventer; Diakonessenhuis, Utrecht; and Sint Franciscus Hospital, Rotterdam.

Twenty healthy controls (HC) with no history of malignancies or autoimmune diseases were enrolled in the study.

Written consent was obtained from all individuals before blood sampling and the study was approved by the ethical committee of the Erasmus Medical Center (MEC-2012-048 (HC) and CCMO: NL33442.042.10 (NSCLC patients))

Isolation of PBMC

Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden) density gradient centrifugation. For this, blood was diluted 1:1 with phosphate-buffered saline (PBS, Gibco, Breda, The Netherlands) before layering onto the Ficoll-Paque PLUS. After centrifugating 20 minutes at 1200xg, PBMC were collected from the plasma-Ficoll interphase. Cells were washed twice with PBS and counted before further analysis.

PBMC were immediately used for flowcytometric analysis or cell sorting. For quantitative RT-PCR, cell pellets of PBMC were snap frozen in liquid nitrogen and stored at -80^{0}C until RNA isolation.

For the experiments where the influence of freezing was tested, part of the PBMC was frozen using RPMI 1640 (Gibco), 40% fetal calf serum (FCS, Sigma Aldrich Chemie GmbH, Steinheim, Germany), 10% dimethyl sulphoxide (DMSO, Sigma Aldrich) and stored at -150^{0}C until use.

Flowcytometry and cell sorting

PBMC were stained with anti-CD15 FITC or PE, anti-CD16 PE or PERCP-Cy5.5, anti-CD124 PE, anti-CD66b PE, anti-CD33 PE Cy7, anti-CD11b APC, anti-HLA-DR

APC-Cy7 (all BD Biosciences), anti-CD14 PE-Texas-Red (Invitrogen, Breda, The Netherlands), and a live/dead marker 4',6-diamidino-2-phenylindole (DAPI, Molecular Probes, Eugene, OR, USA) to analyze MDSC. Staining with anti-CD4 FITC, anti-CD8 APC, anti-CD19 PERCP-Cy5.5 (all BD Biosciences), anti-CD3 APC-eFluor 780 (eBioscience, San Diego, CA, USA), and DAPI was performed for the analysis of T- and B cells.

Cells were washed with FACS buffer (PBS, 0.25% BSA, 5 mM EDTA, 0.05% NaN$_3$) and stained for 30 min at 4^{0}C with the above mentioned antibodies, appropriately diluted in FACS buffer supplemented with 2% normal human serum.

Acquisition of 5 to 8 color samples was done on a LSRII flowcytometer (BD Biosciences). Cell sorting was performed on a FACS Aria (BD Biosciences). Analysis of the data was done using FlowJo software (Treestar, San Carlos, CA, USA).

Detection of reactive oxygen species (ROS)

The oxidation sensitive molecule 2',7'-dichlorofluorescein diacetate (DCFDA, Sigma) was used to measure ROS production by MDSC. Cells were incubated at 37^{0}C in RPMI 1640 in the presence of 0.5 μM DCFDA for 10 minutes and washed twice with cold FACS buffer. Cells were subsequently stained for flowcytometry as described above.

Real-time quantitative RT-PCR

Frozen cell pellets were homogenized and RNA was isolated using RNAqueous micro kit (Ambion Inc, Austin, TX, USA) for sorted cell populations or RNeasy mini kit (Qiagen, GmbH, Hilden, Germany) for PBMC, followed by DNAse I treatment. RNA (100 ng) was reverse transcribed using RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas GmbH, St. Leon-Rot, Germany) with random hexamer primers according to the manufacturers' protocol.

Quantitative RT-PCR for Arg-1 was performed using maxima SYBR Green qPCR mastermix (Fermentas). The following primers were used: β-Actine, forward: CTGTG-GCATCCACGAAACTA, reverse: AGTACTTGCGCTCAGGAGGA; and for Arginase-1, forward: GTTTCTCAAGCAGACCAGCC, reverse: GCTCAAGTGCAGCAAAGAGA.[14] PCR conditions were 2 min at 50^{0}C, 10 min at 95^{0}C, followed by 40 cycles of 15 s at 95^{0}C and 60^{0}C for 1 min using an ABI PRISM 7300 (Applied Biosystems, Carlsbad, CA, USA). PCR amplification of the house keeping gene β-actin was performed during each run for each sample to allow normalization between samples.

T cell suppression assay

PBMC were isolated from a buffy coat of a healthy donor (Sanquin, Amsterdam, The Netherlands) using Ficoll gradient centrifugation as described above. CD8$^+$ T

cells were isolated using a CD8 T cell isolation kit (Miltenyi Biotec B.V., Leiden, The Netherlands) according to manufacturers' protocol. For labeling with carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes), CD8$^+$ T cells were washed twice with serum-free RPMI 1640 and subsequently labeled with 5 µM CFSE in serum-free medium for 10 min at 37^{0}C. The reaction was stopped by adding an excess of ice-cold RPMI 1640 supplemented with 10% FCS.

CFSE labeled CD8$^+$ T cells were stimulated using anti-CD3/anti-CD28 beads (Invitrogen) and co-cultured in a 5:1 ratio with FACS sorted PMN-MDSC in RPMI 1640 supplemented with 20% of pleural effusion of a cancerous patient to prevent further differentiation of MDSC. At day 4, cells were harvested and stained for flowcytometry using anti-CD3 APC-eFluor780 (eBioscience), and anti-CD8 APC (BD Biosciences). Cell division was quantified based on serial halving of CFSE intensity, algorithms provided by FlowJo software (Treestar) were used. Data are shown as percentage of T cells recruited into cell division, calculated as previously described.[15]

Statistical analysis

Differences between healthy controls and NSCLC patients were analyzed by using the Mann–Whitney U test. Correlations were assessed by using the Spearman's rho correlation test. Statistical analysis was performed using the statistical program SPSS (version 17.0, SPSS Inc, Chicago, USA). All p-values were two-sided and p-values below the conventional level of significance (p<0.05) were considered statistically significant. Figures were made in GraphPad Prism (version 7.0, GraphPad Software, San Diego, CA, USA).

RESULTS

Characteristics of study subjects

Table 1 shows the characteristics of the 185 study participants and 20 healthy controls. An approximately equal distribution in gender was seen in the NSCLC group, while most of the healthy controls were female (80%). The mean age in the NSCLC group was 60 years and in the control group 54 years. Adenocarcinoma was the most common histological type (85.4%), followed by large cell carcinoma (14.1%) and adenocarcinoma in situ (0.5%). Most NSCLC patients had a WHO performance score of 0 (47.0%) or 1 (49.3%) at time of inclusion in this study. All healthy controls had a WHO performance score of 0.

Table 1: Subject characteristics of 20 healthy controls and 185 NSCLC patients.

	Healthy controls	NSCLC patients
Number of subjects	20	185
Age (years) (Mean ± SD)	54 ± 7.5	60 ± 8.5
Gender (%)		
Male	4 (20)	93 (50.3)
Female	16 (80)	92 (49.7)
WHO performance score (%)		
0	20 (100)	87 (47.0)
1		91 (49.2)
2		7 (3.8)
Histologic subtype (%)		
Adenocarcinoma		158 (85.4)
Large cell carcinoma		26 (14.1)
Brochoalvoolar carcinoma		1 (0.5)

Phenotypical characterization of MDSC subsets

MDSC populations in peripheral blood were characterized using flowcytometric analysis after density gradient centrifugation. A broad panel of markers was used to detect MDSC, including CD14, CD15, CD16, CD33, CD11b, HLA-DR, and DAPI as a live/dead marker, because unique markers are not identified yet.[6] Two MDSC subsets were identified in blood: PMN-MDSC and M-MDSC.[11] The gating strategy is
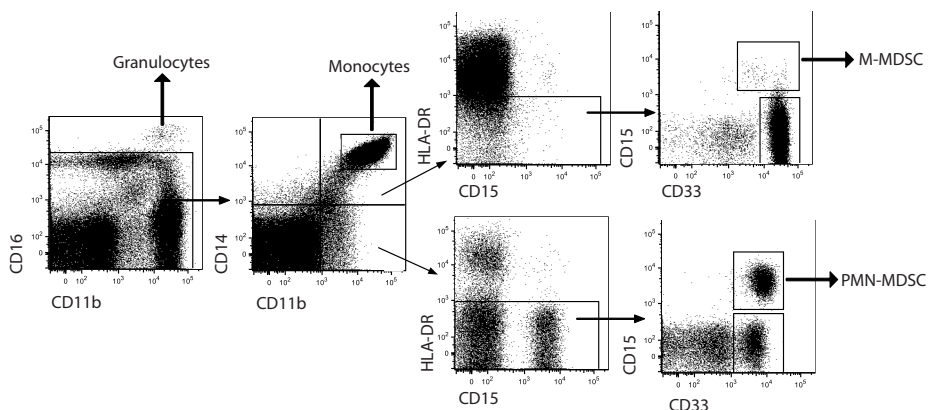
Figure 1: Gating strategy used for the identification of PMN- and M-MDSC. PBMC were isolated from peripheral blood by density gradient centrifugation and subsequently stained for flowcytometric analysis. After excluding debris and death cells, the CD16 low cells were selected. Then CD11b+ CD14+ or CD11b+ CD14- cells were gated, followed by selecting the HLA-DR negative population. The final gate is CD33+ CD15+ cells, markers expressed on both PMN-MDSC and M-MDSC populations.

shown in figure 1. Initial experiments showed a distinct population of PMN-MDSC (mean: 0.95% of alive) in contrast to M-MDSC which were hardly detectable in blood of stage IV NSCLC patients (mean: 0.009% of alive).

CD124 (IL-4Rα) on MDSC, as described in several studies[16,17], was detectable at a very low level on PMN-MDSC. However the same expression was seen on other myeloid cells (data not shown) so this marker has no additional value to discriminate between PMN-MDSC and other PBMC.

Functional characterization of PMN-MDSC

PMN-MDSC express diverse factors that contribute to their immune suppressive activity, including ROS and Arg-1, which can suppress T cell function.[3] ROS production by PMN-MDSC was measured by flowcytometry. The high fluorescent DCF signal demonstrated that a high amount of ROS was present within PMN-MDSC (figure 2a) compared to the negative control.

To evaluate the production of Arg-1 by PMN-MDSC, the mRNA level was measured by qRT-PCR. Figure 2b shows that Arg-1 is mainly produced by PMN-MDSC. This confirms that high Arg-1 mRNA expression is characteristic for PMN-MDSC (figure 2b).

To assess the suppressive capacity of MDSC[16,20], sorted PMN-MDSC were co-cultured with CFSE labeled CD8+ T cells. In the absence of PMN-MDSC 55% of the CD8+ T cells were recruited into cell division. This percentage decreased to 5.8% when PMN-MDSC were added to the culture (figure 2c). This demonstrates that the sorted PMN-MDSC strongly inhibit T cell proliferation *in vitro*.

Effect of cryopreservation on the recovery of PMN-MDSC

In our study, PBMC from NSCLC patients from several hospitals in the Netherlands were analyzed. Therefore, the recovery of the PMN-MDSC after cryopreservating PBMC was analyzed to test whether the blood could be cryopreserved before analysis.

PBMC were divided into two portions; one was immediately used for FACS analysis, while the other was frozen in 10% DMSO and stored at -150⁰C. As shown in figure 3a and 3b, the percentage of PMN-MDSC was strongly reduced in the frozen/thawed samples (n = 10). Only 11% of the cells was recovered after cryopreservation (figure 3a and 3b).

Overnight storage of blood for PMN-MDSC analysis at 4°C

After our finding that frequencies of PMN-MDSC dramatically decrease after cryopreservation, the question raised whether it is possible to store the blood overnight (o/n) at 40C or at room temperature (RT) without influencing the frequency of PMN-MDSC.
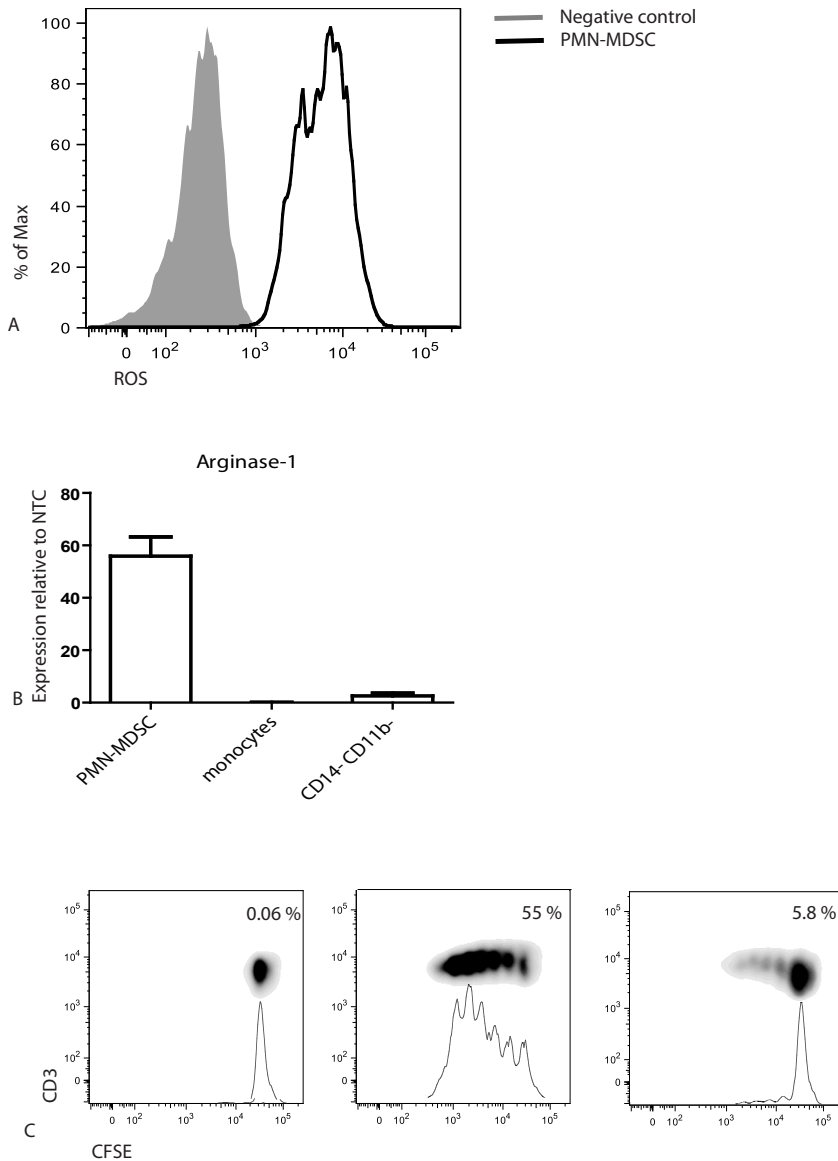
Figure 2: Functional characterization of PMN-MDSC **2A**) Expression of ROS by PMN-MDSC. PBMC were incubated with DCFDA, which is converted into fluorescent DCF in the presence of ROS. The ROS expression in PMN-MDSC is compared to PMN-MDSC not exposed to DCFDA. **2B)** mRNA levels of Arg-1. PMN-MDSC, monocytes and CD14-CD11b+ cells were sorted from 4 patients and used for RNA isolation. The Arg-1 level is 1000x lower in monocytes and 22x lower in CD11b-CD14-cells compared to PMN-MDSC. **2C**) PMN-MDSC were sorted from PBMC and tested for their ability to suppress T cell proliferation. PMN-MDSC and CFSE-labelled T cells were co-cultured in a 1:5 ratio and stimulated with anti-CD3/anti-CD28 beads. After 4 days T cell proliferation was measured. Percentages indicated in the plots represent the percentage of cells recruited into cell division. Representative results from one out of three experiments are shown.
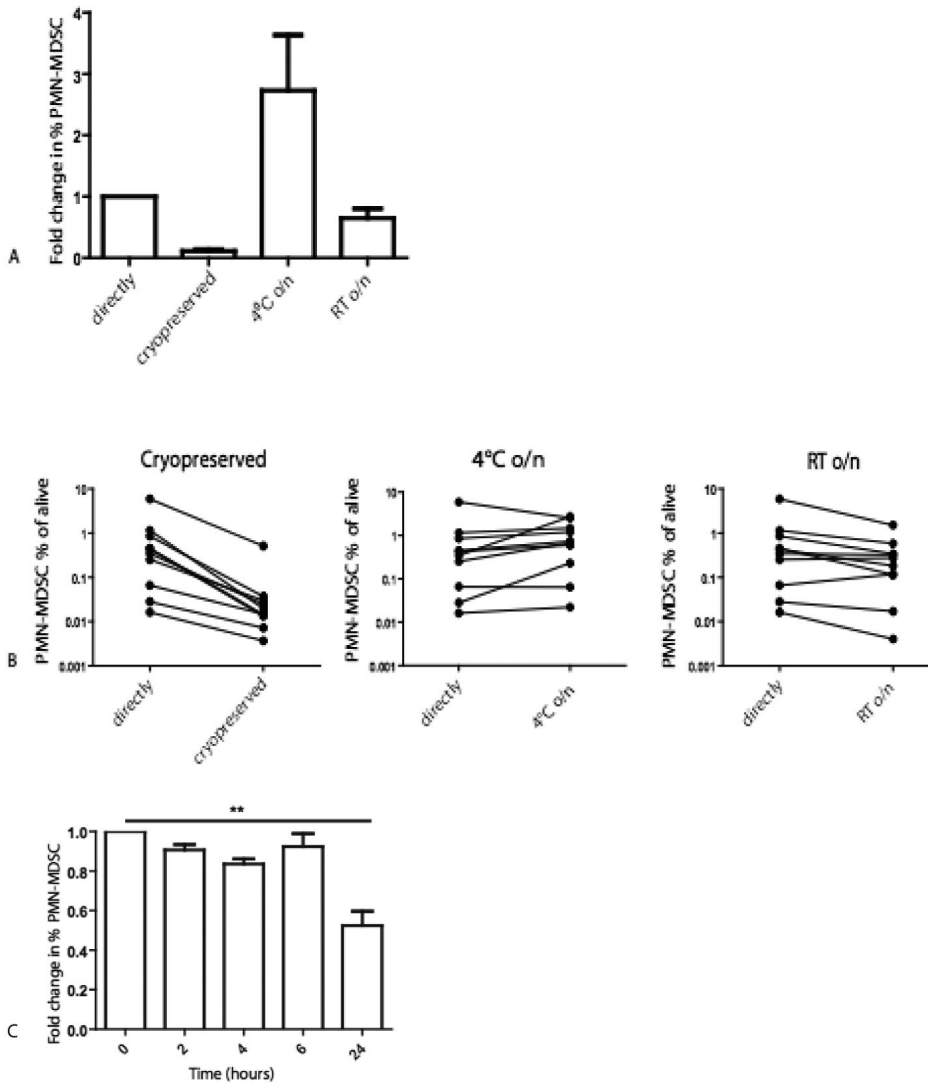
Figure 3: Frequency of PMN-MDSC in directly isolated PMBC compared to the frequency in cryopreserved or overnight stored blood samples (n=10). **3A)** Fold change in frequency of PMN-MDSC after cryopreservation and o/n storage at 4^{0}C or RT. **3B)** Changes in frequency of PMN-MDSC after cryopreservation, o/n 4^{0}C or RT per patient. **3C)** PBMC were isolated from peripheral blood directly after blood was drawn or after storage for 2, 4, 6, and 24 hours at RT (n = 4). No significant differences were seen in PMN-MDSC frequencies until 6 hours after the blood was drawn. However, after 24 hours of storage, the number of PMN-MDSC was significantly decreased (p = 0.0073).

For this experiment, three blood tubes of 10 ml were obtained per patient (n = 10). One tube was used for analysis within two hours after the blood was drawn, the other two were stored o/n at 40C or at RT. PBMC isolation and FACS analysis were performed the next day.

As shown in figure 3a and b, the percentage of PMN-MDSC is 2.7 fold higher when blood is stored o/n at 40C, compared to the freshly analyzed samples. Also the total amount of PBMC per ml blood is increased, which can be caused by a difference in cell density. Storage of blood at 40C can result in an overestimation of the percentage of PMN-MDSC. Overnight storage at RT leads to a 1.6 fold decrease in PMN-MDSC percentage (figure 3a and 3b).

Short-term storage of blood for PMN-MDSC analysis

To determine if variations in PMN-MDSC percentage are introduced over time, due to differences in processing time caused by transportation, we studied the influence of short-term storage on the recovery of PMN-MDSC. Blood samples of NSCLC patients samples were analyzed immediately or after 2, 4, 6, and 24 hours after the blood was drawn. Figure 3c shows that the percentage of PMN-MDSC remains constant at room temperature in the first six hours. A reduction of 50% was found when the blood is stored overnight.

After our finding that frequencies of PMN-MDSC dramatically decrease after cryo-preservation, the question raised whether it is possible to store the blood overnight (o/n) at 4^0C or at room temperature (RT) without influencing the frequency of PMN-MDSC.

For this experiment, three blood tubes of 10 ml were obtained per patient (n = 10). One tube was used for analysis within two hours after the blood was drawn, the other two were stored o/n at 4^0C or at RT. PBMC isolation and FACS analysis were performed the next day.

As shown in figure 3a and b, the percentage of PMN-MDSC is 2.7 fold higher when blood is stored o/n at 4^0C, compared to the freshly analyzed samples. Also the total amount of PBMC per ml blood is increased, which can be caused by a difference in cell density. Storage of blood at 4^0C can result in an overestimation of the percentage of PMN-MDSC. Overnight storage at RT leads to a 1.6 fold decrease in PMN-MDSC percentage (figure 3a and 3b).

Frequencies of PMN-MDSC and M-MDSC are increased in PBMC of NSCLC patients

Using gradient density centrifugation and flow cytometry, the frequency of PMN-MDSC and M-MDSC was analyzed in the PBMCs of 185 NSCLC patients and 20 healthy controls. Table 2 and figure 4a show the percentages, expressed as % of alive cells, and absolute numbers, expressed as cells/mL blood of circulat-
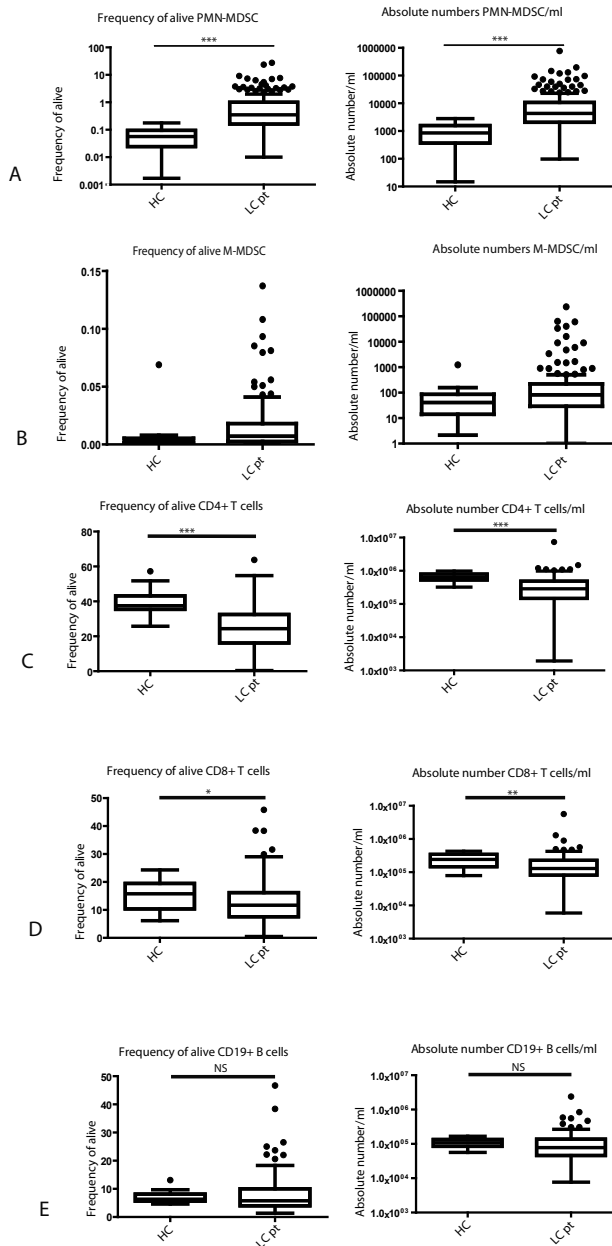
Figure 4: Frequency of PMN-MDSC, CD4+, CD8+ T cells and CD19+ B cells in healthy controls (HC) and NSCLC patients (LC pt). **4A)** Data showing the difference between HC and LC pt in the percentage of alive of PMN-MDSC (left) and the absolute number per ml of PMN-MDSC (right) in the peripheral blood. Data are presented as box-and-whisker plots (1-99 percentile) showing the median, quartiles and outliers. **4B)** The difference between HC and LC in percentage of alive (left) and absolute number per ml (right) of M-MDSC. **4C)** The percentage of alive (left) and absolute numbers per ml blood (right) of CD4+ T cells, **4D)** CD8+ T cells and **4E)** CD19+ B cells.

Table 2: Differences in MDSC populations, T cells and B cells between healthy controls (HC) and NSCLC patients. The table shows the mean percentage of alive of five pivotal immunological cell types in HC and NSCLC patients. The p-values were assessed by using a Mann–Whitney *U* test.

Cell type (mean percentage of alive)	Healthy controls	NSCLC patients	p-value
MO-MDSC (± SD)	0.006 (± 0.02)	0.150 (± 1.19)	0.002
PMN-MDSC (± SD)	0.064 (± 0.05)	1.212 (± 2.97)	< 0.0001
CD4+ T cell (± SD)	39.1 (± 7.33)	24.3 (± 11.87)	< 0.0001
CD8+ T cell (± SD)	15.1 (± 5.61)	12.6 (± 7.36)	0.050
CD19+ B cell (± SD)	6.9 (± 2.12)	7.9 (± 6.13)	0.575

ing PMN-MDSC (CD16^{low},CD11b$^+$,CD14$^-$,HLA-DR$^-$,CD15$^+$,CD33$^+$) and M-MDSC (CD16^{low},CD11b$^+$,CD14$^+$,HLA-DR$^-$,CD15$^+$,CD33$^+$). Analysis of circulating PMN-MDSC revealed that there was a significantly higher percentage, 1.212 vs 0.064 (p < 0.001), and absolute number (p < 0.001) of PMN-MDSC in the peripheral blood of NSCLC patients compared to healthy controls (figure 4b). Also the absolute number and percentage of M-MDSC was significantly higher (p =0.036 and p = 0.002) in patients.

Frequency of T cells is decreased in PBMC of NSCLC patients

To further investigate differences in PBMC between patients and healthy controls percentages and absolute numbers of circulating CD4$^+$ T cells, CD8$^+$ T cells and CD19$^+$ B cells, were measured (table 2). CD4$^+$ T cells and CD8$^+$ T cells in the peripheral blood of NSCLC patients were significantly decreased compared to healthy controls (p < 0.0001 and p = 0.05, respectively of frequency of alive)(figure 4c/d). Also the absolute numbers of CD4$^+$ T cells and CD8$^+$ T cells were significantly decreased (p < 0.001 and p = 0.018, respectively). The frequency of alive and the absolute numbers of CD19$^+$ B cells were similar between healthy controls and patients (figure 4e).

Arginase-1 mRNA expression correlates with frequency of PMN-MDSC

Arg-1 activity is one of the suppression mechanisms of MDSC. To investigate the differences in Arg-1 mRNA expression in HC and NSCLC patients, the Arg-1 mRNA expression was measured in both groups using a qRT-PCR. As shown in figure 5a, the expression in NSCLC was significantly higher compared to the HC (p < 0.0001).

As shown in our results above, Arg-1 mRNA is mainly expressed by PMN-MDSC in peripheral blood. To confirm this, Arg-1 mRNA levels in PBMC were correlated with the frequency of PMN-MDSC in the peripheral blood of NSCLC patients. A significant correlation between these two measures was seen (Spearman's rho: 0.797, p-value < 0.0001).
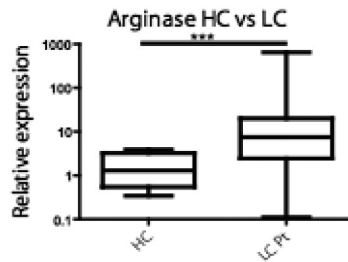
Figure 5: Arginase-1 (Arg-1) mRNA expression is increased in the peripheral blood of NSCLC patients (LC pt) Figure **5** shows the difference in mRNA expression of Arginase-1 in PBMC between healthy controls (HC) and LC pt (p < 0.0001).

DISCUSSION

It has been described that MDSC are present in most cancer patients.[6] To our knowledge, this is the first large study with 185 patients that demonstrates that MDSC are present in Caucasian NSCLC patients. Earlier, MDSC have been reported in a relatively small (n = 10) heterogeneous population of lung cancer patients[23] and in two other studies that investigated MDSC in a genetically different population of Asian lung cancer patients.[9,24] Both studies used a limited set of markers to define the MDSC population. This phenotypic diversity of MDSC, as well as the lack of common markers to study these cells, has generated ambiguity in their characterization. Consequently, the described suppressive activity of the MDSC can be due to the influence of other cells, for instance, suppressive $CD16^+$ granulocytes. In this study an extensive set of all known markers for MDSC was included to characterize these cells.

In the present study, we demonstrate a significant increase in circulating MDSC in patients compared to healthy controls, while, on the contrary, the populations of $CD4^+$ T cells and $CD8^+$ T cells were decreased. Furthermore, there is a high expression of Arg-1 mRNA which correlated with the percentage of PMN-MDSC and suppression assays showed that PMN-MDSC have the ability to suppress $CD8^+$ T cell activity. No differences in mRNA expression between males and females (data not shown) were seen. Therefore, selection bias is not likely.

As there was no clear consensus whether PBMC cryopreservation influences the phenotype, frequency and functionality of MDSC, PMN-MDSC were compared before and after cryopreservation, as cryoperservation is useful for large multicenter studies and reduces the day-to-day variability. Our data indicate that cryopreservation of PBMC had a strong impact on the PMN-MDSC recovery and led to a significant reduction in their frequency (figure 3a).[22] Therefore, when PMN-MDSC are studied in human blood samples it is essential to use fresh, not cryopreserved cells. In addition, it is recommended to analyze the blood within 6 hours.

In this study we have also shown that the numbers of CD4^{+} and CD8^{+} T cells were significantly decreased in NSCLC patients compared with healthy controls. It has been described that in cancer patients the decreased number of lymphocytes is (partially) due to the suppressive effects of MDSC and Treg on T cells.[28] In addition, lower thymic output and a higher apoptosis rate can cause lower frequencies of lymphocytes.[29] Decreased numbers of lymphocytes in peripheral blood are associated with worse survival of lung cancer.[24,27] As described in literature, no differences in B cell numbers were found.[41]

There are multiple immunosuppressive mechanisms that can be used by MDSC.[3,6,10,11] L-arginine depletion by enzymatic activity of Arg-1 is probably one of the most important pathways to be reported in human MDSC.[12,17,30] In the current study we showed Arg-1 is mainly produced by PMN-MDSC and the percentage of PMN-MDSC correlates with the Arg-1 levels in the peripheral blood of NSCLC patients (figure 5). In addition, we suggest that Arg-1 could be used as a surrogate marker for the frequency of MDSC in PBMC. This could have major advantages; first, Arg-1 expression levels can be measured by qRT-PCR instead of six non-specific markers that are needed to identify the MDSC population with flowcytometry. As a result, no expensive flowcytometers are needed to investigate this cell population. Next to this, Arg-1 can be reliably measured in the PBMC fraction that is snap frozen after ficoll isolation. This is useful for large patient multicenter studies, because Arg-1 can be measured at a later time point, in contrast to measuring MDSC on FACS. This might be a valuable tool to reduce the day-to-day variability.

The main effects of Arg-1 expression on T-cell dysfunction are caused by L-arginine depletion.[3,32] It has been described that T cells cultured in medium lacking L-arginine showed a decrease of CD3zeta expression, an activation marker for T-cells. The loss of CD3zeta expression fully reestablished after replacement of L-arginine and citrulline.[31,33,34] The other mechanism by which Arg-1 can cause T cell dysfunction is L-arginine deprivation that can specifically inhibit the cell cycle progression leading to decreased T-cell proliferation.[3,35] The addition of L-arginine to the L-arginine deprived cells without L-arginine recovered the cell cycle progression.[35]

In the future, lung cancer treatment might be tailored to the cellular make-up of each patient individually, based on tumoral and immunological characteristics.[36,37] A strategy could be to intervene with the immunosuppressive pathways used by MDSC.[3,28] In case of the Arg-1 immunosuppressive mechanism, the addition of arginase inhibitors N-Hydroxy-nor-L-Arg (Nor-NOHA) and N-Hydroxy-L-Arg (NOHA) or exogenous L-arginine partially prevented the reduction of L-arginine in culture and reversed the loss of CD3zeta on T-cells.[31,32,38] The same was seen *in vivo* in Lewis lung carcinoma bearing mice; inhibition of Arg-1 decreased the tumour growth.[39] We found high mRNA levels of Arg-1 in NSCLC patients compared to healthy controls;

Therefore, Arg-1 inhibitors may represent a target for new therapies in lung cancer patients.

In conclusion, there are significant differences in MDSC and lymphocyte populations between NSCLC patients and healthy controls. We found that Arg-1 mRNA is mainly expressed in PMN-MDSC and correlates with the frequency of PMN-MDSC in the PBMC fraction of peripheral blood. Therefore, we propose that qRT-PCR on Arg-1 might be useful as an additional or alternative method for flowcytometry to determine the levels of PMN-MDSC in peripheral blood of cancer patients.

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Chapter 9

Pre-treatment immune profile
correlates to performance status
and stage of the disease in patients
with non-small cell lung cancer;
Sub-analysis of the NVALT12-study

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Chapter 10

History of tuberculosis as an
independent prognostic factor
for lung cancer survival

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ABSTRACT

Introduction It is well known that pulmonary tuberculosis is associated with an increased risk of lung cancer. We investigated whether a history of pulmonary tuberculosis is an independent risk factor for lung cancer survival in Caucasian patients.

Methods The data of the prospective population-based cohort of The Rotterdam Study were used. During a mean follow-up time of 18 years, there were 214 incident cases of pathology-proven lung cancer in a source population of 7983 study participants. History of tuberculosis was assessed at baseline by interviewers using standardized questionnaires. Associations of lung cancer survival with the occurrence of pulmonary tuberculosis were assessed using Cox's proportional hazard regression analysis adjusted for age, gender, pack-years, educational level and tumor stage.

Results A history of tuberculosis was reported in 13 of the 214 subjects with lung cancer. The survival of patients with lung cancer was significantly shorter in subjects with a history of pulmonary tuberculosis (HR = 2.36, CI95%: 1.1-4.9), than in subjects without a history of pulmonary tuberculosis with a mean difference of 311 days.

Conclusion: The presence of a history of pulmonary tuberculosis may be an important prognostic factor in the survival of lung cancer.

KEY WORDS

Lung cancer, tuberculosis, prognostic factor, survival

INTRODUCTION

Worldwide, lung cancer is the most common cause of cancer mortality with a five-year survival rate of only 15%.[1] Recently, it has been described that a strong increase in the incidence rate of lung cancer is expected in developing countries.[2] The incidence of tuberculosis is over 9 million new cases per year worldwide, with an uneven distribution. Developing countries harbor a large burden of tuberculosis. In South Africa, for example, there was a six-fold increase of tuberculosis cases over the past two decades.[3] Given the large numbers of morbidity and mortality worldwide of both lung cancer and tuberculosis, associations between these epidemics deserve investigation. It is well known that pulmonary tuberculosis is associated with an increased risk of lung cancer.[4-6]

Although it is not completely understood what the underlying mechanism for this increased risk is, it has been reported that scarring of the lung after tuberculosis predisposes to the development of lung cancer in these patients, especially adeno-carcinoma.[4-6] Recent data suggest that ongoing inflammatory reactions in the lungs of these patients are the primary cause of scar formation and may be the cause of malignant transformation of cells in these areas.[4-6]

Apart from the fact that tuberculosis scar formation has been identified as a risk factor for lung cancer development, there is evidence that a history of tuberculosis might be an additional prognostic factor for lung cancer survival[5,7], next to other well-known prognostic factors such as disease stage and World Health Organization (WHO) performance status.[8] It has been reported that patients with active pulmonary tuberculosis at the time of lung cancer diagnosis or pulmonary tuberculosis development within 2-10 years before or after the diagnosis of lung cancer have a shorter survival time than those without pulmonary tuberculosis in the same periods.[5,7] However, these retrospective studies had a number of methodological limitations, notably selection- as well as information bias. So far, no population-based prospective cohort studies have been performed to investigate whether there is a difference in mortality of lung cancer in patients with and without a life long history of pulmonary tuberculosis.

Therefore, the objective of our study was to investigate whether a history of pulmonary tuberculosis is an independent risk factor for lung cancer survival in a prospective population-based cohort study of Caucasian patients in the Netherlands.

MATERIAL AND METHODS

Source population

Data were obtained from The Rotterdam Study[9], a population-based prospective cohort study in Rotterdam, the Netherlands. The main objective of The Rotterdam Study is to investigate chronic diseases and their risk factors in an elderly population. All inhabitants of the Rotterdam suburb Ommoord aged ≥ 55 years were invited to participate in the study, which started with a baseline interview between July 1989 and July 1993. Of the 10,215 eligible subjects, 7,983 (78%) agreed to participate.[9] The Medical Ethics Committee of the Erasmus Medical Center approved the study and all participants gave written informed consent. Participants were visited at home at the start of the study for a standardized interview on health state and socio-economic characteristics. Afterwards, an extensive physical examination and blood assessment followed at the research center. Since the start of the study, cross-sectional surveys have been carried out periodically. In addition, participants are continuously monitored for major events, including cancer, which occurred during follow-up, through automated linkage with files from general practitioners, and laboratories. Information on their vital status is obtained regularly from public health authorities and general practitioners in Rotterdam.

Study population

From January 1st, 1990 to December 31st 2008, 214 pathologically confirmed incident lung cancer cases were diagnosed in our cohort. The diagnoses of lung cancer were obtained through information from the general practitioners and by linkage with a nationwide pathology registry (PALGA). Two research physicians independently validated lung cancer cases on the basis of medical records of the general practitioner, discharge letters, and pathology reports and assessed the first date and diagnosis of lung cancer. All events were classified according to the International Classification of Disease (ICD) tenth edition.[10] The start date of a participant in this study was defined as the date of first diagnosis of lung cancer. Patients were followed until death, or end of the study period (December 31st, 2008), whichever came first. Causes of death were defined using information obtained from the death certificate or from the clinics.

Information on tobacco use was collected during the home interview. Smoking status was repeatedly assessed during follow-up every 3- to 4-years. So, people were classified as current smokers, past smokers and never smokers on a continuous basis. Moreover, the number of pack-years was calculated.

Exposure definition

Pulmonary tuberculosis was assessed by standardized home-interviews at baseline by the questions: "Did you ever experience tuberculosis?", "Did you have a course of treatment for tuberculosis?", "Was your tuberculosis treated with drugs?" and "What was your age at the time of first diagnosis of tuberculosis?"

Statistical analysis

The patient survival time for lung cancer was calculated as the period between the first date of the pathologically confirmed diagnosis of lung cancer and death, or end of the study period. Associations between pulmonary tuberculosis and lung cancer survival were assessed by using Cox's proportional hazard regression analysis and expressed as hazard ratios with 95% confidence limits (95%CI). The results were adjusted for age, gender, pack-years of smoking, smoking status, level of education, histology of the tumor and tumor stage, because these factors have been described to influence the lung cancer survival and were considered as potential confounders. Information about smoking status was repeatedly updated every 3- to 4 years. Statistical analysis was performed using the statistical program SPSS (version 15.0, SPSS Inc, Chicago, USA). All p-values were two-sided and p-values below the conventional level of significance (p<0.05) were considered statistically significant.

RESULTS

Table 1 shows the characteristics of the 214 study participants of whom 13 had a history of tuberculosis. All patients had developed their tuberculosis before the diagnosis of lung cancer. Approximately two third of the 214 subjects participating in this study were male (p = 0.113). The mean age at diagnosis of lung cancer was 77 years (p = 0.094). Most subjects currently smoked tobacco or had a history of smoking (86.4%) with a mean of 36.8 pack-years (p = 0.293). In the tuberculosis group, large cell carcinoma was the most common histological type (38.5%), followed by adenocarcinoma (30.8%) and small cell lung carcinoma (SCLC) (7.7%), as shown in table 1. In the non-tuberculosis group, large cell carcinoma was the most common histological type (28.4%), followed by squamous cell carcinoma (23.9%), SCLC (14.4%) and adenocarcinoma (12.4%) (p = 0.521). 30.8% of the tuberculosis cases had at least intermediate vocational education against 38.4 % of the non-tuberculosis group (p = 0.278).

Table 2 shows the crude and the adjusted hazard ratios (HR) for time to death within lung cancer patients. The hazard ratios (HR) divide the death rate in lung cancer patient with the characteristic by the death rate in lung cancer patients without

Table 1 shows the subject characteristics of 214 lung cancer patients with and without a history of tuberculosis.

| Characteristic	Number (% of total)	History of tuberculosis number (%)	No history of tuberculosis (%)	p-value
Number of subjects	214 (100)	13 (6.1)	201 (93.9)	
Follow-up (days) (Mean ± SD)	473 ± 733	182 ± 180	493 ± 751	<0.001
Age at diagnosis (years) (Mean ± SD)	77 ± 9.1	73 ± 8,3	77 ± 9.1	0.094
Gender				
Male	144 (67.3)	11 (84,6)	133 (66.2)	0.113
Female	70 (32.7)	2 (15.4)	68 (33.8)	
Smoking Status				
Never	20 (9.3)	0 (0)	20 (10.0)	0.003
Current	119 (55.6)	9 (69.2)	110 (54.7)	
Former	66 (30.8)	4 (30.8)	62 (30.8)	
Unknown	9 (4.3)	0 (0)	9 (4.5)	
Pack-years (Mean ± SD)	36.8 ± 27.8	48.7 ± 26,3	36 ± 27.8	0.293
Stage of lung cancer at diagnosis				
I	4 (1.9)	0 (0)	4 (2.0)	0.626
II	13 (6.1)	1 (7.7)	12 (6.0)	
III	34 (15.9)	3 (23.1)	31 (15.4)	
IV	139 (65.0)	8 (61.5)	131 (65.2)	
Unknown	24 (11.2)	1 (7.7)	23 (11.4)	
Histologic subtype				
Squamous cell	48 (22.4)	0 (0)	48 (23.9)	0.521
Adenocarcinoma	29 (13.6)	4 (30.8)	25 (12.4)	
Large cell	62 (29.0)	5 (38.5)	57 (28.4)	
SCLC	30 (14.0)	1 (7.7)	29 (14.4)	
Unknown	45 (21)	3 (23.1)	42 (20.9)	
Highest education				
Primary	76 (35.5)	6 (46.2)	70 (34.8)	0.278
Lower vocational	33 (15.4)	2 (15.4)	31 (15.4)	
Lower secondary	20 (9.3)	1 (7.7)	19 (9.5)	
Intermediate vocational	59 (27.6)	4 (30.8)	55 (27.4)	
General secondary	12 (5.6)	0 (0)	12 (6.0)	
Higher vocational	10 (4.7)	0 (0)	10 (5.0)	
Unknown	4 (1.9)	0 (0)	4 (2.0)	

Table 2 shows the crude and the adjusted hazard ratios (HR) for time to death within lung cancer patients. The hazard ratios (HR) divide the death rate in lung cancer patient with the characteristic by the death rate in lung cancer patients without the characteristic (reference group) as a measure of relative risk.

Characteristic*	Crude HR	Adjusted HR
	(95%CI)	(95%CI)
History of tuberculosis	1.75 (1.0-3.1)	2.36 (1.13-4.91)
Age at diagnosis (years)	1.00 (0.99-1.01)	0.98 (0.96-1.01)
Gender		
Male	1.08 (0.8-1.5)	1.09 (0.70-1.71)
Female	1.00 (reference)	1.00 (reference)
Smoking Status		
Never	1.00 (reference)	1.00 (reference)
Current	0.82 (0.5-1.3)	0.85 (0.5-1.4)
Former	1.46 (0.7-2.9)	1.52 (0.8-3.0)
Pack-years		
0	1.00 (reference)	1.00 (reference)
1-10	1.76 (0.2-13.6)	2.27 (0.26-19.98)
11-20	2.10 (0.3-16.3)	3.74 (0.43-32.75)
>20	1.44 (0.2-10.4)	2.38 (0.28-20.03)
Stage of lung cancer at diagnosis		
I	1.00 (reference)	1.00 (reference)
II	1.31 (0.4-4.7)	1.47 (0.35-6.24)
III	3.81 (1.0-14.6)	2.68 (0.60-11.96)
IV	4.83 (1.5-15.3)	4.73 (1.22-13.92)
Histologic subtype		
SCLC	1.00 (reference)	1.00 (reference)
Squamous cell	2.13 (1.42-3.21)	2.71 (1.54-4.77)
Adenocarcinoma	2.70 (1.65-4.42)	1.94 (0.91-4.18)
Large cell	1.27 (0.77-2.08)	3.00 (1.54-5.86)
Highest education		
Primary	1.00 (reference)	1.00 (reference)
Lower vocational	0.69 (0.25-1.97)	0.16 (0.05-0.48)
Lower secondary	1.20 (0.42-3.40)	0.22 (0.07-0.70)
Intermediate vocational	0.89 (0.31-2.52)	0.22 (0.07-0.70)
General secondary	1.36 (0.46-4.00)	1.40 (0.45-4.37)
Higher vocational	0.72 (0.26-2.01)	0.24 (0.08-0.66)

* See table 1 for absolute numbers.

the characteristic (reference group) as a measure of relative risk. The crude HR of history of tuberculosis shows a 1.75-fold increased risk (95%CI: 1.0-3.1) and the HR of history of tuberculosis adjusted for age, gender, disease stage, histological subtype, highest education, smoking status and pack-years, shows a 2.36-fold increased risk (95%CI: 1.1-4.9) with a mean difference of 311 days.

Figure 1 shows the differences in survival of lung cancer in patients with a history of tuberculosis and patients without a history of tuberculosis. The survival time is defined as the time period between date of first diagnosis of lung cancer and death due to lung cancer, or end of the study period (December 31st, 2008), whichever came first. The total number of deaths due to lung cancer was 208 out of 214. Six patients without a history of tuberculosis were still alive at the end of the study period whereas those with a history of tuberculosis had all died.
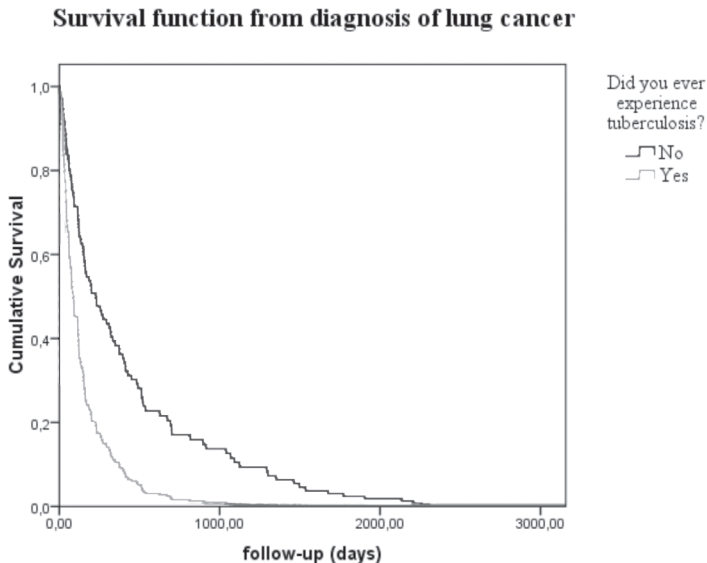
Figure 1 Survival function from diagnosis of lung cancer. The figure shows the differences in cumulative survival in patients with and without a history of tuberculosis. The x-axis is the follow-up time (fup) in days, which is defined as the time period between the date of first diagnosis of lung cancer and death, or end of the study period (December 31st, 2008), whichever came first.

DISCUSSION

To our knowledge, this is the first study that demonstrates that a history of pulmonary tuberculosis is an independent poor prognostic risk factor for lung cancer survival. Earlier, it has been reported that patients with active pulmonary tuberculosis at the time of lung cancer diagnosis or pulmonary tuberculosis up to more than 10 years

before the diagnosis of lung cancer have a shorter survival time than those without pulmonary tuberculosis.[5, 7] The first study was a case-control study performed fifteen years ago in Taiwan.[5] A limitation of this study is that patients were described only if they were diagnosed with tuberculosis up to 2 years before they were diagnosed with lung cancer, or patients who were diagnosed with lung cancer and tuberculosis at the same time. This might have introduced selection bias as well as information bias. Another limitation of that study is that the survival analysis was confounded by a pivotal prognostic factor of lung cancer, i.e. stage of disease. The second study was a cohort study performed among farmers living in the rural county of Xuanwei, China.[7] These farmers had the highest lung cancer mortality rates among the country; therefore, in our opinion, these results indicate a lack of external validity.[4-7, 11]

It is well known that there are two major prognostic factors for lung cancer: WHO performance status and disease stage, but there are also differences in survival of lung cancer between the different histological subtypes.[10-13] The distribution of histological subtype in the group of patients without a history of tuberculosis is similar to what is found in the general population in the Netherlands.[14] An epidemiological study in a large group of tuberculosis patients already demonstrated that the relative risk for lung cancer subtypes in patients with a history of tuberculosis compared to non-tuberculosis patients was highest for adenocarcinoma.[45] Adenocarcinomas are more slowly growing tumors, with a better prognosis than small cell carcinoma and squamous cell carcinomas.[10, 12-13] Although, in the group with a history of tuberculosis, large cell carcinomas and adenocarcinomas were the most common histological types of lung cancer, the mortality risk in this group was higher than the risk in the non-tuberculosis group, with more small cell carcinomas and squamous cell carcinomas.

We hypothesize that the explanation for the poor survival in the tuberculosis group is of immunological origin. After infection with tuberculosis there is an active immunological response that may lead to the development of granulomas in the lungs. These granulomas contain mycobacteria and numerous lymphoid and myeloid cells.[15-16] As a response to the presence of mycobacteria, inflammatory cells will release factors to eradicate the pathogen and enhance the immune reactions.[15-16] This ongoing reaction to the mycobacterial products can result in an accumulation of inflammatory cell types within the infected area in the lung. These inflammatory cells produce large amounts of chemokines, cytokines, lytic enzymes and other substances, like reactive oxygen species (ROS).[15-19] Release of these substances will eventually cause DNA damage, genetic alterations of epithelial cells and extensive fibrosis of lung tissue.[16, 19-21] Tissue repair is associated with cellular proliferation, a process that may lead to further DNA mutations. Lung cancers originating from areas of pulmonary fibrosis are also referred to as scar carcinomas. Several studies have shown that lung cancer patients with a history of pulmonary fibrosis have a decreased

survival, despite the fact that lung cancers originating from these areas of fibrosis are usually adenocarcinomas, as in our study.[22-23]

Furthermore, the decreased survival of lung cancer patients with a history of tuberculosis could be explained by inflammatory conditions that might not only induce malignant cell growth, but may also enhance tumor progression, as described by Mantovani et al.[23-25] The accumulation of macrophages, myeloid derived-suppressor cells (MDSC) and regulatory T cells (Tregs) in these areas impair innate and adaptive immune responses against tumor cells via the production of immune suppressive cytokines and the induction of T cell tolerance. Pro-inflammatory cytokines, such as TNF and IL-6 may lead to up regulated expression of anti-apoptotic genes through the NF-κB pathway.[26] Moreover, the production of substances, like vascular endothelial growth factor (VEGF), matrix metallopeptidases (MMP) by tumor-associated macrophages (M2 macrophages) and MDSC contributes to angiogenesis (a common feature of tissue repair and essential for tumor growth) and tumor invasion.[24-25, 27]

With an intact immune system it is estimated that not more than 10% of tuberculosis infections will progress to active disease. Impairment of the immune system can increase the risk of reactivating a latent tuberculosis, for example in HIV-infected patients to up to 10% per year. An intrinsic impairment of the immune system could therefore be another explanation for our observation that lung cancer patients with a history of tuberculosis have a worse prognosis. Less data support the tumor suppressive effect of a pro-inflammatory status. From animal and human models we know that, between other factors, macrophages, CD4+ and CD8+ cells, interferon-γ, tumor necrosis factor-α, interleukins and apoptosis of infected cells are important components of the immunity to Mycobacterium tuberculosis (*M. tuberculosis*).[15] Macrophages play a central role in the immune response to infection with *M. tuberculosis*. Tumor-associated macrophages represent the major inflammatory component of tumors, affecting different aspects of tumor growth. Macrophage activation has been described as a spectrum, with the 2 extremes described as M1 and M2. Different from M2, M1 is associated with tumor suppression. Infection with *M. tuberculosis* will result in a classical M1 activation with cytotoxic activity both to ingested intracellular micro-organisms like *M. tuberculosis*, and to tumor cells. Less M1 activity might result in both reactivation of tuberculosis infection and in less cytotoxic antitumor response and faster tumor growth. Evidence for this hypothesis comes from studies, which found that the presence of a low M1 macrophage density in the tumor is associated with an impaired lung cancer prognosis.[28-29] This demonstrates that M1 macrophages play a pivotal role in the overall survival of cancer.

Because of the prospective cohort design and high participation rate of our study, selection and information bias are less likely while we adjusted for most known confounders. Our findings cannot be generalized to the total population because all

study members were 55 years or older. However, the effect of this restriction is likely to be modest as 75-85% of the lung cancer arises in people aged above 55 years.[30-31]

Notably, we cannot exclude some misclassification of the exposure due to self report of tuberculosis. Not all subjects may remember their respiratory tuberculosis, or they may be unaware that they have had tuberculosis in the past. Though, we were able to confirm eleven out of the thirteen respiratory tuberculosis cases on the basis of medical records; for the remaining two cases no data were available. However, such non-differential misclassification of exposure is usually random which leads to an underestimation of a true association. This might mean that our results are conservative.

Another limitation is that we did not have data available on the prognostic factor WHO performance status of our lung cancer cases. It would have been preferential if we could have adjusted for this aspect in the survival comparison, because together with disease stage it is one of the most important prognostic factors for lung cancer survival. However, there is no evidence to assume that a history of tuberculosis at least 16 years before lung cancer diagnosis will influence the performance status of the patients at time of diagnosis. This is supported by the comparable findings in characteristics between the two groups in age at diagnosis, stage of disease, histological subtype and pack-years. Therefore, we hypothesize that a history of tuberculosis might cause changes in the microenvironment of the lung tissue and that these immunological changes lead to a shorter survival of lung cancer.

Unfortunately, we were not aware of the treatment of the patients. However, treatment of lung cancer is rarely curative, and there are no established therapies, which convincingly increase survival after adjusting for type and stage of the lung cancer. As we adjusted for type and stage, it is not likely that treatment confounded our results.

The number of participants included in the cohort of the Rotterdam Study is large (n = 7,983), all patient data are well documented, and the study design is unique for world-wide cohorts. However, due to relatively low incidences of both lung cancer and tuberculosis in The Netherlands it is not realistic to double or triple this cohort to reaffirm our data. Nevertheless, the results obtained were striking in the way that a significant correlation between the survival of lung cancer and a history of tuberculosis infection was found.

In conclusion, a history of pulmonary tuberculosis seems to be a poor prognostic factor for lung cancer survival in Caucasian patients. Because of the low numbers in our study, replication of our results in independent cohorts is warranted. Apart from epidemiological studies, more research is needed to elicit common immunological pathways in the etiology of tuberculosis and lung cancer.

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Part IV

General discussion and summary

Chapter 11

General discussion and summary

GENERAL DISCUSSION

This thesis describes the role of early detection of lung cancer by screening programs and the influence of the immune system on the course of lung cancer treatment. Survival of lung cancer can be improved by intervening on two pivotal prognostic fac-tors: disease stage and treatment. In the following sections we elaborate on the main findings from this thesis and discuss the implications these might have on the detec-tion and treatment of lung cancer. At the end we will speculate about future research.

Evaluating the role of lung cancer screening

In the studies described in chapter IV and V, we evaluate the use of lung cancer screening programs. As early stages of lung cancer have a better prognosis, early diagnosis by screening programs could lead to reduction of lung cancer mortality. However, important biases, including lead-time bias, length time bias and overdiagnosis should be taken into account (**Chapter V**).

Recently, the National Lung Screening Trial (NLST)[1] showed that lung cancer death rates can be reduced by 20% when a high-risk group is screened with a low-dose CT scan compared to chest radiography. Because this study used strict inclusion criteria we investigated whether the results of the NLST are generalizable (**Chapter IV**). When we applied the relative reduction in mortality in the lung cancer cases found by the NLST to the Rotterdam Study, we found different results; a relative reduction in mortality of 20%, as found in the screening trial, would lead to a reduction in mortality in the Rotterdam Study population of lung cancer of 6.0%. We therefore concluded that screening of lung cancer with the present inclusion criteria reduces lung cancer death rate compared to standard of care. But because only a minority of lung cancer patients complies with these criteria we should be cautious in generalizing findings to the total patient population.

An important aspect that should be considered in generalizing the results of screening studies is the therapeutic options for patients with a positive screening, as lung cancer treatment is an important prognostic factor. In recent years, peri-operative mortality has decreased by the introduction of Video Assisted Thoracoscopy (VATS) and better peri-operative management.[2] Early stage patients who are not eligible for surgery are frequently treated with radiotherapy with curative intent.[3] Novel radiotherapy techniques, such as stereotactic ablative radiotherapy do increase survival and also adjuvant chemotherapeutic regimens have been shown to increase survival especially in resected patients with stage II and IIIA disease. These regimens are expensive and therefore the results of the NLST can only be applied to countries with health care systems with a quality comparable to the US.[4]

Additionally, a concern in lung cancer screening is whether it is ethical to limit screening programs to individuals with >30 pack years (PY) of smoking, while approximately one-third of the population of lung cancer patients has smoked less than this threshold.[5] Screening programs may therefore unintentionally give the reassurance that there is no need to stop smoking, leading to continuation of smoking.

Also the cost effectiveness of low dose CT screening must be considered. The use of three annual CT screenings, the follow-up of the participants and the additional clinical procedures in response to positive screening is very expensive: $725,000 to prevent one death from lung cancer.[6] Several research groups modelled the cost-effectiveness analyses of CT screening with varying conclusions.[7,8] Wisnivesky et al.[7] conclude that the cost-effectiveness ratio of a baseline CT scan is within the range of clinical practice and health policy acceptability, while Mahadevia et al.[8] state that lung cancer screening is unlikely to be highly cost-effective, because of the low substantial reductions in mortality, low rates of adherence, high rates of overdiagnosis, and high costs per screening test. This contradiction should be investigated in more detail. The Dutch Belgian lung cancer screening study NELSON will also give important information on this.[9,10]

Taken together, early detection of lung tumors by lung cancer screening programs has led to more curative therapeutic options for patients and to a mortality reduction. However, results from the trial may only apply to individuals with access to high quality lung cancer care. In addition, screening trials for lung cancer only include a minority of lung cancer patients as most patients do not comply with these criteria. Therefore, clinicians should be cautious in generalizing findings to the total population of lung cancer patients, as until now, the use of lung cancer screening in clinical practice may have limitations in reducing lung cancer mortality. Nevertheless, screening programs will be very important in improving survival by earlier detection and subsequent treatment of lung cancer cases.

The contribution of the immune system in lung cancer

Multiple studies have shown that chronic inflammation predisposes to different forms of cancer, including lung cancer.[11,12] For instance, it has been shown that the risk of developing lung cancer is higher among smokers with COPD compared to smokers without COPD.[13] In addition, an inflammatory component is present in the microenvironment of most neoplastic tissues, including those not causally related to an obvious inflammatory process.[12,14,15]

In recent years it has been established that the immune system plays an important role in carcinogenesis and makes an essential contribution to the anti-tumor effects of traditional therapies.[16-18] Modulation of the patient's immune system by immunotherapy either as monotherapy or combined with conventional cancer treatments

offers the prospect of tailoring treatments much more precisely and could lead to a better response to treatment and overall survival of NSCLC patients.[19] However, before immunotherapy can be successfully applied to lung cancer patients, more knowledge about the immune system and cancer-related inflammation is needed.

The tumor environment contains a wide variety of recruited host immune cells. These tumor infiltrating immune cells influence anti-tumor responses in opposing ways and emerge as critical regulators of tumor growth.[20] In this thesis we describe that the presence of inflammation, in the form of a history of pulmonary tuberculosis may be an important prognostic factor in the survival of lung cancer. We hypothesize that the explanation for the poor survival in the tuberculosis group is of immunological origin (**Chapter X**)[21]; as a response to the presence of mycobacteria, inflammatory cells will release factors to eradicate the pathogen and enhance the immune reactions. The eradication may fail in tuberculosis, resulting in ongoing inflammatory reactions in the lungs of these patients that can lead to scar formation.[22-24] Although it is not completely understood what the underlying mechanism for this increased risk is, it has been reported that scarring of the lung after tuberculosis predisposes to the development of lung cancer in these patients, especially adenocarcinoma.[22-24] We hypothesize this is also caused by the ongoing immune related inflammation present in these scares. When a tumor is formed, macrophages, myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg) can accumulate and this can lead to impaired innate and adaptive immune responses against tumor cells via the production of immune suppressive cytokines and the induction of T cell tolerance.[21] Pro-inflammatory cytokines, such as tumor necrosis factor (TNF) and interleukin (IL)-6 may lead to up regulated expression of anti-apoptotic genes through the nuclear factor kappa beta (NF-κB) pathway.[25] Moreover, the production of substances, like vascular endothelial growth factor (VEGF), matrix metallopeptidases (MMP) by tumor-associated macrophages (M2 macrophages) and MDSC contributes to angiogenesis (a common feature of tissue repair and essential for tumor growth) and tumor invasion.[11,26,27] So, inflammatory conditions might not only induce malignant cell growth, but may also enhance tumor progression. Gaining insight into the functions of the different immune cells that play a role in lung cancer improves the understanding of a substantial part of the mechanisms that regulate lung cancer development and progression. In addition, knowledge about these mechanisms could be used to tailor lung cancer treatment to a patient's specific needs and requirements, or to optimize the condition of the patient before treatment.

We evaluated the influence of the relevant immunological cell types and their complex and dynamic roles within lung cancer patients (**Chapter VI and VII**). In this thesis we focussed on CD4+ and CD8+ T cells and MDSC, because these cell populations

play a pivotal role in the tumor-immunology and the knowledge of the presence and functions of these cells in lung cancer patients is still limited.

We showed that there were significant differences in MDSC and lymphocyte populations between NSCLC patients and healthy controls (**Chapter VIII**); MDSC levels were significantly higher in patients, while CD4+ and CD8+ T cells were significantly decreased in lung cancer patients compared to healthy controls. These differences are correlated with WHO performance status and stage of the disease. As these two are well known prognostic factors in lung cancer, we hypothesize that these cells could play an important role in the prognosis of lung cancer patients.

MDSC (**Chapter I, VI-IX**) consist of a heterogeneous group of immature myeloid cells and myeloid progenitor cells that can accumulate under pathological conditions at the tumor site, in the lymphoid organs and in peripheral blood.[28,29,30] They can suppress T cell responses by different mechanisms, dependent on the context of the microenvironment.[31] MDSC are characterized by different cell markers, including immature myeloid markers and mature myeloid markers, high arginase activity, and the production of reactive oxygen species (ROS). Two major subpopulations of MDSC were defined based on the difference in expression of CD14: polymorphnuclear (PMN) MDSC and monocytic (M) MDSC. PMN-MDSC are characterized as CD16^{low},CD11b$^+$,CD14$^-$,HLA-DR$^-$,CD15$^+$,CD33$^+$ cells, while M-MDSC are characterized by CD16^{low},CD11b$^+$,CD14$^+$,HLA-DR$^-$,CD15$^+$,CD33$^+$.[32,33,34] In addition, PMN-MDSC and M-MDSC differ in function and quantity; M-MDSC use different suppressive mechanisms and are present in much smaller amounts in the peripheral blood of non-small cell lung cancer (NSCLC) patients than PMN-MDSC.[35]

As described above, the characterization of MDSC in humans is challenging because the lack of specific markers. In chapter VIII we showed that arginase-1 (Arg-1) mRNA is mainly produced by PMN-MDSC and the percentage of PMN-MDSC correlates with the Arg-1 levels in the peripheral blood of NSCLC patients. We anticipate that Arg-1 is a useful surrogate marker for the frequency of MDSC in PBMC. This could have major advantages; first, Arg-1 mRNA expression levels can be measured by qRT-PCR instead of six markers that are needed to identify the MDSC population with flowcytometry. As a result, no expensive flowcytometers are needed to investigate this cell population. Next to this, Arg-1 mRNA can be reliably measured in the PBMC fraction that is snap frozen after ficoll isolation. This is useful for large patient multicenter studies, because measurements can be performed at later time points, in contrast to measuring MDSC by flowcytometry freshly. This might be a valuable tool to reduce the day-to-day variability. The question remains however, how these immunological data can be used to improve the response to treatment and the related survival.

Cancer immunotherapy attempts to activate the host's immune system to recognize and destroy small tumor nodules or the residual lung cancer cells that conventional therapy misses (**Chapter I, VI and IX**). CD8+ T cells play an important role in attacking tumor cells, and therefore many immunomodulatory agents and technologies intend to increase the number and efficacy of CD8+ T cells. Immunotherapy that tries to activate CD8+ T cells is presumed to be most effective in early stages of lung cancer.[36] This raises the question whether this could be based on immunological differences between the stages of lung cancer or the WHO performance status categories.

We showed that MDSC were significantly correlated with stage of disease. In addition, CD3+ and CD8+ T cells are significantly negatively correlated with WHO performance status, while the number of PMN-MDSC showed a strong positive trend (**Chapter IX**). We also provided evidence that there are large differences within stage IV lung cancer patients, for instance, in the numbers of CD8+ T cells (**Chapter IX**). Some patients have similar levels of CD8+ T cells as healthy controls, while other patients have a significantly decreased number of CD8+ T cells. So, there are large differences in the composition of the immune system between patients of different stages and even between patients within the same stage. These findings might explain why only a small group of lung cancer patients at present benefit from immunotherapy. We anticipate that immunotherapeutic agents that attempt to activate CD8+ T cells are only effective when CD8+ T cells are actively present.[37] Patients with a limited number of CD8+ T cells, which are mostly seen in advanced stage patients, are not likely to benefit from these therapies without modulation of the immune system beforehand.

The response to immunotherapy probably depends on the composition of the immune system, which is different in every patient. Therefore, we anticipate that the response to treatment could improve significantly if the treatment is tuned to the cellular make-up of each patient individually. This patient tailored treatment should be based on both tumoral and immunological characteristics, rather than by the stage of disease or histology. This individualized, multi-targeted approach will be able to restore the balance towards efficacious antitumor responses that can improve the overall survival of lung cancer patients. We anticipate that the future standard of care of lung cancer patients will imply patient tailored conventional therapy combined with immunotherapeutic approaches based on the patient's unique immune profile, driven by the increasing understanding of the immune system in the cancer cell's environment.

This pre-treatment profile should divide patients into different groups based on the immunological cells types that are present in the host. Immunological cells that can be taken into account are CD4+ and CD8+ T cells and MDSC. CD4+ T-helper cells and CD8+ cytotoxic T cells represent the strong effectors of the adaptive immune

response against cancer and MDSC are immature immunosuppressive cells that inhibit T cells and are involved in angiogenesis and metastatic spread. Patients with low T cell numbers would benefit if they first receive dendritic cell vaccination or adoptive T-cell transfer therapy to increase the number of T cells, resulting in a better response to immunomodulatory antibodies, such as anti-CTLA-4 (ipilimumab).[37] In patients with high MDSC levels, immunotherapy will probably have better results when MDSC first eliminated for instance with drugs like gemcitabine, 5-fluorouracil (5FU) or VEGF blockers (sunitinib).[38-40] In addition, the suppressive mechanisms of MDSC could be targeted by arginase-1 inhibitors, for instance N-Hydroxy-nor-L-Arg (Nor-NOHA), N-Hydroxy-L-Arg (NOHA) or by exogenous L-arginine suppletion[41-43], or by blocking the ILT3 receptor[44] (**Chapter VII and VIII**). These inhibitors are already used in the clinic in other diseases; it has been shown that arginase inhibition markedly improves endothelial function in patients with coronay artery disease and type 2 diabetes mellitus.[45] In addition, by giving lung cancer patients Nor-NOHA or NOHA, the suppressive effects of MDSC will be attenuated. This could lead to a stronger attack of the tumor cells by the immune system.

We hypothesize that these interventions are likely to increase the number of patients that will benefit from immunotherapy. Additionally, we think that these pretreatment profiles could also play an important role in conventional therapy, as the response to conventional therapy depends on the composition of the immune cells. Future research should be focused on which therapy has the best results based on the presence and activity of immune cells of the host.

Methodological considerations

The patients described in this thesis are embedded in three studies: the Rotterdam-study, the NVALT-12 study and the Intrimthom study. These studies are subject to common issues that reflect on the validity and generalizability. The Rotterdam Study[46] is a population-based prospective cohort study in Rotterdam, the Netherlands. All inhabitants of the Rotterdam suburb Ommoord aged ≥ 55 years were invited to participate in the study, which started with a baseline interview between July 1989 and July 1993. Of the 10,215 eligible subjects, 7,983 (78%) agreed to participate.[46] Because of the prospective design and high participation rate of this study, selection and information bias are not likely. However, the findings from this study cannot be generalized to the total population because all study members were 55 years or older. The NVALT-12 study (trial number NCT01171170) is a randomized phase II multicentre study on the effect of a nitroglycerin patch or placebo in patients with stage IV non-squamous NSCLC treated with carboplatin paclitaxel and bevacizumab. Blood samples were collected at baseline from 185 patients who were not applicable for treatment with curative intent. The Intrimthom study (trial number MEC-2010-384)

is a multicenter observational study that aims to investigate the influence of immune cells in thoracic malignancies as a prognostic factor. Patients from the NVALT-12 study and the Intrimthom study are also part of a selected population, i.e. stage IV non-squamous NSCLC and patients treated in an academic hospital, respectively. Selection bias and generalization bias are likely in both studies.

Cell populations were measured on a LSRII flowcytometer. This technology allows to identify a large number of different cells in one sample, based on a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. Because the different blood samples were not processed on the same day, there is the possibility of day-to-day variability of the instrument. Nevertheless, this variability is reduced by acalibration on a regular basis..

Implications for further research

We have stored blood and serum samples at three different time points of the patients from the NVALT-12 study: before chemotherapy (baseline), and after the first cycle and second cycle of chemotherapy. In the INTRIMTHOM study, bloodsamples are collected in patients before and after curative interventions and afterwards every 3 months. A next step in evaluating the influence of the immune system in lung cancer is to investigate the presence of other cell types and cytokines rather than T cells and MDSC, and to correlate the immunological data with the response to treatment and the survival of the patients. In addition, a more detailed subdivision of T cells, for instance, Treg or the state (e.g. exhaustion) of T cells will be made. Moreover, it would be interesting to investigate whether adapting the composition of the immune system of a lung cancer patient by immunotherapy based on a pre-treatment profile will lead to an increased survival.

Other cell types and cytokines

As described in chapter V, there are many different cell types that play an important role in lung cancer. Investigating other relevant cell types, for instance Treg and TAM could be valuable for fine-tuning patient tailored treatment. In future research it would be interesting to investigate whether there are differences in these cell types between lung cancer patients and if these cells correlate with survival.

Treg are a subpopulation of $CD4^+CD25^+$ T lymphocytes that are produced during maturation in the thymus (natural Treg) or induced in the periphery (induced Treg). They are further characterized by the expression of forkhead box P3 (Foxp3), glucocorticoid-induced TNF-receptor-related-protein (GITR), lymphocyte activation gene-3 (LAG-3), cytotoxic T-lymphocyte-associated antigen 4 (CTLA4), and a down-regulation of CD127 (IL-7R). Tregs are increased in tumor tissue[47] and in peripheral blood[48] of NSCLC patients compared to healthy volunteers. This increase in Tregs was

found to promote tumor growth and correlated with lymph node metastasis.[14,49] There is substantial interest for overcoming Treg induced immunesuppression to enhance the efficacy of cancer immunotherapy.

Macrophages in tumors are usually referred to as TAM and their presence can be substantial (10 to 65% of the tumor stroma). Macrophages can be divided into M1 macrophages ('classical activation') or M2 macrophages ('alternative activation').[50] M1 macrophages attract and activate cells of the adaptive immune system and have anti-tumor and tissue destructive activity, while the M2 phenotype is more focused on tissue repair, tissue remodeling and immunoregulation. In a small tumor, the TAM mainly consist of M1 macrophages and later in the process, when the tumor starts to invade and vascularize, there is a skewing towards the M2 phenotype. It has been shown that high numbers of M1 macrophages infiltrating the tumor are correlated with improved survival.[51,52] There are currently no agents for clinical use available that specifically target TAM[53] but several strategies are currently investigated that attack macrophages at several levels. For example, blockade of factors and cytokines secreted by tumor or immune cells to limit the induction of M2 macrophages are investigated, these include inhibition of prostaglandin E2 synthesis (cox-2 inhibitor)[54], anti-CCL2, anti-TGF-β, anti-IL-6 (Siltuximab)[55], agonists for TLR and NOD (Imiquimod)[56], however this results in loss of typical M2 markers but not their function.[57] It has been shown that inhibiting IκB kinase (IKK) reprogrammes the M2 phenotype to the M1 subset.[58,59] Also CD40 therapy seems to skews tumor-infiltrating (not the resident) macrophages towards the M1 phenotype.[60] The effects of bisphosphonates (Zoledronic acid) on TAM were investigated.[61,62] Influencing the attraction, the polarization or the activation of M2 macrophages may improve survival when combined with standard or other immunotherapeutic regimens.

Tumor cells produce an offensive repertoire of factors that can lead to the recruitment and activation of bone-marrow-derived cells to the tumor site. In general, these include cytokines, chemokines, such as transforming growth factor (TGF), tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), insulin-like growth factor (IGF), and different types of interleukins (IL)-1, IL-6 and IL-8, amongst many others.[20] In future research it would be interesting to see whether there are differences in these cytokines and chemokines in the serum of lung cancer patients compared to healthy controls. These factors can easily be detected by enzyme-linked immuno sorbent assay (ELISA). In addition, it would be interesting to see whether these factors are correlated with the different immune cells and other prognostic factors.

Response to treatment and survival of patients in the nVaLT12 study

Response to treatment and survival data from the NVALT12 study are not available yet. As soon as these are available, the different pivotal immunological cell types and relevant cytokines of the lung cancer patients are correlated with outcome (response to treatment and/or overall survival).

Predicting the best treatment per patient and the survival based on the immunological profile could lead to major advances in survival. Next to this, patients who are less likely to get a response to treatment will not unnecessarily undergo treatment and suffer from the associated side effects.

Future recommendations to improve lung cancer survival

Modulation of the patient's immune system by immunotherapy either as monotherapy or combined with conventional cancer treatments offers the prospect of tailoring treatments much more precisely and could lead to a better response to treatment and overall survival of NSCLC patients. Still, patients will have the best prognosis if lung cancer is detected in an early stage of disease, for instance by lung cancer screening. In this early stage, the immune system of the patient is mostly still sufficient and can more easily be adapted by therapy.

The ideal situation would be that a high-risk population for the development of lung cancer is identified by non-invasive screening of lung cancer biomarkers, for instance by blood biomarkers, exhaled breath measurements or sputum cytology, which should be highly sensitive and specific. Currently these are not available. If this screening is positive a CT scan should be made. When subjects are diagnosed with lung cancer they will get a pre-treatment profile based on the composition of different immunological cells. Based on the stage of disease and this immunological profile, the best patient-tailored treatment will be chosen.

The future of cancer therapy lies in combining conventional treatment, such as surgery, radiotherapy or systemic therapy, with immunotherapy in order to consolidate the effects of the single treatment. This will evolve alongside our understanding of the immune system in tumorogenesis. We hypothesize that conventional treatments options combined with immunotherapy, based on a pretreatment profile of the immune system of the host, could be a valuable tool to increase the survival of patients with early stage NSCLC.

SUMMARY

- Lung cancer screenings trials only apply to individuals with access to high quality lung cancer care. In addition, screening trials for lung cancer only include a minority of lung cancer patients, as most patients do not comply with the inclusion criteria of these screening trials.
- At this point the use of lung cancer screening in clinical practice has limitations in reducing lung cancer mortality, as the results of screenings trials are not generalizable to the total population of smokers.
- The presence of a history of pulmonary tuberculosis is an important prognostic factor in the survival of lung cancer and plays an important role in lung cancer development.
- The immune system differs significantly between lung cancer patients and healthy controls.
- The frequencies of immune cells are correlated to the two most important prognostic factors: disease stage and world health organisation (WHO) performance status.
- Arginase-1 (Arg-1) mRNA is mainly expressed by myeloid derived suppressor cells MDSC and the level of Arg-1 mRNA in peripheral blood mononuclear cells (PBMC) correlates with the frequency of MDSC in PBMC. qRT-PCR on Arg-1 might therefore be useful as an alternative method to determine the levels of PMN-MDSC in peripheral blood of cancer patients.
- The immune suppressive immunoglobulin-like transcript (ILT)-3 is present on MDSC. This protein is an attractive candidate for immunotherapy, as blocking ILT3 could inhibit the immune suppressive functions of ILT3.
- The limited number of advanced lung cancer patients that benefit from treatment is probably caused by the coherence of a restricted number of CD8+ T cells and an increased number of MDSC caused by the tumor microenvironment.
- Patient tailored immunotherapies that optimize tumor-specific CD8+ T cell function seem an essential step to increase the efficacy of lung cancer treatment. It might be advantageous to make a pre-treatment prediction based on an immunological profile which patients are likely to benefit from therapy.

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Nederlandse samenvatting

LONGKANKER

Longkanker is de belangrijkste oorzaak van overlijden door kanker. De 5-jaars overleving is slechts 16%. Een van de redenen voor deze uiterst slechte overleving is dat longkanker meestal wordt gediagnosticeerd in een vergevorderd stadium. Dit komt door het gebrek aan klinische symptomen, zoals vermoeidheid, kortademigheid, pijnklachten en bloed ophoesten tijdens de vroege stadia. Daarnaast is longkanker vaak een agressieve ziekte, waardoor er snel metastasen ontstaan. Gemetastaseerde longkanker is momenteel een ongeneeslijke ziekte. De standaard chemotherapie leidt slechts tot geringe verbetering in de overleving. Minder dan 30% van de patiënten met gevorderde stadia van longkanker hebben een response op chemotherapie. Terwijl dit de meest effectieve eerstelijns behandeling van uitgezaaide longkanker is. Er zijn twee belangrijke vormen van longkanker: niet-kleincellig long carcinoom (NSCLC) (85% van alle gevallen) en klein-cellig longcarcinoom (15% van alle gevallen). NSCLC kan worden onderverdeeld in vier histologische subtypes: plaveiselcel carcinoom, adenocarcinoom, grootcellig longcarcinoom en ongedifferentieerd longcarcinoom. Roken is de belangrijkste risicofactor voor longkanker. Plaveiselcel carcinoom is een aan roken gerelateerde kanker die zich meestal ontwikkelt vanuit bronchiale epitheelcellen in de centrale luchtwegen. Indien longkanker zich ontwikkeld bij mensen die niet gerookt hebben is dit meestal adenocarcinoom, en ontwikkelt de kanker zich vanuit basale bronchiale cellen.

Sreeningsonderzoek naar longkanker

Longkanker screeningsonderzoek is erop gericht om longkanker in een vroeg stadium op te sporen, zodat de kans op genezing groter is en zo te zorgen voor een verbetering van de overleving van deze ziekte. Er zijn verschillende studies uitgevoerd die onderzochten of longkanker vroegtijdig te diagnosticeren is met behulp van een röntgenfoto (X-thorax), maar helaas werd daar geen overlevingsvoordeel mee aangetoond. De meest recente studies onderzoeken met behulp van een lage stralingsdosis computed tomografie (CT) scan of longkanker eerder opgespoord kan worden, waardoor de kans op overleving vergroot wordt. Een CT scan is gevoeliger voor het detecteren van afwijkingen in de thorax dan een standaard röntgenfoto.

Bij screeningsonderzoeken moet echter altijd rekening worden gehouden met bias (vertekening van de resultaten). Belangrijke vormen van bias zijn: length time bias en lead time bias. Length time bias kan optreden wanneer er, door langdurige screenings intervallen, vooral ziekten worden gedetecteerd met een langdurig ziektebeloop. Als er door het screenen dan alleen langzaam groeiende longtumoren worden gevonden kan dit de indruk geven dat screening leidt tot een verbetering in overleving, terwijl dit in werkelijkheid niet zo hoeft te zijn. Lead time bias is als een ziekte door

screening in een vroeger stadium wordt gedetecteerd zonder dat dit uiteindelijk leidt tot een verbetering van de overleving. De overlevingsduur is dan wel toegenomen, maar dit komt niet doordat het beloop van de ziekte is veranderd, maar puur omdat de tumor eerder is gediagnosticeerd. Daarnaast kan er door screening overdiagnose optreden. Dit houdt in dat er een ziekte wordt gediagnosticeerd door screening, die anders tijdens het leven van de patiënt nooit voor symptomen zou zorgen en de patiënt door andere oorzaken overlijdt. Deze detectie kan echter wel zorgen voor veel psychologische stress en onnodige behandeling van de patiënt, waardoor de kosten voor de gezondheidszorg verder stijgen.

De Nationale Lung Screening Trial (NLST) heeft echter recent aangetoond dat een jaarlijkse low-dose CT screening van een op roken en leeftijd geselecteerde groep mensen voor een daling in longkanker sterfte zorgt van 20%. Dit komt doordat de longkanker door de screening in een vroeger stadium wordt ontdekt en dan nog curatief behandeld kan worden, waardoor de overleving toeneemt. De resultaten van het onderzoek zijn echter door strenge selectiecriteria van de NLST alleen toepasbaar op een geselecteerde groep mensen. De bevindingen kunnen daarom op dit moment nog niet gegeneraliseerd worden naar de hele bevolking. Desondanks zorgen long-kanker screeningsprogramma's voor een vroege detectie van de ziekte en kunnen ze in de toekomst erg belangrijk zijn om de longkanker overleving te verbeteren.

Longkanker en het immuunsysteem

Bij het ontstaan van kanker speelt het immuunsysteem een belangrijke rol. Verschil-lende onderzoeken hebben aangetoond dat chronische ontsteking een risicofactor vormt voor het ontstaan van longkanker. Het risico op het krijgen van longkanker onder rokende mensen met chronisch obstructieve longziekten (COPD) is bijvoor-beeld veel hoger dan bij rokende mensen zonder COPD.

Daarnaast is het aangetoond dat de tumoromgeving veel verschillende immuuncel-len bevat. Een deel van deze immuuncellen, zoals cytotoxische T cellen (CTLs) kun-nen de tumor aanvallen, waardoor deze kleiner wordt of zelfs helemaal weggaat. Er zijn echter ook immuuncellen, zoals myeloid derived suppressor cells (MDSC), die er voor kunnen zorgen dat de tumor sneller kan groeien, doordat deze cellen bijvoorbeeld de CTLs onderdrukken.

Inzicht krijgen in de functies van de verschillende immuuncellen die een belangrijke rol spelen bij longkanker kan ervoor zorgen dat de mechanismes verantwoordelijk voor het ontstaan van longkanker en de progressie van de ziekte duidelijker worden. Deze kennis kan vervolgens gebruikt worden om de behandeling van longkanker meer af te stemmen op de individuele kenmerken van de patiënt, waardoor de kans op respons op de behandeling groter wordt en de overleving toeneemt.

Doel van het onderzoek

In de afgelopen decennia is er veel onderzoek gedaan naar het verlagen van de sterfte van patiënten met longkanker. Helaas is tot nu toe de verbetering in de lange termijn overleving beperkt en heeft longkanker nog steeds een slechte prognose. Recente onderzoeken tonen een afname aan van de sterfte aan longkanker door screenings-programma's. Het is niet de verwachting dat door verbeteringen in de standaard behandelingsstrategieën de prognose van longkanker patiënten veel zal verbeteren. Daarnaast zou modulatie van het immuunsysteem van een longkanker patiënt door immunotherapie, hetzij als monotherapie of in combinatie met conventionele kan-kerbehandelingen, kunnen leiden tot een meer nauwkeurigere behandeling. Dit zou kunnen zorgen voor een betere respons op de behandeling en een langere overleving.

Aangezien slechts kleine verbeteringen in overleving kunnen worden verwacht in uitgezaaide longkanker met gebruik van de al bestaande therapieën, zou meer onderzoek gericht moeten zijn op longkanker screeningprogramma's en tevens voor de patiënt toegesneden immunotherapie met of zonder conventionele therapieën.

Dit proefschrift evalueert de rol van het immuunsysteem in longkanker patiënten. Bovendien wordt de rol van CT screening, om zo longkankerpatiënten in een vroeg stadium te detecteren, onderzocht. Het doel van het onderzoek was om te zoeken naar nieuwe strategieën om de overleving van longkanker te verbeteren.

Het proefschrift is opgedeeld in vier delen. Het eerste deel beschrijft longkanker in het algemeen. Het tweede deel gaat over longkanker screening en het derde deel over de rol van het immuunsysteem bij longkanker. Het vierde deel bestaat uit de discussie en samenvatting.

Hoofdstuk I geeft een algemene introductie over longkanker, het ontstaan van de ziekte en de therapieën. Daarnaast wordt besproken hoe longkanker screening en modulatie van het immuunsysteem mogelijk kunnen zorgen voor een verbeterde overleving van longkanker patiënten.

Hoofdstuk II geeft het overzicht en het doel van dit proefschrift weer. De over-leving van longkanker is de afgelopen jaren niet erg verbeterd. In **hoofdstuk III** wordt beschreven dat de overleving van longkanker verbeterd kan worden door longkanker in een vroeger stadium op te sporen en de behandeling aan te passen aan de immuuncellen die aanwezig zijn in de patiënt. Wij vinden dat er daarom meer onderzoek zou moeten worden gedaan op het vlak van longkanker screening en patiënt-specifieke therapie.

In **Hoofdstuk IV** worden de inclusiecriteria van longkanker screening besproken en wordt onderzoek verricht naar de generaliseerbaarheid van de bevindingen van grote screenings studies naar de totale populatie van longkanker patiënten. Wij vonden dat

als de inclusie criteria van de NLST worden toegepast op de mensen uit de Rotterdam Studie, dat de afname in sterfte door screening echter lager is dan de getallen van de NLST. De 20% afname in longkanker sterfte is alleen toepasbaar in een geselecteerde groep mensen die al een sterk verhoogd risico hebben voordat ze gescreend worden.

Hoofdstuk V bediscussieert het belang van longkanker screening in meer detail. Longkanker screening heeft als voordeel dat je de kanker in een vroeg stadium detecteert, maar belangrijk is om rekening te houden dat de resultaten van verbeterde overleving door screening niet in elk land gelden. Dit is onder andere afhankelijk van de gezondheidszorg in dat land. Daarom moeten artsen voorzichtig zijn om de bevinding dat screening leidt tot verbeterde overleving van longkanker te generaliseren.

Hoofdstuk VI geeft een overzicht van de relevante immunologische celtypes die een rol spelen in longkanker. De rol van het immuunsysteem is tweezijdig. Aan de ene kant zorgt het immuunsysteem ervoor dat kanker cellen aangevallen worden, maar het immuunsysteem kan er ook voor zorgen dat de kanker juist sneller kan groeien. Dit hoofdstuk beschrijft de complexe en dynamische functies van de celtypes in de tumor omgeving.

In **hoofdstuk VII** beschrijven we een nieuwe receptor op een belangrijke populatie van immuun onderdrukkende cellen, MDSC. Deze receptor heet immunoglobulin-like transtcript-3 (ILT3) en speelt een rol in onderdrukking van T cellen. Wij denken dat de ILT3 receptor een van de vele manieren is waarmee een MDSC een immuun-suppressief is wat ertoe kan leiden dat een tumor sneller groeit.

Hoofdstuk VIII beschrijft de immunologische karakteristieken van 185 gemetasta-seerde NSCLC patiënten. We beschrijven de karakterisatie en optimale omstandigheden om MDSC te analyseren en we onderzochten hun aanwezigheid en functie in het perifere bloed van gemetastaseerde NSCLC patiënten. We vonden dat MDSC verhoogd voorkomen, terwijl T-cellen juist in verlaagd voorkomen in het bloed van longkanker patienten in vergelijking met gezonde mensen. Daarnaast vonden we dat de hoeveelheid messenger-RNA van Arginase-1 correleert met het aantal MDSC dat aanwezig is in het bloed van longkanker patienten.

In **hoofdstuk IX** onderzoeken we het verband tussen de belangrijkste immuuncellen, zoals CD8+ T cellen en MDSC in het perifere bloed van longkanker patiënten met verschillende ziekte stadia. We vonden verschillen in immuuncel samenstelling tussen patiënten met verschillende ziekte stadia van longkanker en patiënten uit verschillende categorieën van de WHO performance status.

Hoofdstuk X beschrijft de relatie tussen ontsteking en longkanker. We vonden dat longtuberculose een onafhankelijke risicofactor is voor de overleving van longkanker. Mensen die in het verleden tuberculose hebben doorgemaakt hebben een slechtere overleving van hun longkanker.

Concluderend, de overlevingskansen van longkanker patiënten zijn op dit moment nog steeds laag. Twee manieren waarmee de overleving verbeterd zou kunnen worden is door het eerder opsporen van longkanker, bijvoorbeeld met behulp van CT screeningsprogramma's of door de therapie beter op elke patiënt afzonderlijk af te stemmen op basis van de verschillen in samenstelling van het immuunsysteem.

Longkanker screeningsprogramma's dragen bij tot een verbetering in overleving van longkanker in landen met een goede kwaliteit van zorg. Op dit moment is echter alleen onderzoek gedaan naar de effectiviteit van screening bij een geselecteerde groep mensen en zijn de resultaten van de onderzoeken dus niet toepasbaar op de hele bevolking.

Daarnaast hebben we aangetoond dat er grote verschillen zijn in de samenstelling van het immuunsysteem tussen gezonde mensen en longkanker patiënten. Deze verschillen zie je ook binnen longkanker patiënten van verschillende stadia en zelfs binnen de groep van stadium IV longkanker patiënten. We hebben laten zien dat Arg-1 mRNA een goede maat is om het aantal MDSC in het bloed van longkanker patiënten te meten. Daarnaast beschrijven we dat de immuunsuppressieve receptor ILT3 verhoogd aanwezig is op de MDSC van longkanker patiënten in vergelijking met gezonde controles.

Er zijn maar weinig patiënten met gemetastaseerde ziekte die reageren op immuuntherapie. Dit komt waarschijnlijk doordat ze een laag aantal CD8+ T cellen en een hoog aantal MDSC in hun bloed hebben.

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Rotterdam, 11 juni 2013

Marlies

About the author

Marlies Heuvers was born on April 27th 1987 in Hoorn, the Netherlands. After completing high school in 2005 (Gymnasium) at the OSG, she started studying medicine at the Erasmus University Rotterdam. During her study she was doing research at the Department of Respiratory Medicine at the Erasmus Medical Center under supervision of dr. P.Th.W. van Hal. She was also an assistant faculty member of the Lowlands Institute of Surgical and Applied Anatomy. In April 2009 she started her PhD project at the department of Respiratory Medicine under the supervision of Prof. dr. H.C. Hoogsteden, dr. J.G.J.V. Aerts, and dr. J.P.J.J. Hegmans. Parallel to her PhD project she did a Master of Science in Clinical Epidemiology at the NIHES institute. Per Februari 25th 2013 Marlies started her clinical internships at the Erasmus Medical Center in Rotterdam. In 2014 she will start with her pulmonary medicine training at the Erasmus Medical Center (Head of department: Prof. dr. H.C. Hoogsteden).

List of publications

LIST OF PUBLICATIONS

Heuvers, M.E., Aerts, J.G., Cornelissen, R., Groen, H. *et al.* Patient-tailored modulation of the immune system may revolutionize future lung cancer treatment. *BMC Cancer* **12**, 580 (2012)

Heuvers, M.E., Hegmans, J.P., Stricker, B.H., Aerts, J.G. Improving lung cancer survival; time to move on. *BMC Pulm Med* **12**, 77 (2012)

Cornelissen R., Heuvers, M.E., Maat, A.P. Hendriks R.W. *et al.* New roads open up for implementing immunotherapy in mesothelioma *Clin Dev Immunol* (2012)

Heuvers, M.E., Wisnivesky, J., Stricker, B.H., Aerts, J.G. Generalizability of results from the National Lung Screening Trial. *Eur J Epidemiol* **27**, 699-672 (2012)

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Cornelissen R., Lievense L.A., Heuvers M.E., Maat, A.P. *et al.* Dendritic cell-based immunotherapy in mesothelioma. *Immunotherapy* **4**, 1011-1022 (2012)

Heuvers, M.E., Aerts, J.G., Hegmans, J.G., Veltman, J.D. *et al.* History of tuberculosis as an independent prognostic factor for lung cancer survival. *Lung Cancer* **76**, 452-456 (2012)

Kan, H.J., Heuvers, M.E., Grijm, K., van Hal, P.Th.W.; Sirolimus related dyspnoea, airway obstruction and pleural effusion after lung transplantation. *Transplant International* **22,** 940-942 (2009)

PhD Portfolio

PHD PORTFOLIO

Summary of PhD training and teaching

PhD Training

Research skills

2009-2011   Master of Science in Clinical Epidemiology, Netherlands Institute for Health Sciences, Rotterdam, the Netherlands

Courses

2011   Molecular Immunology course, Molecular Medicine Postgraduate School, Erasmus MC Rotterdam, the Netherlands

2010   NWO masterclass creative thinking, The Hague, the Netherlands

2009   Molmed excel cursus

2009-2013   Research seminars, department of Epidemiology and Pulmonary Medicine, Erasmus MC Rotterdam, the Netherlands

(Inter) national scientific presentations

2013   Molecular Medicine Day, Rotterdam, the Netherlands (poster)

2012   Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout, the Netherlands (poster)

2012   American Association for Cancer Research (AACR), Chicago, USA, (2 posters)

2011   World Conference on Lung Cancer (WCLC), Amsterdam, the Netherlands (poster)

2011   Molecular Medicine Day, Rotterdam, the Netherlands (poster)

2010   Annual Meeting Dutch Society for Immunology (NVVI), Noordwijkerhout, the Netherlands (poster)

2010   Thomas L. Petty Lung Conference, Aspen, USA (oral)

2010   European Respiratory Society (ERS) Annual Conference, Vienna, Austria (poster)

Inter(national) conferences attended

2010   CHARGE meeting, Houston, USA

2009   CHARGE meeting, Rotterdam, the Netherlands

2009   World Conference on Lung Cancer (WCLC), San Francisco, USA

Teaching activities

2012	Teaching clinical skills (PKV) for second-year medical students
2011	Supervising master of science-student. Molecular Medicine postgraduate School, Erasmus MC Rotterdam
2010	Data-analysis in pharmacoepidemiology, NIHES, Rotterdam, the Netherlands
2009-2013	Study courses for fifth- and sixt-grade VWO students, Erasmus University, Faculty of Medicine
2009-2013	Journal courses for first-year medical students, Erasmus University, Faculty of Medicine