Minimally invasive diagnostics and immunotherapy of lung cancer

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Colophon

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Minimally invasive diagnostics and immunotherapy of lung cancer

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Chapter 1

General introduction,
aim and outline of the thesis

LUNG CANCER: EPIDEMIOLOGY AND CLASSIFICATION

Lung cancer has a major impact on the global burden of disease. In 2012, it caused the death of approximately 1,5 million persons, which makes lung cancer the most common cause of cancer-related mortality in males (24%) and the second leading cause in females (14%) worldwide[1,2]. In the Netherlands, 10.357 lung cancer related deaths occurred (6.179 men and 4.178 women) in 2014. In 2015, 12.217 new cases of lung cancer were been reported, 6.861 males (56%) and 5.356 females (44%). When analyzing trends in incidence and mortality of lung cancer in the Netherlands in the past 25 years (**Figure 1 and Figure 2**), an evident but moderate decline is observed in males with respect to the number of newly diagnosed lung cancer cases and number of deaths caused by lung cancer. However, the opposite is the case for women, in whom a sharp increase in incidence and mortality is observed. This relative increase of lung cancer prevalence and mortality is caused by the increase in tobacco smoking by Dutch women. Smoking is the single most predominant risk factor responsible for lung cancer. Although the number of smokers is gradually decreasing in the Netherlands, almost a quarter of the Dutch population of 12 years and older still smoked in 2015, of whom 74% on a daily basis[3].

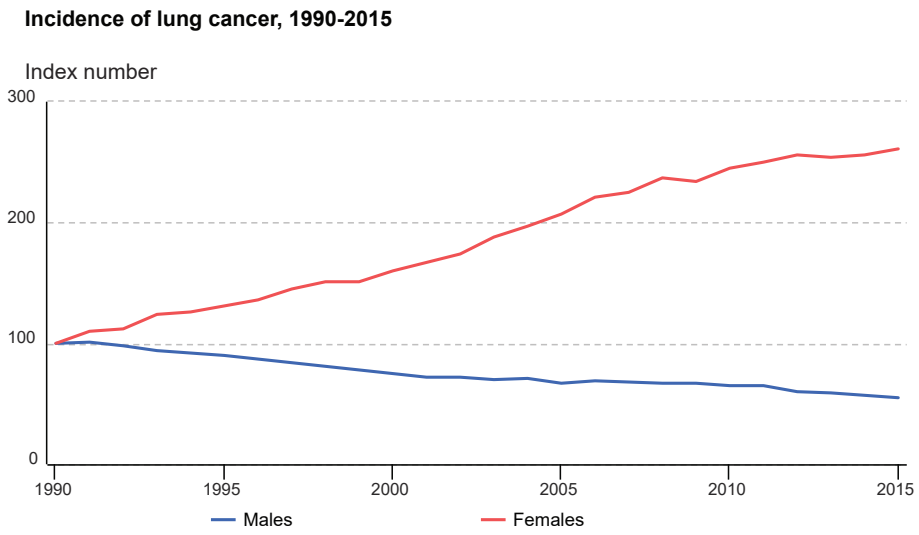

Figure 1. Incidence of lung cancer in the Netherlands from 1990 – 2015
(Source: volksgezondheidenzorg.info)

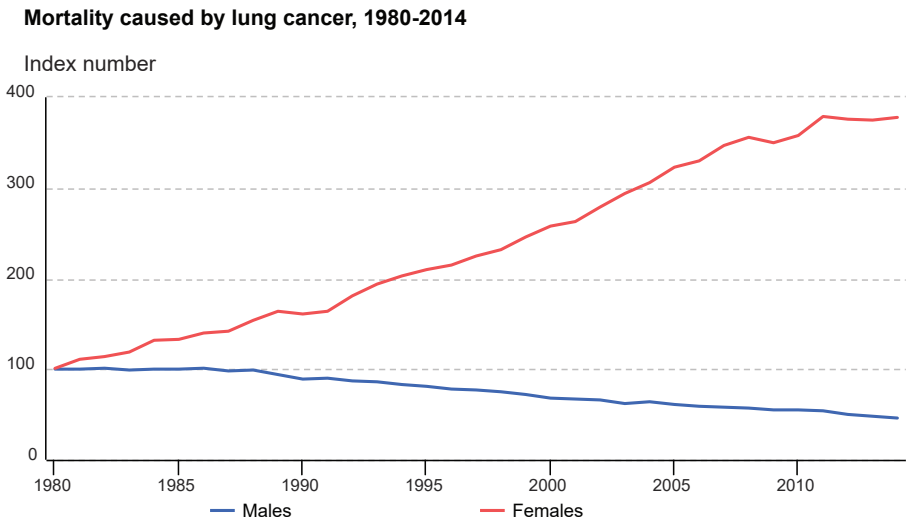

Figure 2. Mortality of lung cancer in the Netherlands from 1990 – 2015 (Source: volksgezondheidenzorg.info)

Lung cancers are classified according to histological type as small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). The majority of cases are NSCLC, which comprises approximately 80% of all lung cancers[4]. NSCLC has three main subtypes: squamous cell carcinoma, large-cell carcinoma and adenocarcinoma, the latter accounting for the majority (40%) of NSCLC cases[5,6]. A routine but critical part of diagnosing lung cancer is to assess the stage of disease in order to select the appropriate treatment and to determine prognosis. Hence, newly diagnosed lung cancer patients undergo multiple diagnostic procedures to determine the extent of disease, such as contrast-enhanced chest computed tomography (CT), fluorodeoxyglucose-positron emission tomography (FDG-PET) scan and minimally invasive endosonography[7,8].

DIAGNOSING NSCLC: THE ROLE OF ENDOSONOGRAPHY

An essential part of preoperative staging of NSCLC is the assessment of the mediastinum for lymph node involvement. Endoscopic ultrasound (EUS) and endobronchial ultrasound (EBUS) are minimally invasive endosonography techniques that operate by placing an ultrasound probe in the esophagus (EUS) or airways (EBUS). They serve to visualize mediastinal lymph nodes and by means of fine needle aspiration (FNA) tissue sampling can be performed. Both EUS and EBUS have a high sensitivity for detecting mediastinal lymph node metastases (89% for both procedures). A combined EUS/EBUS approach was shown to reduce the number of unnecessary thoracotomies (18% vs 7%,

p=0.02), while displaying a comparable sensitivity compared to surgical staging (85% vs 79%, p=0.47), which was considered the gold standard of mediastinal tissue staging before the introduction of endosonography in pulmonary oncology[7,9]. In recent international guidelines, endosonography procedures (EUS and/or EBUS) are advocated as the initial tissue sampling procedure in NSCLC staging[8,10,11].

CURRENT TREATMENT OF NSCLC

With the use of non-invasive and (minimally) invasive staging modalities, lung cancer patients can be staged according to international guidelines on TNM Classification for Lung Cancer[12] which serve to select the appropriate therapy modality for NSCLC patients. Patients with stage I/II NSCLC are treated by surgical resection of the tumor with systematic lymph node dissection which is mandatory to verify the extent of disease and to plan adjuvant treatment[13]. Stereotactic body radiotherapy (SBRT) can be an alternative treatment for early stage NSCLC, especially in those patients who are not considered medically fit to undergo surgery[14]. In case of locally advanced disease (stage III), patients are generally treated with chemoradiotherapy, preferably in a concurrent fashion[15]. In case of advanced NSCLC (stage IV), patients are treated with palliative chemotherapy. As first-line treatment, a platinum-based compound combined with a third generation cytotoxic agent is recommended[16]. For advanced NSCLC patients with tumors that carry epidermal growth factor receptor mutations or anaplastic lymphoma kinase gene rearrangements, small-molecule kinase inhibitors have shown a clear beneficial clinical effect and a substantial improvement of prognosis[17]. When first line treatment with palliative chemotherapy or a small-molecule compound is successful (clinical response or stable disease), patients are eligible for maintenance therapy, either by switching to or continuation of a single compound of the combination chemotherapy (e.g. pemetrexed, a multifolate inhibitor) or a small-molecule inhibitor[15,18,19].

IMMUNOTHERAPY OF NSCLC

Despite the current improvements in NSCLC staging and the availability of the aforementioned therapies, survival rates for NSCLC remain exceptionally poor. In the Netherlands, an analysis from a 20 year period (1989 -2009) indicated that only a very modest improvement from 14.8% to 17.4% in five-year survival for all NSCLC patients has been achieved[20]. An important contributing factor to this dismal survival statistic is the fact that the majority of NSCLC patients have locally advanced or metastatic disease (stage III/IV) at first presentation. In general, these patients cannot be cured and are only

eligible for palliative treatment[15,16]. Hence, there is a clear medical need to explore novel therapies for NSCLC in order to improve current clinical outcomes.

One new anticancer therapy that has emerged in recent years is active immunotherapy which is directed at eliciting and reinforcing T-cell-mediated antitumor responses. Studies on the tumor microenvironment have shown that NSCLC, like many immunogenic cancers, is infiltrated by a plethora of immune cells (mainly T cells, macrophages and mast cells) with both tumor-promoting and suppressive effects[21]. A dense infiltrate of M1-macrophages[22,23], CD4+ T-cells and CD8+ T-cells[24,25] is associated with an improved survival rate, which indicates that the immune system is actively involved in keeping NSCLC at bay. This immunogenicity of NSCLC is an important prerequisite for active immunotherapy and is exploited by novel immunotherapeutic treatments aimed at interrupting the action of T cell regulatory molecules[26]. In particular, infusion of antibodies that target programmed death 1 (PD-1) receptor and its ligand PD-L1, has shown very promising clinical effects in NSCLC[27], which has led to the approval of the PD-1 blocking antibody nivolumab for treating metastasized non-small cell squamous carcinoma[28,29].

AIM AND OUTLINE OF THE THESIS

The first aim of this thesis is to investigate how the implementation of minimally invasive endoscopic ultrasound techniques (EUS and EBUS) in the staging algorithm of NSCLC can be optimized (**Part I**). The routine use of EUS-FNA in the preoperative staging of unselected patients with NSCLC is evaluated in **Chapter 2**. An assessment is made with respect to diagnostic test performance (sensitivity, predictive value and accuracy), the number of prevented surgical staging procedures and the value of additional mediastinoscopy if EUS-FNA fails to detect nodal metastases. An in-depth analysis of predictors for false negative EUS and/or EBUS findings in NSCLC patients is made in **Chapter 3**. These predictors may elucidate when it is justified to directly proceed to thoracotomy and omit additional surgical procedures when the mediastinum is staged negative after EUS and/or EBUS.

The second aim of this thesis focuses on the role and potential of immunotherapy in NSCLC patients (**Part II**). An overview of recent developments in the field of therapeutic peptide vaccination of cancer (with a focus on NSCLC) is presented in **Chapter 4**. The impact of immunity on the clinical outcome of NSCLC was studied by assessment of the numbers of intra-tumoral CD8+ T cells and the expression of classical and non-classical HLA molecules. The association of these parameters with overall survival is described in **Chapter 5**. Since it was shown in animal models that chemotherapy and radiotherapy have beneficial effects on the immune system in tumor bearing hosts, the systemic effects of these conventional NSCLC therapies were investigated in NSCLC patients with

respect to the composition and function of circulating immune cells in **Chapter 6.** This provides insight on whether these standard-of-care NSCLC therapies can also be combined with novel immunotherapeutic drugs in the human setting. An investigation into the expression and immunogenicity of XAGE-1b, a novel tumor antigen of the cancer testis antigen family that is preferentially expressed in NSCLC, is presented in **Chapter 7**. With this study on spontaneously induced XAGE-1b-specific humoral and cellular responses, vital background knowledge was obtained for starting a phase I clinical trial in which a XAGE-1b overlapping peptide vaccine is tested in advanced NSCLC patients. A study protocol and rationale of this study together with preliminary results are provided in **Chapter 8**. Finally, **Chapter 9** provides a general discussion and summary of this thesis.

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Chapter 2

EUS-FNA in the preoperative staging of non-small cell lung cancer

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ABSTRACT

Background

According to current guidelines, transesophageal ultrasound-guided fine needle aspiration (EUS-FNA) can be performed as an alternative for surgical staging to confirm mediastinal metastases in patients with non-small cell lung cancer (NSCLC). To date however, data regarding the routine use of EUS-FNA in the preoperative staging of unselected patients with NSCLC are limited.

Aims and Objectives

1. To evaluate the diagnostic value of EUS-FNA in consecutive patients with NSCLC regardless of nodal size at CT.
2. To determine the impact of EUS-FNA on the prevention of surgical staging procedures.
3. To assess the accuracy of mediastinal staging by combining EUS-FNA and mediastinoscopy.
4. To investigate whether a subgroup of patients exists that can be accurately staged by EUS-FNA alone.

Methods

152 consecutive operable patients with proven or suspected NSCLC who underwent EUS-FNA were retrospectively analyzed. In the absence of mediastinal metastases, mediastinoscopy and/or thoracotomy with lymph node dissection was performed.

Results

The prevalence of mediastinal metastases was 49%. Sensitivity, negative predictive value (NPV) and accuracy of EUS-FNA for N2/N3 disease were 74%, 73% and 85%, respectively, whereas these values for the combined staging of EUS-FNA and mediastinoscopy were 92%, 85% and 95%. Additional surgical staging in patients staged N0 at EUS-FNA reduces the false negative EUS-findings by 55%. The NPV of EUS-FNA for left-sided tumors was 68%. EUS-FNA prevented surgical staging procedures in 60 of 152 patients (39%). No major complications occurred during EUS-FNA.

Conclusion

Routine use of EUS-FNA in unselected patients with NSCLC reduces the need for surgical staging procedures in nearly half of patients. Additional surgical staging in patients without nodal metastases at EUS-FNA reduces the false negative EUS-FNA findings considerably regardless of the location of the primary lung tumor.

INTRODUCTION

Preoperative staging of non-small cell lung cancer (NSCLC) defines the anatomic extent of the disease at the time of the diagnosis and will determine treatment recommendations and prognosis. The basis for NSCLC staging is the TNM system [1]. In the absence of distant metastases, regional lymph node status is critical for determining treatment options. Contrast enhanced computed tomography (CT) scanning of the chest is useful in providing anatomic detail, but the accuracy of chest CT scanning in differentiating benign from malignant lymph nodes in the mediastinum is limited[2]. Therefore, tissue verification of mediastinal lymph nodes is indicated to ensure accurate nodal staging.

Mediastinoscopy is considered the reference standard for invasive staging of patients with potentially operable NSCLC. Though invasive, mediastinoscopy is an accurate staging method, but it has limited access to posterior subcarinal lymph nodes and the lower mediastinum[3]. Transesophageal ultrasound-guided fine needle aspiration (EUS-FNA) is a safe and minimally invasive staging procedure with a diagnostic reach complementary to mediastinoscopy. EUS-FNA added to mediastinoscopy in the preoperative staging of lung cancer has been shown to result in improved nodal staging and to prevent futile thoracotomies [4,5]. EUS-FNA is useful for confirming mediastinal metastases, but has its limitations regarding its negative predictive value (73–83%) [6,7]. EUS-FNA has been shown to prevent surgical staging procedures in 50–70% of patients by demonstrating the presence of lymph node metastases [8,9]. Therefore, EUS-FNA has been advocated in recent guidelines as the first mediastinal staging test to provide tissue confirmation (but not exclusion) of nodal metastases [3,6]. The impact and accuracy of EUS-FNA have mostly been investigated in cohorts of selected patients with nodal enlargement on CT or with positron emission tomography (PET) positive lymph nodes[7]. To date, however, its merits in the routine preoperative staging of unselected patients with NSCLC are as yet unclear.

Therefore, we evaluated a lung cancer staging strategy involving consecutive patients with potentially operable lung cancer who were initially staged by EUS-FNA. Sensitivity, NPV and accuracy of EUS-FNA in the preoperative nodal staging of NSCLC were assessed. We also investigated whether a subgroup of lung cancer patients exists that can be accurately staged by EUS-FNA alone without subsequent surgical staging. Finally, we determined the impact of EUS-FNA on the prevention of surgical staging procedures.

MATERIALS AND METHODS

Design and patients

We retrospectively evaluated a lung cancer staging strategy over a 3.5-year period (between August 2003 and February 2007) in which patients with operable lung cancer

were initially staged by EUS-FNA. Consecutive patients with (suspected) NSCLC who were medically fit to undergo surgical resection of the lung tumor were discussed at the weekly Lung Oncology Board meeting of the Leiden University Medical Center. All patients had previously undergone a contrast enhanced chest computed tomography (CT) scan and fiberbronchoscopy. CT reports were examined to establish the location of the lung tumor and presence of enlarged (>10mm on the short axis) mediastinal lymph nodes. PET scans were not part of the standard staging protocol and did not influence the inclusion of patients.

EUS-FNA was used as the first mediastinal tissue staging procedure and was performed regardless of mediastinal nodal size at chest CT. Patients without locoregional nodal metastases (N2/N3) after EUS-FNA either underwent subsequent surgical staging by mediastinoscopy or direct thoracotomy with lymph node dissection. The choice of which subsequent procedure was to be performed was based on a consensus decision of the Lung Oncology Board. Negative EUS-FNA findings were compared to surgical-pathological staging. All complications which occurred during EUS-FNA and subsequent surgical procedures were recorded.

Procedures: EUS-FNA

The EUS-FNA examinations were performed in an ambulatory setting at the Department of Pulmonary Medicine, Leiden University Medical Center. A Pentax FG 34 UX echoendoscope (Pentax GmbH, Hamburg, Germany) was used with a longitudinal convex ultrasound transducer and an adjustable ultrasonic frequency of 5, 7.5 or 10MHz in combination with a Hitachi EUB 6500 ultrasound scanner (Hitachi Medical Systems Ltd., Reeuwijk, the Netherlands). Patients were under conscious sedation of midazolam intravenously. All mediastinal lymph node stations within diagnostic reach of EUS-FNA (stations 2L, 4L, (5), and 7–9) were evaluated in a standardized fashion. All visible lymph node stations were checked for certain features such as size, shape, sharp demarcated borders and echotexture. The decision on which mediastinal lymph nodes were to be aspirated, was made by the endoscopist based on this information. Enlarged, hypoechoic nodes with a round shape and well-demarcated borders were always aspirated while very small elongated flat nodes with an isoechoic texture and vague borders were mostly not. A mean number of 2.2 (range 0–6) different mediastinal lymph node stations were sampled. Aspiration of mediastinal lymph nodes was performed under ultrasound guidance from the esophagus with a 22-gauge needle and vacuum (Hancke/Vilmann type, GIP/Medi-Globe Inc., Tempe, Ariz). On-site staining and examination was done to determine whether representative material was obtained. Afterwards, all lymph node aspirates were judged by an experienced cytopathologist. Patients were observed for 2 h after the procedure and were instructed to contact the hospital if chest or other discomfort occurred.

Procedures: Surgery

Mediastinoscopy was performed according to current guidelines stating that at least both the lower paratracheal (4L and 4R) and the subcarinal (7) lymph node stations are to be biopsied. All mediastinoscopies (n = 40) were successful in targeting these lymph node stations and were therefore considered to be adequate. A mean number of 4.2 (range 3–6) different mediastinal lymph node stations were sampled at mediastinoscopy. Resected mediastinal lymph node tissue was examined according to standard procedures (in lymph nodes <1 cm, sliced once in the midline; in lymph nodes >1 cm, lamination of the lymph node and staining with hematoxylin-eosin).

When thoracotomy was performed, the primary tumor was resected by lobectomy or pneumectomy. A complete and systematic dissection of regional lymph node stations was performed according to the current guidelines[3]. A mean number of 5.9 (range 2–10) lymph node stations were sampled at thoracotomy.

Outcome measures

The main outcome measures were EUS-FNA test results such as sensitivity (the ability to detect mediastinal metastases), negative predictive value (the proportion of patients that do not have mediastinal metastases among all negative tested patients) and diagnostic accuracy (the total of correctly classified cases by EUS-FNA as a proportion of the whole cohort). They were also determined for specified subgroups such as (a) patients with metastases in lymph nodes within reach of EUS-FNA, (b) patients who underwent EUS-FNA and mediastinoscopy combined, and (c) patients who underwent EUS-FNA as the only mediastinal staging procedure. Furthermore, all patients were categorized according to tumor location and their lobe-specific diagnostic values were calculated accordingly. Finally, the number of surgical procedures prevented by EUS-FNA was determined. Surgery was defined as prevented in all cases in which EUS-FNA demonstrated the locoregional metastasis of lung cancer or in which EUS-FNA yielded a diagnosis other than lung cancer. All statistical analyses were performed using SPSS version 16.0 (SPSS, Chicago, IL, USA).

RESULTS

A total of 152 patients were included in this study. There were 101 males and 51 females with a median age of 66 years (range 38–82). Contrast enhanced CT scans of the chest showed the primary lung tumor to be located in the LUL (n = 44), LLL (n = 30), lingula (n = 1), central left lung (n = 6), RUL (n = 31), ML (n = 8), RLL (n = 28) and central right lung (n = 3). There was 1 patient with a double tumor on CT. 103 of 152 patients were shown to have nodal enlargement (short axis >10mm) on CT. Based on CT, 32% of patients were

staged as I/II and 68% as stage III. Final diagnoses and stage of all patients are presented in **Table 1**. 140 patients were diagnosed with NSCLC; 50 patients had a squamous cell carcinoma, 51 patients had an adenocarcinoma, 31 patients had an undifferentiated NSCLC, 4 patients had a neuroendocrine carcinoma and 4 patients had adenosquamous, metaplastic, sarcomatoid and mixed carcinoma, respectively.

Table 1. Overview of final diagnoses and tumor stage of the 152 patients with (suspected) NSCLC who underwent EUS-FNA as the initial mediastinal tissue staging procedure.

Final diagnoses (n=152)	
NSCLC	140
Stage I/II	40
Stage IIIA	50
Stage IIIB	41
Stage IV	9
SCLC	5
Carcinoid tumor	1
Metastasized extrathoracal cancer	2
Lymphoreticular malignancy	1
Sarcoidosis	1
Chondroid hamartoma	1
Infectious	1

EUS-FNA outcomes

It was feasible to perform a standardized examination in all 152 patients who were scheduled for EUS-FNA. In 56 patients (37%) EUS-FNA provided tissue proof of locally advanced stage III or stage IV NSCLC. Mediastinal lymph node metastases (N2/N3) were found in 51 patients (34%). Distant metastases (M1), localized either in the left adrenal gland (n = 2), the contralateral lung (n = 2) or celiac lymph nodes (n = 1), were proven by EUS-FNA in 5 patients (3%). In 7 patients (5%) with suspected NSCLC, EUS-FNA revealed a diagnosis other than NSCLC. In 4 patients the lymph node aspirate indicated the presence of small cell lung cancer. In 1 patient a metastasis of a carcinoid tumor was found in the mediastinal lymph nodes. Two patients were diagnosed with metastasized renal cell carcinoma and sarcoidosis, respectively.

Surgical-pathological staging

No mediastinal metastases (N0) nor distant metastases (M0) were found at EUS-FNA in 89 patients (59%). In 40 patients mediastinoscopy was subsequently performed as a second mediastinal staging method (**Fig. 1**). In 27 of these 40 patients the tumor location was right-sided: RUL (n = 12), ML (n = 3), RLL (n = 11) and central right lung (n = 1).

13 patients had a left-sided tumor: LUL (n = 7), LLL (n = 4), lingula (n = 1) and central left lung (n = 1). CT showed nodal enlargement in 26 of 40 patients (65%).

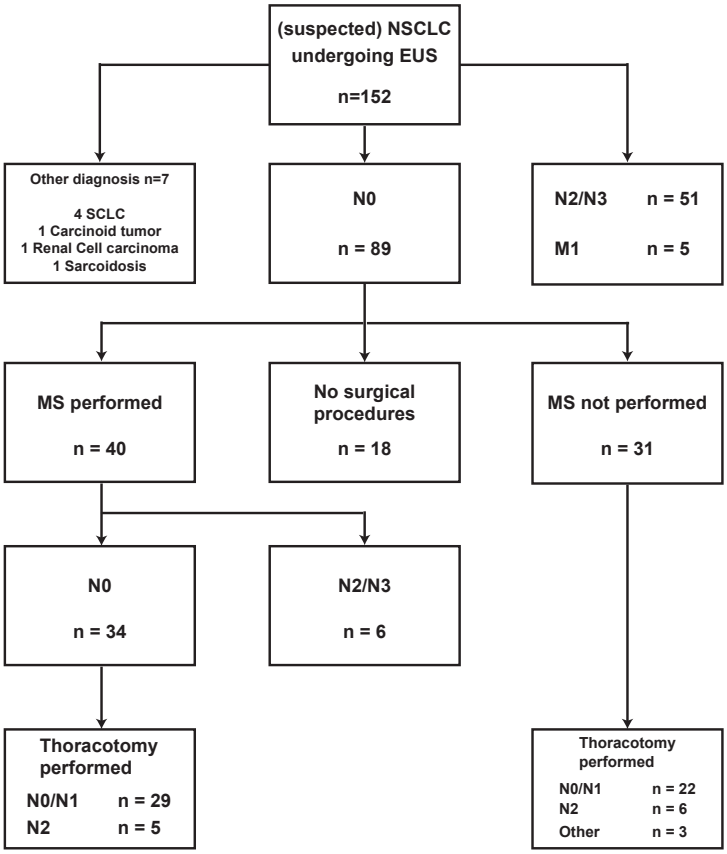

Figure 1. Flow chart of 152 consecutive patients with (suspected) NSCLC who underwent EUS-FNA in the preoperative mediastinal staging
Abbreviations: MS Mediastinoscopy

In 6 out of 40 patients (15%) with prior negative EUS-FNA, mediastinoscopy detected mediastinal metastases (N2/N3). These were located in lymph node stations 4L (n = 1), 4R (n = 2) and 7 (n = 3). The remaining 34 patients, in whom both EUS-FNA and mediastinoscopy did not detect nodal metastases, underwent thoracotomy with lymph node dissection. In 29 patients the absence of mediastinal metastases was confirmed. However, in 5 patients mediastinal metastases were found that were neither detected by EUS-FNA nor by mediastinoscopy. These metastases were located in lymph node stations 8 (n=1), 7 (n = 3) and 5/6 (n = 1).

31 patients who were staged N0 by EUS-FNA underwent thoracotomy with lymph node dissection without additional surgical staging (**Fig. 1**). 5 of these 31 patients had a right-sided tumor: RUL (n = 4) and RLL (n = 1). 26 patients had a left-sided tumor: LUL (n = 18), LLL (n = 7) and central left lung (n = 1). CT showed nodal enlargement in 12 of 31 patients (39%).

In 22 of the above 31 patients, the absence of mediastinal metastases was confirmed. However, in 6 patients mediastinal metastases were detected by thoracotomy. These were located in lymph node station 9 (n=3), 5 (n=1) and 7(n = 1). In 1 patient tumor cells were found in multiple lymph node stations (2L, 5, 6, and 8). In 3 patients histological examination of resected lung tissue revealed a diagnosis other than NSCLC. They had a pulmonary chondroid hamartoma, a cryptococcal infection and a metastasis of a resected sigmoid carcinoma, respectively.

No surgical verification of EUS-FNA findings

18 patients staged N0 by EUS-FNA did not undergo surgical procedures (**Fig. 1**). In 2 patients, surgery was not performed because endobronchial ultrasound revealed mediastinal metastases. One patient was staged N2 after detection of tumor cells in lymph node stations 5 and 7; the other was diagnosed with small cell lung cancer. In 8 patients, EUS-FNA ultrasound images were highly suspicious for invasion of the primary lung tumor in either the mediastinum or centrally located vessels (T4). These patients were staged IIIb and were not considered to be candidates for surgical treatment. Five patients were not considered for further surgical procedures due to the strong suspicion of T4 or M1 disease after additional non-invasive staging methods (MRI, PET). In 1 patient with suspected lung cancer a lymphoreticular malignancy was diagnosed after several diagnostic procedures. Two patients died shortly after the EUS-FNA procedure due to a deterioration of their clinical condition. There was no relation between the deterioration and the EUS-FNA procedure.

False negative EUS-FNA results

In this cohort, there were 89 patients in whom EUS-FNA found no mediastinal metastases. For 71 patients there was a surgical-pathological reference standard available. In 19 patients, EUS-FNA findings turned out to be false negative. Mediastinal metastases were detected by endobronchial ultrasound (n = 2), mediastinoscopy (n = 6) or thoracotomy (n = 11). Of these 19 patients, 13 patients had a left-sided tumor and 6 had a right-sided tumor. An overview of EUS-FNA false negative findings can be found in **Table 2**.

In 5 of 19 EUS-FNA false negative findings, nodal metastases were found in lymph node stations that are not within reach of EUS-FNA. Lymph node station 4R can often not be visualized due to intervening air in the trachea. Furthermore, lymph node stations

Table 2. Overview of false negative EUS-FNA findings.

Tumor localization	MS performed	Nodal metastasis detected by	Malignant lymph node	EUS-FNA type of error
LUL	Yes	Thor	7	Detection error
LUL	No	Thor	2L, 5,6, 8	Sampling error
LUL	Yes	Thor	5,6	Not within reach
LUL	Yes	Thor	7	Sampling error
LUL	No	Thor	5	Not within reach
LUL	Yes	MS	4L	Detection error
LUL	Yes	MS	7	Detection error
LUL	Yes	MS	4R	Not within reach
LLL	No	Thor	9	Detection error
LLL	No	Thor	7	Sampling error
LLL	No	Thor	9	Detection error
LLL	No	Thor	9	Interpretation error
LLL	Yes	EBUS	5,7	Sampling error
RUL	Yes	MS	7	Detection error
RUL	Yes	Thor	7,8	Detection error
RUL	Yes	MS	4R	Not within reach
RUL	No	EBUS	4R	Not within reach
RLL	Yes	Thor	7,8	Sampling error
RLL	Yes	MS	7	Sampling error

Types of error:

- Not within reach: the false negative finding is located in a lymph node station (4R,5 and 6) not within the diagnostic reach of EUS-FNA
- Detection error: the false negative finding is located in a lymph node station within reach of EUS-FNA, but has nevertheless not been detected by EUS-FNA.
- Sampling error: the false negative lymph node station has been detected by EUS-FNA. However, it was either not targeted because it was considered to be not suspect, or it was targeted but the aspirated lymph node failed to demonstrate tumor metastasis.
- Interpretation error: the false negative lymph node station was echographically suspect and located adjacent to the primary tumor. The aspirate demonstrated tumor cells but failed to show any lymphocytes, which made it unclear whether a mediastinal lymph node or the primary tumor was targeted.

Abbreviations: LUL left upper lobe, LLL left lower lobe, RUL right upper lobe, RLL right lower lobe

5 (aorto-pulmonary window) and 6 (para-aortal) are, even when visualized, difficult to target due to the intervening pulmonary artery (station 5) and aorta (station 6).

In 7 patients, EUS-FNA false negative findings were located in mediastinal lymph nodes that were principally within reach but had not been detected by EUS-FNA. Therefore, these false negative test results are considered to be detection errors.

In 6 patients the malignant lymph node was detected by EUS-FNA, but malignancy could not be confirmed. In 2 patients the malignant lymph node was not biopsied because it was echographically considered not to be suspected. In 4 patients the

malignant lymph node was targeted, but the aspirate demonstrated only lymphocytes without tumor metastasis. These 6 EUS-FNA false negative findings are considered to be sampling errors.

In 1 patient the malignant mediastinal lymph node was located adjacent to the primary tumor. Despite echographic visualization and cytological tumor diagnosis, it was unclear if the biopsied structure was a lymph node or the primary tumor. To avoid the possibility of a EUS-FNA false positive finding, this patient underwent subsequent thoracotomy despite doubts about possible presence of malignant mediastinal lymph nodes. This EUS-FNA false negative finding is considered an interpretation error.

Diagnostic values

The prevalence of mediastinal metastases in this cohort of 152 patients was 49%. EUS-FNA test results for the assessment of mediastinal lymph nodes were determined in a subgroup (n = 125) which is formed by NSCLC patients staged N2/N3 by EUS-FNA (n = 51), by suspected NSCLC patients who were diagnosed with SCLC (n = 4), by NSCLC patients staged N0 by EUS who underwent endobronchial ultrasound (n = 2) and by NSCLC patients staged N0 by EUS who underwent surgical procedures (n = 68). These test results are presented in **Table 3**. EUS-FNA had a sensitivity, NPV and diagnostic accuracy of 74%, 73% and 85%, respectively in the detection of all mediastinal lymph node metastases. These values do not take into account the fact that certain lymph node stations cannot be detected (station 4R) or safely biopsied (stations 5 and 6) by EUS-FNA. Therefore we also determined these EUS-FNA test results for the subgroup of patients (n = 120) with mediastinal metastases in lymph node stations located within reach of EUS-FNA. These were 80%, 78% and 88%, respectively. These test results were also determined for the subgroup of patients (n = 87) with nodal enlargement on CT. They were 83%, 74% and 89%, respectively.

Table 3. EUS-FNA diagnostic values for the detection of malignant mediastinal lymph nodes in patients with lung cancer. Sensitivity, NPV and accuracy of EUS-FNA are determined for the following subgroups:

	Sensitivity (%)	NPV (%)	Accuracy (%)
EUS-FNA overall (n=125)	74	73	85
EUS-FNA with nodal enlargement on CT (n=87)	83	74	89
EUS-FNA without nodal enlargement on CT (n=38)	40	71	76
EUS-FNA within reach (n=120)	80	78	88

- EUS-FNA overall: all mediastinal lymph nodes
- EUS-FNA with nodal enlargement on CT (short axis of > 10 mm)
- EUS-FNA without nodal enlargement on CT (short axis of < 10 mm)
- EUS-FNA within reach: for those mediastinal lymph nodes that are within the diagnostic reach of EUS (lymph node stations 2L, 4L, 7,8,9)

In the subgroup of patients (n = 40) who underwent EUS-FNA and mediastinoscopy combined, sensitivity, NPV and diagnostic accuracy were 92%, 85% and 95%, respectively. In the subgroup of patients (n = 31) in which EUS-FNA was the only staging method performed prior to thoracotomy, EUS-FNA had a sensitivity, NPV and diagnostic accuracy of 89%, 79% and 92%, respectively.

Several subgroups were identified in this cohort based on tumor location and nodal enlargement on CT. Sensitivity, NPV and diagnostic accuracy were calculated for all subgroups. These diagnostics values are shown, together with the prevalence of metastases and number of false negatives in the respective subgroups, in **Supplementary Table 1**.

Complications

152 patients were staged by EUS-FNA. Only two minor complications occurred. In 1 patient, the procedure was prematurely stopped due to a tachycardia of 150 beats per minute and restlessness. In the other patient, a small blood extravasate appeared surrounding the aspirated lymph node after EUS-FNA. However, the patient did not report any complaints and hospital admission was not required.

A total of 40 mediastinoscopies were performed. One procedure was complicated by right pulmonary artery hemorrhage requiring conversion to thoracotomy. The patient died later due to septic shock caused by double sided pneumonia. Another patient required sternotomy to treat an azygos vein hemorrhage.

A total of 65 thoracotomies were performed. Two patients died within 30 days of surgery. One patient died due to cardiac failure following completion pneumonectomy for extensive mediastinal and thoracic wall invasion. Autopsy showed dilating cardiomyopathy. Another patient died 6 weeks after bilobectomy due to bronchopleural fistula and empyema.

There were 3 cases of bronchopleural fistula (including the patient mentioned above), 2 following bronchial sleeve resection. One patient had paraplegia after lobectomy with extensive chest wall resection.

DISCUSSION

In this cohort of 152 consecutive operable patients with (suspected) NSCLC who were unselected by PET or CT, EUS-FNA had a sensitivity, NPV and accuracy of 74%, 73% and 85% for detecting malignant mediastinal lymph nodes. For the subgroup of 40 patients who were staged by both EUS-FNA and mediastinoscopy, these test results were 92%, 85% and 95%. Based on EUS-FNA findings, surgical staging procedures were prevented in 60 of 152 patients (39%) with lung cancer due to tissue proof of locally advanced disease (N2/N3 (n = 51), M1 (n = 5) or small cell lung cancer (n = 4)). Finally, the present

study demonstrated that it was feasible and safe to perform a standardized EUS-FNA examination routinely as the initial mediastinal tissue staging procedure in patients with NSCLC.

In a recent meta-analysis, a pooled EUS-FNA sensitivity and NPV of 83% and 78% was found[7]. In the present study, several factors accounted for the slightly lower test results. Firstly, the prevalence of mediastinal metastases in our study (49%) was relatively low compared to studies included in the meta-analysis (33%–85%)[7]. NPV is known to improve when the prevalence of mediastinal metastases in a given cohort is high. Secondly, sensitivity and NPV increase when NSCLC patients with nodal enlargement on CT are enrolled. In our unselected cohort of (suspected) lung cancer patients the proportion of patients with nodal enlargement on CT (68%) is also relatively low. EUS-FNA has been shown to have a low sensitivity (29–61%) for NSCLC patients without nodal enlargement on CT [10,11]. When sensitivity, NPV and accuracy are calculated for the subset of patients with nodal enlargement on CT (n = 82), these values improve to 83%, 74% and 89%, respectively. Thirdly, the overall EUS-FNA test results do not take into account the limited diagnostic reach of EUS-FNA. Lymph node stations 4R (due to intervening air) as well as 5 and 6 (due to intervening vascular structures) are notoriously difficult to visualize and target. When sensitivity, NPV and accuracy are determined for lymph node stations within reach of EUS-FNA, these test results improve to 80%, 78% and 88%, respectively.

For the subgroup of patients in which EUS-FNA and mediastinoscopy were combined, sensitivity, NPV and accuracy were 92%, 85% and 95%, respectively. Additional surgical staging in patients staged N0 at EUS-FNA reduces the false negative rate by half. The data from this retrospective study confirm our previous report in which we found that EUS-FNA added to mediastinoscopy improves the preoperative mediastinal staging of patients with NSCLC [4].

In a recent study, a high EUS-FNA sensitivity was found for left-sided tumors [12]. Left-sided tumors predominantly metastasize to lymph node stations 4L and 7 that can accurately be assessed by EUS-FNA. It could be argued that patients with left-sided tumors without nodal enlargement on CT could be staged accurately by EUS-FNA without the need for subsequent surgical staging. However, EUS-FNA sensitivity, NPV and accuracy for left-sided tumors in this cohort were only 65%, 68% and 80%, respectively (**Supplementary Table 1**). We could not identify a subgroup of patients, defined by tumor localization or nodal enlargement, for which EUS-FNA as the only mediastinal staging method could suffice.

EUS-FNA prevented additional surgical staging in 60 of 152 unselected patients (39%). This high proportion is in concordance with other studies in which patients were included who were selected by CT or PET [4,9,12]. Our retrospective study thus confirms that

EUS-FNA, as a minimally invasive alternative to surgical staging, reduces the need for surgical procedures.

In our study, only two minor complications occurred during EUS-FNA which did not require hospital admission or treatment. EUS-FNA has already established itself as a safe and minimally invasive modality for the staging of lung cancer [4,6,9,12]. One patient died after mediastinoscopy, suggesting a high mortality rate for mediastinoscopy. Including this patient however, the mortality rate after a mediastinoscopy in our hospital is 0.5% (3 deaths out of 590 mediastinoscopies) over the last 15 years, which is a lower mortality rate than reported in literature [6]. Morbidity rate for mediastinoscopy in our study (2.5%) as well as morbidity and mortality rate for thoracotomy correspond to rates reported in other studies [6,13].

This study has several limitations. Firstly, this is a retrospective analysis with all its inherent drawbacks. A lung cancer staging strategy was investigated which reflects actual clinical practice. The staging protocol that was in effect in the 3.5-year study period did not contain fixed rules indicating when patients staged N0 by EUS-FNA had to undergo additional surgical staging or when to proceed directly to thoracotomy with lymph node dissection. In a multidisciplinary meeting by the Lung Oncology Board the clinical condition and all preoperative staging methods for each patient were discussed and a consensus decision was made. Secondly, PET scans were not part of the standard staging protocol and did not influence the inclusion of patients. Finally, 18 patients who were staged N0 at EUS-FNA, did not undergo surgical procedures. These EUS-FNA results, indicating absence of mediastinal metastases, could not be verified by a surgical reference standard.

In conclusion, routine performance of EUS-FNA detects advanced disease in nearly half of patients with (suspected) NSCLC and therefore qualifies as a minimally invasive alternative for surgical staging. In patients without nodal metastases at EUS, additional surgical staging is indicated regardless of the location of the primary tumor or mediastinal nodal size.

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Supplementary Table 1. Subgroup analysis of EUS-FNA based on tumor location and nodal enlargement on CT. Sensitivity, NPV and diagnostic accuracy are shown, together with the prevalence of metastases and number of false negative.

Tumor localization	EUS-FNA Sensitivity (%)	EUS-FNA NPV (%)	EUS-FNA Accuracy (%)	Prevalence of metastases (%)	False negatives
Left sided (n=67)	68	68	81	40/67 (60%)	n=13
Left sided with nodal enlargement (n=41)	79	67	85	29/41 (71%)	n=6
Left sided without nodal enlargement (n=26)	37	68	73	11/26 (42%)	n=7
Right sided (n=58)	81	82	90	31/58 (53%)	n=6
Right sided with nodal enlargement (n=44)	85	81	91	27/44 (61%)	n=4
Right sided without nodal enlargement (n=14)	50	83	86	4/14 (29%)	n=2
LUL (n=40)	65	68	80	23/40 (58%)	n=8
LUL with nodal enlargement (n=25)	74	55	80	19/25 (76%)	n=5
LUL without nodal enlargement (n=15)	25	79	80	4/15 (27%)	n=3
LLL (n=23)	69	58	78	16/23 (70%)	n=5
LLL with nodal enlargement (n=12)	89	75	92	9/12 (75%)	n=1
LLL without nodal enlargement (n=11)	43	50	64	7/11 (64%)	n=4
RUL (n=26)	69	76	85	13/26 (50%)	n=4
RUL with nodal enlargement (n=17)	78	80	88	9/17 (53%)	n=2
RUL without nodal enlargement (n=9)	50	71	78	4/9 (44%)	n=2
RLL (n=24)	86	83	92	14/24 (58%)	n=2
RLL with nodal enlargement (n=19)	86	71	89	14/19 (74%)	n=2
RLL without nodal enlargement (n=5)	-	100	100	0/5 (0%)	n=0

Abbreviations: LUL left upper lobe, LLL left lower lobe, RUL right upper lobe, RLL right lower lobe

Chapter 3

Endosonography for lung cancer staging: predictors for false-negative outcomes

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ABSTRACT

Objectives

Non-small cell lung cancer (NSCLC) guidelines recommend endosonography (endobronchial [EBUS] and/or transesophageal ultrasound [EUS]) as the initial step for mediastinal tissue staging. Identifying predictors for false negative results could help establish which patients should undergo confirmatory surgical staging.

Materials and Methods

775 NSCLC patients staged negative by EBUS, EUS or combined EUS/EBUS were retrospectively analyzed. Predictors of false-negative outcomes were identified by logistic regression analysis.

Results and Conclusion

Three predictors for false-negative outcomes were identified: central location of the lung tumor (OR 3.7/4.5/3.6 for EBUS, EUS and EUS/EBUS respectively, $p<0.05$), nodal enlargement on CT (OR 3.2/2.5/4.9 for EBUS, EUS and EUS/EBUS respectively, $p<0.05$) and FDG-avidity of N2/N3 lymph node stations on PET (OR 4.2/4.0/7.5 for EBUS, EUS and EUS/EBUS respectively, $p<0.05$). One subgroup (peripheral lung tumor, nodal enlargement on CT without FDG-avidity for N2/N3) had a low predicted probability (7.8%) for false-negative EUS. For combined EUS/EBUS, two subgroups were identified: peripheral located tumor with nodal enlargement on CT but without FDG-avidity for N2/N3 (predicted probability 4.7%) and centrally located tumor without affected lymph nodes on CT or PET (predicted probability 3.4%). In conclusion, for specific well-defined subsets of NSCLC patients the low predicted probability of metastasis after negative endosonography might justify omitting confirmatory surgical staging.

INTRODUCTION

Lung cancer is the most common cause of cancer mortality in men in the developed world and one of the leading causes in women[1]. NSCLC comprises about 80% of all lung cancers[2]. Clinical TNM staging is pivotal because it forms the basis for treatment and has prognostic value[2]. Non-invasive staging methods such as computed tomography (CT) and positron emission tomography (PET) are valuable diagnostic tools that provide information about the size and location of the primary tumor as well as an indication of the presence of local and distant metastases. Tissue sampling of mediastinal lymph nodes is required for confirmation when imaging techniques such as CT and PET show signs of suspected nodal metastatic involvement since nodal status dictates treatment when distant metastases are absent[3].

Endosonography is a minimally invasive mediastinal tissue staging technique that allows sampling of intrathoracic lymph nodes under ultrasound guidance either by the airways (endobronchial ultrasound-guided transbronchial needle aspiration [EBUS-TBNA]) or the esophagus (transesophageal ultrasound-guided fine needle aspiration [EUS-FNA])[4]. Endosonography is advised as the test of choice for mediastinal nodal assessment in current guidelines[3,5,6] due to its high sensitivity of 83% (EUS) and 88% (EBUS)[7,8]. Additionally, combined endosonography (EUS-FNA and EBUS-TBNA) has been shown to improve sensitivity and predictive value compared to EUS or EBUS alone[3,9,10]. In the ASTER randomized clinical trial, combined EUS/EBUS was compared with surgical staging, which was previously considered as the gold standard for mediastinal tissue staging. Combined endosonography showed comparable sensitivity to surgical staging (85% vs 79%, p=0.47) but led to fewer unnecessary thoracotomies (18% vs 7%, p=0.02). Furthermore, it was shown that eleven patients needed to undergo mediastinoscopy in order to detect one single patient with N2 disease missed by combined EUS/EBUS[11].

An important question is whether patients should routinely undergo confirmatory surgical staging after negative endosonography or proceed directly to surgical resection of the primary tumor with systematic lymph node dissection. False-negative endosonography findings lead to suboptimal staging and treatment. However, unnecessary mediastinoscopies are associated with morbidity, treatment delay and higher health care costs[12]. In this study, we aim to identify patient-, tumor- and procedure-related predictors for false-negative endosonography results. Subsequently, we identify specific subgroups of NSCLC patients at risk of having a false-negative endosonography outcome (thus justifying confirmatory surgical staging) as well as subsets of patients for whom additional surgical staging has limited value.

MATERIALS AND METHODS

After examination of patient records from 1999-2013 by reviewing Endosonography databases from the Leiden University Medical Center (LUMC, The Netherlands), Leeuwarden Medical Center (MCL, The Netherlands) and Ghent University Hospital (GUH, Belgium), patients were retrospectively included based on the following criteria:

- intrapulmonary lesion suspected of or histologically confirmed as primary NSCLC
- endoscopic staging procedures (EUS, EBUS or both) performed without detection of mediastinal nodal metastases (cN0/N1)
- availability of a surgical mediastinal nodal reference standard
- final diagnosis of NSCLC.

Of all patients, gender and age was noted. Based on computed tomography (CT) and positron emission tomography (PET) reports, several tumor characteristics were recorded, such as tumor location, nodal enlargement on CT (short axis > 10mm) and FDG-avidity of mediastinal lymph nodes (N2/N3) on PET scans. Furthermore, it was determined whether the tumor was located centrally (inner third of the thorax) or at the periphery of the lung. When available, a histological diagnosis before endosonography procedures was determined based on patients' pathology reports.

Procedures

EUS-FNA, EBUS-TBNA or combined procedures were performed in three hospitals (LUMC, MCL, GUH) with linear echo-endoscopes using 22-gauge needles, as previously described[11,13,14]. The mediastinum was assessed in a standardized fashion [15]. From all endosonography procedures, the number of needle passes and the short axis of the largest lymph node were noted. Furthermore, it was recorded whether mediastinal lymph nodes with echographic features suggestive of malignancy (either short axis >10 mm, round shape, sharp demarcation, or a diffusely hypoechoic ultrasound pattern) were detected.

Surgical staging was performed by mediastinoscopy with systematic assessment of left and right high and lower paratracheal and subcarinal nodes. Parasternal mediastinotomy (MT) or video assisted thoracoscopic surgery (VATS) was used in case of a suspected lymph node metastasis in lymph node station 5 or 6. Thoracotomy was performed by (bi)lobectomy or pneumectomy with dissection of regional lymph node stations according to current guidelines[3,16]. Based on pathology reports, a final diagnosis of NSCLC was determined.

Data analysis

The outcome measure of this study was either the presence or absence of false negative endosonography (EUS, EBUS or combined EUS/EBUS) findings. False-negative findings

(N2/N3) were defined as mediastinal nodal metastases missed by endosonography that were found during either surgical staging (mediastinoscopy, parasternal mediastinotomy or VATS) or surgical resection with systematic lymph node dissection. False-negatives were classified as either detection errors (lymph node metastasis not detected by endosonography) or sampling errors (a missed metastasis despite lymph node sampling during endosonography). True negative endosonography findings (N0/N1) were defined as negative endosonography results that were confirmed by surgical sampling of mediastinal lymph nodes during surgical staging procedures, thoracotomy or both.

Since the outcome measure is binary, all potential predictors were assessed by multivariable logistic regression, thereby adjusting for potentially confounding variables. Subsequently, we performed an automatic variable selection procedure with a p-value of >0.10 based on the log likelihood ratio test (with backward selection). Hereby, the model was reduced which led to the strongest predictors that remained in the final model. Results were reported as odds ratios (ORs) with 95% confidence intervals (95% CI). Based on the predicted probabilities of the final model, an area under the ROC curve (c-statistic) was calculated for each dataset (EUS, EBUS, EUS/EBUS combined) to assess the discriminative ability of the model. A Hosmer–Lemeshow test was done to determine goodness-of-fit of the final logistic regression model. All analyses were conducted with SPSS Statistics for Windows, Version 20.0 (SPSS Inc., Chicago, IL).

RESULTS

Data were retrieved from NSCLC patients who underwent EBUS (n=182), EUS (n=471), or a combined EUS/EBUS procedure (n=122) for diagnostic or staging purposes. A summary of patient, tumor and procedure-related characteristics of all three cohorts is displayed in **Table 1**.

EBUS-TBNA

From 2004 to 2013, a total of 182 patients were identified in whom EBUS-TBNA did not demonstrate nodal metastases and who underwent subsequent surgical verification of mediastinal nodal status. 109 patients underwent surgical staging by mediastinoscopy (n=101), parasternal mediastinotomy (n=5) or VATS (n=3). These procedures showed histologically proven mediastinal metastases (N2/N3) in 26 patients (23.9%). In 83 patients, no mediastinal metastases were detected, of whom 80 patients subsequently underwent thoracotomy. Surgical resection of the tumor with lymph node dissection showed the presence of metastases in mediastinal lymph node stations (N2/N3) in 15 patients (18.8%), whereas 65 patients were confirmed to be free of mediastinal metastases (N0/

Table 1. Patient characteristics

Study characteristics	EBUS (n= 182)	EUS (n= 471)	EUS/EBUS (n= 122)
Hospital			
Leiden University Medical Center	130	296	94
Ghent University Hospital	52	39	28
Leeuwarden Medical Center	0	136	0
Age, mean (SD), y	66 (9)	65 (10)	65 (9)
Sex, No. (%)			
Male	117 (64)	330 (70)	85 (70)
Female	65 (36)	141 (30)	37 (30)
Indication for staging, No. (%)			
Suspected NSCLC	76 (42)	132 (28)	30 (25)
Staging NSCLC			
Adenocarcinoma	33 (18)	81 (17)	33 (27)
Squamous cell carcinoma	45 (25)	153 (33)	32 (26)
Large cell carcinoma	7 (4)	24 (5)	9 (7)
NSCLC NOS	19 (11)	81 (17)	18 (15)
Tumor localization, No. (%)			
Left lower lobe	19 (10)	88 (19)	20 (16)
Left upper lobe	27 (15)	183 (39)	31 (25)
Right upper lobe	86 (47)	111 (24)	44 (36)
Middle lobe	7 (4)	15 (3)	4 (3)
Right lower lobe	43 (24)	72 (15)	23 (19)
Central right	0 (0)	2 (0)	0 (0)
Central tumor on CT, No. (%)			
Yes	76 (42)	223 (47)	43 (35)
No	104 (57)	242 (52)	79 (65)
Unknown	2 (1)	5 (1)	0 (0)
Nodal status PET, No. (%)			
N0/N1	72 (39)	140 (30)	61 (50)
N2/N3	70 (39)	162 (34)	58 (48)
No PET	40 (22)	169 (36)	3 (3)
Nodal enlargement on CT, No. (%)			
Yes	77 (42)	191 (41)	52 (43)
No	105 (58)	280 (59)	70 (57)
Short axis of largest LN on CT, mean (SD), mm	11 (6)	12 (5)	11 (4)
Lymph node suspect during E(B)US			
Yes	67 (37)	155 (33)	49 (40)
No	91 (50)	308 (65)	71 (58)
Missing	24 (13)	8 (2)	2 (2)
Number of needle passes, mean (range), No.	3 (0-8)	3 (0-9)	5 (0-14)

Table 1. Patient characteristics (continued)

Study characteristics	EBUS (n= 182)	EUS (n= 471)	EUS/EBUS (n= 122)
Short axis of largest LN during E(B)US, mean (SD), mm	11 (7)	8 (6)	11 (5)
Final diagnosis, No. (%)			
Adenocarcinoma	72 (40)	155 (33)	46 (38)
Squamous cell carcinoma	62 (34)	233 (50)	46 (38)
Adenosquamous	3 (2)	11 (2)	2 (2)
Large cell carcinoma	7 (4)	16 (3)	6 (5)
Neuroendocrine	7 (4)	3 (1)	1 (1)
Carcinoid	3 (2)	1 (0)	0 (0)
NSCLC NOS	28 (15)	52 (11)	21 (17)

Abbreviations: CT computed tomography, EBUS endobronchial ultrasound-guided transbronchial needle aspiration, EUS transesophageal ultrasound-guided fine needle aspiration, LN lymph node, NOS not otherwise specified, NSCLC non-small cell lung cancer, SD standard deviation

N1). For 73 patients, thoracotomy was the next step after negative EBUS findings. Mediastinal metastases (N2/N3) were found in 11 patients (15.1%), whereas 62 patients did not have mediastinal metastases (N0/N1). A flowchart of this cohort is presented in **online supplementary Figure 1**.

A total of 52 false-negatives occurred in this cohort (**Table 2**). False-negative findings were classified as either detection errors (n=24) or sampling errors (n=28). Lymph node stations 2R, 4R, 2L, 4L and 7 are considered to be within the diagnostic reach of EBUS. Nine false-negative outcomes (17%) occurred in lymph node stations outside the reach of EBUS. Lymph node stations 4R (n=15) or multiple stations (n=18) were most frequently affected. In the latter group of multiple affected lymph node stations, only two cases involved stations outside the reach of EBUS (stations 5, 6, 8 and 9).

All potential predictive variables (age, sex, central location, nodal enlargement on CT, FDG-avidity for N2/N3 on PET, tumor location, tumor histology, number of needle passes, enlarged LN during EUS, suspect LN during EUS) were included in the multivariable logistic regression analysis. After reduction of the model by backward selection, seven predictors (age, sex, tumor location, tumor histology, number of needle passes, enlarged LN during EUS, suspect LN during EUS) failed to reach statistical significance ($p > 0.05$). Three variables remained strongly associated with false-negative EBUS outcomes (**Table 3**): central location of the lung tumor (OR 3.7, CI 95% 1.5-8.9, p= 0.004), nodal enlargement on CT (OR 3.2, CI 95% 1.3-7.8, p= 0.009) and FDG-avidity for N2/N3 on PET (OR 4.2, CI 95% 1.6-10.7, p=0.003). The c-statistic (area under the ROC curve), based on the predicted probabilities of the final model, was 0.782, indicating good discriminative ability of this model. The Hosmer-Lemeshow test was non-significant (p=0.488), indicating good calibration of this model.

Table 2. False-negative endosonography outcomes. Stations 2R, 4R, 2L, 4L and 7 are considered within the reach of EBUS. Stations 2L, 4l, 7, 8 and 9 are considered within the reach of EUS.

	EBUS (n= 52)	EUS (n= 112)	EUS/EBUS (n= 18)
Number of FN, No. (%)			
Detection error	24 (46)	70 (63)	7 (39)
Sampling error	28 (54)	42 (37)	11 (61)
Detected by MS/MT/VATS	26 (50)	51 (46)	9 (50)
Detected by Thoracotomy	26 (50)	61 (54)	9 (50)
FN within reach of test (%)	43 (83)	53 (47)	13 (72)
FN outside reach of test (%)	9 (17)	59 (53)	5 (28)
FN stations			
2R	1	3	1
2L	0	1	0
3	0	0	1
4R	15	21	5
4L	4	9	2
5 and/or 6	6	35	5
7	7	23	0
8R	0	0	2
8L	0	2	1
9	1	4	0
Multiple stations	18	14	1

Detection error: tissue-proven mediastinal nodal metastasis not detected by endosonography.
Sampling error: a tissue-proven mediastinal nodal metastasis missed by endosonography despite sampling
Abbreviations: EUS transesophageal ultrasound-guided fine needle aspiration, EBUS endobronchial ultrasound-guided transbronchial needle aspiration, FN False negative, MS mediastinoscopy, MT parasternal mediastinotomy; VATS video assisted thoracoscopic surgery

EUS- FNA

From 1999 to 2013, a total of 471 patients were identified who had undergone EUS-FNA for mediastinal staging of (suspected) NSCLC without detection of mediastinal metastases and in whom a surgical reference standard was available. 289 patients proceeded to surgical staging by cervical mediastinoscopy (n=276), parasternal mediastinotomy (n=7) or VATS (n=5). Surgical staging showed histologically proven mediastinal metastases (N2/N3) in 51 patients (17.6%). In 238 patients, no mediastinal metastases were found, of whom 225 patients subsequently underwent surgical resection of the tumor. Lymph node sampling during thoracotomy showed 34 patients (15.1 %) with metastases in mediastinal lymph node stations (N2/N3), whereas 191 patients were confirmed to be free of mediastinal metastases (N0/N1). For 182 patients, the clinical decision was made to

Table 3. Predictors of false-negative EBUS, EUS and combined EUS/EBUS results.

EBUS			
Variable	***Odds Ratio***	***95% CI***	***P value***
Central location of lung tumor	3.7	1.5 - 8.9	0.004
Nodal enlargement on CT	3.2	1.3 - 7.8	0.009
FDG-avidity for N2/N3 on PET	4.2	1.6 - 10.7	0.003
EUS			
Variable	***Odds Ratio***	***95% CI***	***P value***
Central location of lung tumor	4.5	2.4 - 8.6	0.000
Nodal enlargement on CT	2.5	1.4 - 4.8	0.004
FDG-avidity for N2/N3 on PET	4.0	2.0 - 8.2	0.000
EUS/ EBUS			
Variable	***Odds Ratio***	***95% CI***	***P value***
Central location of lung tumor	3.6	1.1 - 11.6	0.036
Nodal enlargement on CT	4.9	1.4 - 17.6	0.015
FDG-avidity for N2/N3 on PET	7.5	1.5 - 36.5	0.013

Abbreviations: CI Confidence Interval, CT computed tomography, EBUS endobronchial ultrasound-guided transbronchial needle aspiration, EUS transesophageal ultrasound-guided fine needle aspiration, PET positron emission tomography

proceed to direct thoracotomy after negative EUS-FNA findings. In 27 patients (14.8 %), mediastinal metastases (N2/N3) were found during thoracotomy, whereas 155 patients were free of mediastinal metastases (N0/N1). A flowchart of this cohort is presented in **online supplementary Figure 2**.

A total of 112 false-negative EUS results occurred (**Table 2**). False-negative findings were classified as either detection errors (n=70) or sampling errors (n=42). Lymph node stations 2L, 4L, 7, 8 and 9 are considered to be within the diagnostic reach of EUS. 59 false-negative outcomes (53%) occurred in lymph node stations outside the reach of EUS, of which stations 5/6 (n=35) and 4R (n=21) were affected in the majority of cases.

All potential predictors were evaluated by multivariable logistic regression analysis. After reduction of the model by backward selection, seven predictors (age, sex, tumor location, tumor histology, number of needle passes, enlarged LN during EUS, suspect LN during EUS) failed to reach statistical significance ($p > 0.05$). Three variables remained strongly associated with false-negative EUS outcomes (**Table 3**): central location of the lung tumor (OR 4.5, CI 95% 2.4-8.6, $p < 0.001$), nodal enlargement on CT (OR 2.5, CI 95%

1.4-4.8, p= 0.004) and FDG-avidity for N2/N3 on PET (OR 4.0, CI 95% 2.0-8.2, p< 0.001). The c-statistic (area under the ROC curve) was 0.773, indicating good discriminative power of this model. The Hosmer-Lemeshow test was non-significant (p=0.989), indicating good calibration of this model.

Combined EUS/EBUS

From 2005 to 2013, a total of 122 patients underwent a combined EUS/EBUS procedure for mediastinal staging of (suspected) NSCLC without detection of mediastinal metastases. 84 patients underwent additional surgical staging by mediastinoscopy (n=79), parasternal mediastinotomy (n=2) or VATS (n=3). These procedures showed histologically proven mediastinal metastases (N2/N3) in 9 patients (10.7%). In 75 patients, no mediastinal metastases were detected, of whom 69 patients subsequently underwent thoracotomy. Surgical resection of the tumor with lymph node dissection showed the presence of metastases in mediastinal lymph node stations (N2/N3) in 7 patients (10.1 %), whereas 62 patients were confirmed to be free of mediastinal metastases (N0/N1). 38 patients proceeded directly to surgical resection after negative EUS/EBUS outcomes. Mediastinal metastases (N2/N3) were found in 2 patients (5.3%), whereas 36 patients did not have mediastinal metastases (N0/N1). A flowchart of this cohort is presented in **online supplementary Figure 3**.

A total of 18 false-negatives occurred in this cohort (**Table 2**). False-negative findings were classified as either detection errors (n=7) or sampling errors (n=11). 5 false-negative EUS/EBUS outcomes (28%) occurred in lymph node stations 5/6, which are difficult to reach even when combining both procedures.

All potential predictive variables were included in the multivariable logistic regression analysis (**Table 3**). Again seven predictors failed to reach statistical significance (p> 0.05), whereas the same three variables as in the previous cohorts remained strongly associated with false-negative EUS/EBUS outcomes: central location of the lung tumor (OR 3.6, CI 95% 1.1-11.6, p= 0.036), nodal enlargement on CT (OR 4.9, CI 95% 1.4-17.6, p= 0.015) and FDG-avidity for N2/N3 on PET (OR 7.5, CI 95% 1.5-36.5, p=0.013). The c-statistic (area under the ROC curve) was 0.832, indicating good discriminative power of this model. The Hosmer-Lemeshow test was non-significant (p=0.923), indicating good calibration of this model.

Predicted probabilities of logistic regression model after backward selection

In all three cohorts (EUS, EBUS and combined EUS/EBUS) the same three variables reached statistical significance (p<0.05) and remained in the logistic regression model after backward selection: central location of the lung tumor, nodal enlargement on CT and FDG-avidity for N2/N3 on PET. This reduced model was used to predict the occurrence of false negative findings for EUS, EBUS and EUS/EBUS combined. **Table 4** displays

the predicted probabilities of false negative occurrence generated by this model for all three cohorts when combining the three main variables.

Table 4. Predicted probabilities of false negative endosonography results in all three cohorts (EBUS, EUS, EUS+EBUS combined). Probabilities were generated based on the logistic regression model using the combination of the three main variables that reached statistical significance (p<0.05). Patient subsets with predicted post-test probability of <5% are indicated in bold.

	EBUS	EUS	EUS/EBUS
Peripheral tumor, no nodal enlargement on CT, PET N0/N1	**0.04253**	**0.032**	**0.00987**
Peripheral tumor, nodal enlargement on CT, PET N0/N1	0.12542	0.078	**0.04656**
Peripheral tumor, no nodal enlargement on CT, PET N2/N3	0.15611	0.119	0.06959
Central tumor, no nodal enlargement on CT, PET N0/N1	0.14059	0.131	**0.03423**
Peripheral tumor, nodal enlargement on CT, PET N2/N3	0.37393	0.255	0.26823
Central tumor, nodal enlargement on CT, PET N0/N1	0.34562	0.277	0.14798
Central tumor, no nodal enlargement on CT, PET N2/N3	0.40523	0.377	0.21012
Central tumor, nodal enlargement on CT, PET N2/N3	0.68748	0.606	0.56589

Abbreviations: CT computed tomography, EBUS endobronchial ultrasound-guided transbronchial needle aspiration, EUS transesophageal ultrasound-guided fine needle aspiration, PET positron emission tomography

DISCUSSION

We retrospectively identified 775 NSCLC patients who were staged N0/N1 by either EBUS (n=182), EUS (n=471) or combined EUS/EBUS (n=122), and underwent subsequent surgical evaluation of the mediastinum. In these three cohorts 52 (28.6%), 112 (23.8%) and 18 (14.7%) false-negative cases occurred respectively, which supports the assertion that endosonography has limitations in excluding mediastinal metastatic disease and that complete mediastinal staging by combined EUS/EBUS has superior test characteristics in comparison to EUS and EBUS alone[9-11,17-20]. The relatively high prevalence of false-negative cases can in part be explained by the diagnostic reach of the techniques. In the EUS cohort 59 false negatives (52.6%) occurred outside the diagnostic reach of this technique (**Table 2**). 24 of these false-negatives were located in right sided paratracheal lymph nodes (stations 2R/4R) within the reach of EBUS, indicating that a combined approach could have reduced the number of false-negatives. When adjusted for the diagnostic reach of these techniques the false-negative rates for EBUS (23.6%), EUS (11.3%) and combined EUS/EBUS (10.7%) are well within the previously reported range[3].

Our study focused on identifying determinants associated with false-negative endosonography outcomes by multivariable logistic regression analysis. In all three cohorts, the same three variables stood out: central location of the lung tumor (OR 4.5/ 3.7/3.6 for EUS, EBUS and EUS/EBUS respectively, p<0.05), nodal enlargement on CT (OR 2.5/3.2/4.9

for EUS, EBUS and EUS/EBUS respectively, p<0.05) and FDG-avidity of N2/N3 lymph node stations on PET (OR 4.0/4.2/7.5) for EUS, EBUS and EUS/EBUS respectively, p<0.05). Several other clinical determinants such as tumor location (left vs right sided lung tumors) failed to reach statistical significance.

The reduced logistic regression model, containing the three main variables (central location of the lung tumor, nodal enlargement on CT and FDG-avidity for N2/N3) was used to predict the occurrence of mediastinal metastases (N2/N3) after negative EUS, EBUS and combined EUS/EBUS outcomes (**Table 4)**. A post-test probability of nodal metastasis of 5% or less was proposed as an acceptable cut-off point for clinicians[21].

For EUS, the subgroup of patients with a peripheral tumor and nodal enlargement on CT without FDG-avidity has a low predicted probability (7.8%) of providing a false-negative result. This might imply that this subgroup has very limited chance of benefiting from additional surgical staging. For EBUS, such a subgroup is difficult to identify. Patients with a peripheral tumor and a normal mediastinum on CT and PET have a low probability (4.2%) of having a false-negative outcome, but since the pre-test probability of a mediastinal nodal metastasis is very low in these patients anyway, they generally proceed directly to surgical resection without further tissue staging[3,22]. For combined EUS/EBUS, two subgroups with low post-test probability of false-negative outcome can be identified. Patients with a peripheral tumor with nodal enlargement on CT but without FDG-avidity have a probability of 4.7% to be false-negative, whereas patients with a central lung tumor but without affected lymph nodes on CT or PET have a post-test probability of 3.4% to be false-negative. The constructed model also predicts low to moderate probability (7.0%) of false-negative outcomes in patients with a peripheral located lung tumor, no nodal enlargement on CT but with FDG-avidity of N2/N3 stations. Clinicians should consider whether additional mediastinoscopy is really beneficial to patients in these subgroups considering that a recent randomized trial established the need for 11 mediastinoscopies to detect one patient with N2 disease missed by combined EUS/EBUS[11]. The current data underlie the importance of combined EBUS/EUS staging which can be achieved by only using the EBUS scope[17].

Several limitations apply to this study. Its retrospective design has inherent drawbacks. In a considerable subset of patients, mainly in the EUS cohort, PET results were not available since these scans were only recently implemented in the routine preoperative staging of NSCLC. Also, a selection bias might be present as from one center (MCL) only EUS results were available and from another center (GUH) data on a surgical reference standard was difficult to retrieve from referring hospitals, which accounts for its relatively modest contribution to sample size. Finally the logistic regression model should, despite its good calibration and discriminative ability, be validated on a prospective cohort of NSCLC patients in order to establish its external validity.

In conclusion, these data show that three variables (central location of the lung tumor, nodal enlargement on CT and FDG-avidity for N2/N3 on PET) are associated with false-negative EUS/EBUS outcomes. By combining these variables in a logistic regression model, we were able to identify subgroups of patients who have a low probability of false-negative endosonography outcomes, which might imply that these patients have limited benefit from additional surgical staging and can proceed directly to surgical resection. However, prospective studies should confirm these data in order to establish external validity of this model.

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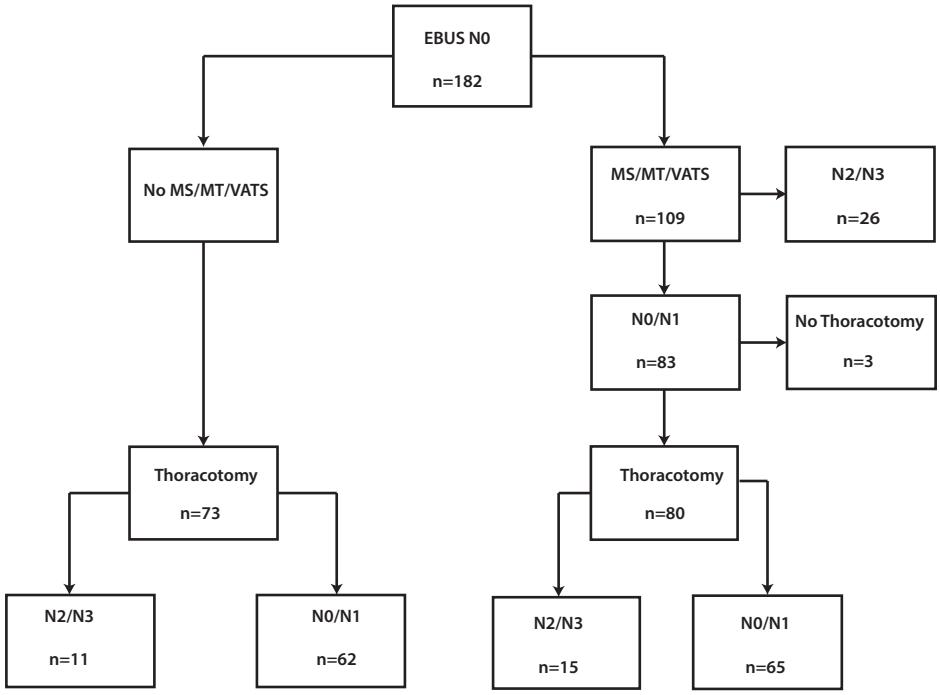
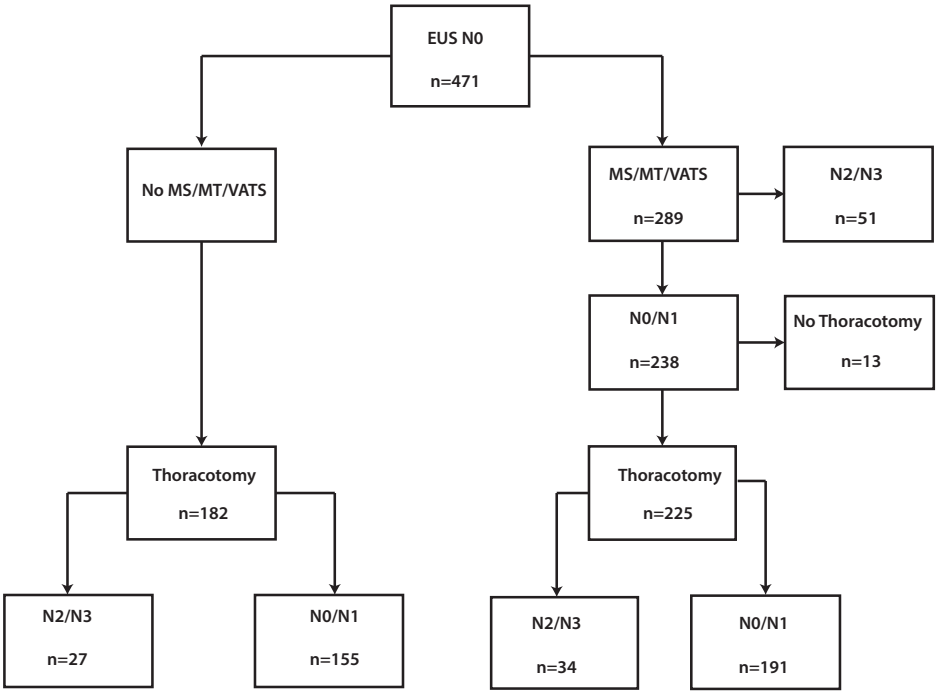
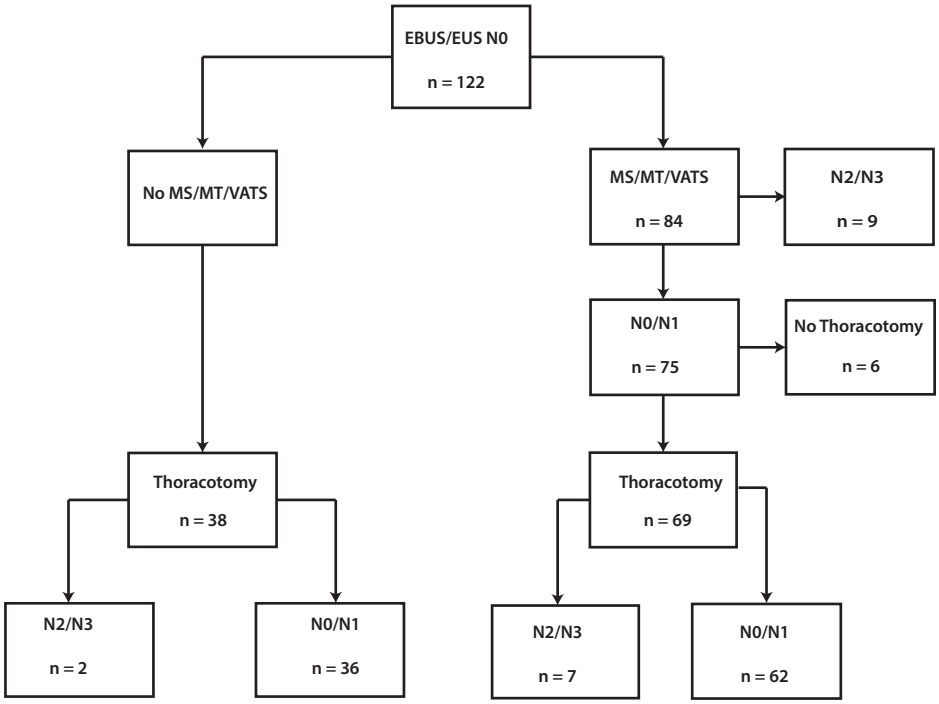


Online supplementary Figure 1. EBUS-TBNA study cohort
Abbreviations: EBUS endobronchial ultrasound-guided transbronchial needle aspiration, MS cervical mediastinoscopy, MT parasternal mediastinotomy, VATS video assisted thoracoscopic surgery



Online supplementary Figure 2. EUS-FNA study cohort
Abbreviations: EUS transesophageal ultrasound-guided fine needle aspiration, MS cervical mediastinoscopy, MT parasternal mediastinotomy, VATS video assisted thoracoscopic surgery



Online supplementary Figure 3. EUS/EBUS-TBNA study cohort
Abbreviations: EUS, transesophageal ultrasound-guided fine needle aspiration, EBUS, endobronchial ultrasound-guided transbronchial needle aspiration, MS cervical mediastinoscopy, MT parasternal mediastinotomy, VATS video assisted thoracoscopic surgery

Chapter 4

Recent progress in peptide vaccination in cancer with a focus on non-small cell lung cancer

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ABSTRACT

Active immunotherapy aimed at the stimulation of tumour-specific T-cells has established itself within the clinic as a therapeutic option to treat cancer. One strategy is the use of so-called peptides that mimic genuine T-cell epitopes as vaccines to activate tumour-specific T-cells. In various clinical trials, different types of vaccines, adjuvants and other immunomodulatory compounds were evaluated in patients with different types of tumours. Here we review the trials published in the last 3 years focusing on the T-cell response, the effect of immunomodulation and potential relationships with clinical outcomes. Furthermore, we would like to make a case for the development of peptide vaccines aiming to treat non-small cell lung cancer, the most common cause of cancer mortality.

INTRODUCTION

Active immunotherapy is emerging as a new, valuable addition to currently existing standard therapies in oncology. It has established itself recently by the FDA approval of sipuleucel-T, a therapeutic vaccine for the treatment of prostate cancer [1] and ipilimumab, a therapeutic antibody against cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) [2]. Moreover, recent studies on the use of antibodies blocking PD-1 (programmed death 1 receptor) and PD-L1 (PD-1 ligand) activity have shown impressive clinical and durable response rates in patients with several solid tumours [3-5]. In a recent phase I trial, ipilimumab was combined with the anti-PD-1 antibody nivolumab. At the maximum doses that were associated with an acceptable level of adverse events, 53% of advanced melanoma patients had an objective clinical response reflected by a tumour reduction of 80% or more. These results are considered as another important example of the role of the immune system in protecting against cancer progression and the clinical potential of active immunotherapy [6].

The immune system plays a major role in cancer [7,8]. Professional antigen presenting cells (APC), predominantly dendritic cells (DC), sample antigen from tumours. These so-called tumour antigens are processed and then presented as peptides by major histocompatibility complex (MHC) class I and II molecules on the DC surface, to be recognized by the T-cell receptor (TCR) of T-cells. MHC class I – peptide complexes are recognized by CD8+ T-cells that become activated and differentiate into effector cytotoxic T lymphocytes (CTL). MHC class II – peptide complexes are recognized by the TCR of the CD4+ T-cells. Upon stimulation, tumour-specific CD4+ T-helper cells deliver essential help for tumour-specific CD8+ T-cells by fully activating DC through the CD40- CD40L signaling pathway as well as by secretion of IL-2 [7,9]. Moreover, the tumour-specific CD4+ T-cells provide help by guiding the homing of CD8+ T-cells and by polarizing the local tumour environment to allow tumour-specific CD8 T-cells to exert their tumouricidal function [10]. Collectively, the close interaction between DC, CTL and T-helper cells is aimed at the control of tumour growth and ultimately may contribute to tumour regression.

Active immunotherapy focuses on the exploitation of T-cell mediated anti-tumour responses. It consists of three major classes (or combination(s) of the following):

1. Antibodies inhibiting key immune checkpoint receptors (e.g. CTLA-4, PD-1, PD-L1) that govern T-cell responses;
2. Adoptive cell therapy, the infusion of T-cells specific for tumour cells;
3. Vaccines, to enhance tumour-specific T-cell responses.

Vaccines, in particular peptide based therapeutic vaccines, aiming at the induction of tumour antigen-specific immune responses, are the subject of extensive research. Our group has conducted a number of trials in which cancer patients were treated with overlapping synthetic long peptide (SLP) vaccines covering the sequence of tu-

mour antigens such as HPV16 E6/E7 and p53. SLP vaccines are safe and have induced robust T-cell responses in several early phase clinical trials [11-14]. Moreover, vaccination of patients with HPV16-induced high-grade vulvar intraepithelial neoplasia (VIN 3) with a HPV16-based SLP vaccine resulted in durable regression of these premalignant lesions [15]. Importantly, the kinetics and phenotype of the immune response (strong and broad vaccine-prompted HPV16-specific proliferative response with T-cells producing higher levels of IFNg and IL-5) were clearly linked with complete regression of the lesion [16]. These results show that immunotherapy based on the use of peptide vaccines can mediate clinical effects.

Peptide vaccines have been studied since the seminal paper of Kast *et al.*, showing that a peptide vaccine could induce protection to an otherwise lethal infection with Sendai virus [17] and the developments of peptide vaccines have been reported by us earlier [9,18,19]. Here, we summarize recent progress in the field of peptide vaccination in solid tumours as published from 2011 to 2013. Data on toxicity, immune response and clinical effect in phase I, II or III trials will be discussed and are summarized in **Table 1**.

Next, we will focus on lung cancer, which is the most common cause of cancer mortality in men in the developed world and one of the leading causes in women[20]. The two major forms of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC comprises about 80 % of all lung cancers [21]. The majority of patients present with advanced disease (stage III/IV), which accounts for high rates of morbidity and mortality in NSCLC [22]. Current therapies provide limited benefit. Interestingly, the immune contexture of NSCLC and the high number of mutations found in these cancers provide a good rationale for strategies involving immunotherapeutic approaches, such as therapeutic vaccines, but studies in this area are limited. Therefore, in the second part of this review, we will discuss current research and successful T-cell based immunotherapy in NSCLC as a rationale to increase the efforts on therapeutic vaccine development for NSCLC.

PEPTIDE VACCINATION IN CANCER

Peptides vaccines aiming to induce tumour-specific CD8 T-cell responses

Vaccines consisting of exact Human Leukocyte Antigen (HLA) class I-binding peptides corresponding to the amino acid sequence of parts of tumour antigens are one of the oldest and most widely studied forms of peptide vaccines [19]. These vaccines are relatively easy to produce but are restricted to particular HLA alleles and require patients to be selected for this purpose.

In a phase II trial, 61 patients with metastatic melanoma were treated with 3 cryptic epitopes of survivin, a member of the inhibitors of apoptosis protein family that is highly

Table 1. Clinical peptide vaccination trials in cancer

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Phase I								
Hayashi *et al* (2013)	9	Solid tumors	OTS11101 (VEGFR1 epitope)	IFA	ELISPOT	NA	2 of 9 responders	5 SD: 2 immune responders and 3 non-responders
Ishikawa *et al* (2013)	6	Advanced gastric cancer	LY6K peptide	Montanide	ELISPOT, DTH	NA	6 of 6 ELISPOT + 4 of 6 strong IR	3 SD, 3 PD All SD showed strong IR
Kameshima *et al* (2013)	6	Pancreatic cancer	Survivin-2B80-88	IFNα + Montanide	ELISPOT Tetramer	NA	4 of 6 responders	Responders had SD
Phuphanich *et al* (2013)	21	(recurrent) GBM	EHR2, TRP2, gp100, MAGE-1, IL13Rα2, AIM-2	None	ICS	NA	5 of 15 peptide-specific IFNγ production	No correlation with IR
Sonpavde *et al* (2013)	14	Metastatic CRPC	Arm A: HLA-I NY-ESO-1 peptide Arm B: HLA-II NY-ESO-1 peptide Arm C: HLA-I and II NY-ESO-1 peptide	Montanide, GM-CSF (discontinued)	ELISPOT, ELISA	NA	Arm A: 3 of 3 ELISPOT+ Arm B: 2 of 3 ELISPOT+ Arm C: 1 of 3 ELISPOT+ 0 of 9 NY-ESO-1 IgG +	No association between slowing of PSA doubling time and IR
Yoshimura *et al* (2013)	18	Metastatic RCC	VEGFR-1-770 peptide or VEGFR-11084 peptide	Montanide	ELISPOT	CMV	Dose 0.5 mg: 5 of 6 ELISPOT+, 2 of 6 DTH+ Dose 1 mg: 5 of 6 ELISPOT+, 1 of 6 DTH+ Dose 3 mg: 5 of 6 ELISPOT+, 3 of 6 DTH+	2 PR (1 low dose, 1 high dose), 8 SD, 8 PD 7 of 8 SD showed VEGFR-1 IR
Zeestraten *et al* (2013)	11	Metastasized colorectal cancer	p53 SLP	Montanide, IFNα	ELISA, ELISPOT, LST, CBA, ICS	MRM	4 of 9 LST + 6 of 9 IFN-y release 9 of 9 ELISPOT + 7 of 8 IgG response	NA

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Akiyama *et al* (2012)	9	Recurrent high grade glioma	α-type I polarized DCs pulsed with WT-1, HER-2, MAGE-A1, MAGE-A3 and gp100	None	ELISPOT, DTH, ICS	KLH, PPD	6 of 8 ELISPOT+ to ≥ 1 peptide 3 of 8 ELISPOT+ to ≥ 3 peptide 4 of 4 DTH+ vaccine peptides	1 SD, 8 PD No clear correlation with IR
Chu *et al* (2012)	10	Ovarian cancer stage IC-IV in remission	**Arm 1**: Her2/neu, hTERT, and PADRE pulsed DCs **Arm 2**: CY+ peptide pulsed DC	None	ELISPOT, tetramer	CRM197, PMA/ionomycin	Arm 1: 3 of 5 ELISPOT+ Arm 2: 4 of 5 ELISPOT+	No difference in OS and PFS between both arms
Koski *et al* (2012)	27	DCIS	Her2 peptide pulsed DCs	IFNγ and LPS	ELISPOT IFNγ release	NA	11 of 13 patients systemic CD8 response 22 of 25 patients systemic CD4 response 21 of 22 patients local CD4 response 13 of 14 patients long lasting ELISPOT response	NA
Obara *et al* (2012)	6	Metastasized Bladder cancer	MPHOSPH1-278 and DEPDC1-294	IFA	ELISPOT	NA	3 of 4 ELISPOT + to MPHOSPH1-278 4 of 6 ELISPOT+ to DEPDC1-294	4 responders showed SD (n=3) or PR (n=1) 2 non-responders with PD

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Ohno *et al* (2012)	28	WT1 + tumors	WT-$1_{235-243}$	Montanide (all) GM-CSF (arm B) CpG-ODN (arm C)	NA	NA	NA	Arm A: 2/10 SD, 8/10 PD Arm B: 2/8 SD, 6/8 PD Arm C 6/10 SD, 4/10 PD
Sabatini *et al* (2012)	28	Advanced ovarian cancer	NY-ESO-1 OLP	OLP (n=4) OLP+Montanide (n=13) OLP+Montanide+ poly-ICLC (n=11)	ELISPOT Tetramer	NA	Integrated CD4/CD8/IgG response: OLP: 1 of 4 OLP+Montanide: 4 of 13 OLP+Montanide+poly-ICLC: 10 of 11	NA.
Sawada *et al* (2012)	33	HCC	$GPC3_{144-152}$ OR $GPC3_{298-306}$ peptide vaccine	Montanide	ELISPOT Dextramer	NA	30 of 33 ELISPOT+	19 SD, 1 PR OS associated with GPC3 dextramer frequency
Sharma et al (2012)	27	DCIS	Her2 peptide pulsed DCs	IFNγ and LPS	NA	NA	NA	5 of 27 patients CR 13 of 22 patients PR
Tarhini *et al* (2012)	20	Melanoma stage III or IV	$Gp100_{209-217}$, MART-1_{26-35} and $tyrosinase_{368-376}$	Montanide, GM-CSF and CpG 7909	ELISPOT	CMV, EBV and influenza virus peptide pool	9 out of 20 + for ELISPOT	1 responder showed PR 5 responders showed SD 3 responders showed PD

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Tel *et al* (2012)	15	Metastatic melanoma	pDC pulsed with gp100 and tyrosinase peptides	None	DTH, tetramer, Th1/Th2 Multiplex kit	FSME, KLH	7/15 $gp100_{154-162}$ tet+ 10/15 DTH +	Improved OS compared to matched controls
Voskens *et al* (2012)	5	Recurrent or metastatic SCCHN	HPV-16 and MAGE-A3 Trojan vaccine	Montanide, GM-CSF	ELISPOT, tetramer, ELISA	Con-A	3 of 5 ex-vivo Trojan ELISPOT + 4 of 5 Trojan ELISPOT + after antigen exposure 4 of 5 HLA-II peptide response 2 of 5 IgG+(MAGE/HPV16)	2 SD, 3 PD
Walter *et al* (2012)	28	Advanced RCC	IMA901 multipeptide vaccine	GM-CSF	ELISPOT multimer	HBV	12 of 27 responses to 1 peptide 8 responses to ≥ 2 peptides.	16 PD 11 SD 1 PR Better DCR when immune response to ≥ 2 peptides
Block *et al* (2011)	19	Melanoma stage II-IV	MART-1a, gp100(207–217), and Survivin	Montanide Arm A: GM-CSF (300μg) Arm B: GM-CSF (300μg)+IL-2 Arm C: GM-CSF (500μg) Arm D: GM-CSF (500μg)+IL-2	Tetramer	NA	11 of 17 MART-1a tet+ 16 of 19 gp100 tet+ 14 of 19 Survivin tet+ No improvement with IL-2	NA.

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Hunger *et al* (2011)	10	Melanoma	hTERT (GV1001 and p520)	GM-CSF (n=10) or Tuberculin (n=6)	DTH proliferation assay	SEC, PPD	GM-CSF group: 3 of 10 DTH + 6 of 10 + proliferation Tuberculin group: 0 IR	NA
Kaida *et al* (2011)	25	Stage III-IV pancreatic or biliary tract cancer	WT-1 peptide (126-134) + gemcitabine	Montanide	DTH, Multimer	CMV tetramer staining	13 of 20 patients tet+ after in vitro stimulation of PBMC 3+ for DTH	9 PD, 16SD, 9 of 16 SD were immune responders
Kakimi *et al* (2011)	10	NY-ESO-1 + tumors	NY-ESO-1_{91-110}	OK-432,Montanide	ELISA, IFN-y capture assay	NA	9 of 10+ IgG response 9 of 10 CD4 response 9 of 10 CD8 response	3 SD 7PD
Kameshima *et al* (2011)	13	Colorectal cancer	Survivin-2B80-88	Montanide with IFNα (n=8) Montanide without IFNα (n=5)	ELISPOT Tetramer	NA	No IFNα: 0 of 5 tet +, 1 of 5 ELISPOT + IFNα : 4 of 8 tet +, 5 of 8 ELISPOT +	No IFNα: 1 SD 1 IR with PD IFNα: 4 SD, 4 PD 4 IR with 2 SD and 2 PD
Kawaguchi *et al* (2011)	21	Unresectable synovia sarcoma	SYT-SSX 9 mer wild-type and modified peptide	Arm A: wt peptide Arm B: modified peptide Arm C: wt peptide +Montanide+IFNα Arm D: modified peptide+Montanide+IFNα	DTH, tetramer	NA	DTH - in all patients Arm A:3 of 5 tet + Arm B: 1 of 4 tet + Arm C: 0 of 6 tet + Arm D: 3 of 5 tet +	7 SD, 14 PD No correlation with tetramer frequency
Kyte *et al* (2011)	25	Melanoma stage IV	GV1001 combined with temozolomide	GM-CSF	Proliferation	NA	18 of 23 + proliferation Durable response in 10 of 12 responders 0 + for DTH	5 immune responders among 5 PR cases

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Lesterhuis *et al* (2011)	43	Stage III/IV melanoma	Intranodal gp100 and tyrosinase pulsed DC vaccine (arm A+C) Intradermal gp100 and tyrosinase pulsed DC vaccine (arm B+D)	Low dose IL-2 (arm A+B) No IL-2 (arm C+D)	DTH, tetramer, CBA	KLH	Arm B+D: 90% tet+ Arm A+C: 70% tet+ DTH site cultured T cells from intradermally vaccinated patients superior in recognizing tumor cells expressing gp100 and tyrosinase No advantage from low-dose IL-2	Relation with IR not assessed
Mittendorf *et al* (2011)	182	Breast Cancer	E75 vaccine (HER2 epitope, HLA-A2/3 restricted) 106 treated, 76 controls	GM-CSF	NA	NA	NA	Improved DFS in vaccinated patients with low grade tumors and low HER2 expression
Miyazaki *et al* (2011)	10	Oral cancer	Survivin-2B80-88	None	DTH, tetramer	NA	6 of 8 tetramer +	1 PR in patient with IR
Noguchi *et al* (2011)	15	CRPC	4 PPV (ITK-1) combined with EMP	Montanide	ELISA (IFN-γ release) bead-based multiplex assay	NA	10 out of 15 T cell response 7 out of 15 IgG response	NA

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Okada *et al* (2011)	22	recurrent malignant glioma	GAA peptide pulsed α-type I polarized DCs	Hiltonol (poly-ICLC)	ELISPOT, tetramer	NA	Dose level 1 ($1x10^7$ DCs): 6 of 10 CD8 response Dose level 2 ($3x10^7$ DCs): 5 out of 9 CD8 response	2 out 21 patients (9%) clinical responses: 1 CR and 1 PR. 9 out 21 SD for at least 12 months. No clear correlation with immune response
Romano *et al* (2011)	36	Stage III/IV melanoma	Either moDCs or LCs pulsed with tyrosinase and gp100	none	DTH, tetramer, ELISPOT	Flu tetramer, KLH	Tyrosinase tetramer reactivity achieved significance in favor of LCs over moDCs (p=0.04)	NA
Rong *et al* (2011)	7	pancreatic ductal adenocarcinoma stage III/IV	DCs pulsed with MUC-1	GM-CSF, IL-4	ELISPOT (IFNγ and granzyme B)	NA	2 out of 7 ELISPOT+	7 patients with PD
Sakakibara *et al* (2011)	8	CRC stage IV	CEA peptide pulsed DC	OK432, prostanoid and IFNα	DTH ELISPOT pentamer	NA	No immune response	1 SD, 7 PD

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Slingluff *et al* (2011)	167	Resected stage IIB to IV melanoma	**Arm A** (n=41): 12 MP + THP **Arm B** (n=41): 12 MP + THP + CY **Arm C** (n=42): 12 MP + 6MHP **Arm D** (n=43): 12 MP + 6MHP +CY	Montanide	ELISPOT	NA	**Arm A**: 78% ELISPOT+ to 12 MP, 93% ELISPOT+ to THP **Arm B**: 71% ELISPOT+ to 12 MP, 90% ELISPOT+ to THP **Arm C**: 19% ELISPOT+ to 12 MP, 48% ELISPOT+ to 6MHP **Arm D**: 28% ELISPOT+ to 12 MP, 56% ELISPOT+ to 6MHP	3 year OS 79% 3 year PFS 79% No difference between all arms
Terasaki *et al* (2011)	12	GBM	PPV for IKT-1 (4 of 14 candidate peptides)	Montanide	ELISA (IFN-y release) bead-based multiplex assay	NA	Dose 1mg: T cell response in 4/6 patients, 1 out of 6 patients IgG + Dose 3mg: T cell response in 5/6 patients, 1 out of 6 patients IgG +	2 PR with broad immune immune responders after after 12^{th} vaccination. 5 SD 5 PD
Yoshikawa *et al* (2011)	14	HCC	$GPC3_{144-152}$ peptide vaccine	Montanide	ELISPOT Dextramer	NA	12 of 14 ELISPOT+	1 PR with strong IR 7 SD of whom 5 IR 6 PD of whom 6 IR

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Phase II								
Camisachi *et al* (2013)	43	Stage II/III melanoma	Arm A: Melan-A (27L), gp100 (210M), NY-ESO-1 (165V), survivin (97M) Arm B: observation	Arm A: Montanide, CY, IL-2 Arm B: observation	ELISPOT. Multimer, ICS, T_{reg} suppression assay	CMV, EBV, Flu	lower Treg freq in LN of pre-treated group IL-2 induced TGF-β Tregs in PBMC Tregs not associated with IR suppression	NR
Miyatake *et al* (2013)	40	WT1 + gynaecological malignancies	$WT\text{-}1_{235\text{-}243}$	Montanide	DTH	NA	27 of 40 DTH +	16 SD, 24 PD Prolonged OS In immune responders
Poelgeest *et al* (2013)	20	Advanced gynaecological carcinoma	HPV16 E6/E7 SLP	Montanide	ELISPOT, LST, CBA	MRM	11 of 13 ELISPOT + 9 of 16 LST +	Strong IR in patients with long survival (>12.6 months)
Aarntzen *et al* (2012)	33	Stage III/IV melanoma	**Arm A**: DCs pulsed HLA-I/II gp100 and tyrosinase peptides **Arm B**:DCs pulsed with HLA-I gp100 and tyrosinase peptides	None	tetramer, CBA	KLH	**Arm A**: 7 of 14 SKIL tet+ 2 of 14 PBMC tet+ 4 of 11 IFNγ production **Arm B**: 6 of 14 SKIL tet+ 0 of 14 PBMC tet+ 6 of 6 IFNγ production	Better DFS and OS in Arm A vs matched controls No difference in DFS and OS in arm B vs matched controls No association IR with survival

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Becker *et al* (2012)	61	Metastatic melanoma	3 survivin peptides (HLA-A1, HLA-A2, HLA-B35 restricted)	Montanide	ELISPOT, multimer	NA	13 of 41 ELISPOT +	11 of 55 patients with PA. Increase in PA and OS in immune responders
De Vos van Steenwijk *et al* (2012)	9	HSIL	Arm A(HPV16 E6/E7 SLP) Arm B (placebo)	Montanide	ELISPOT,LST, CBA, DTH	MRM	Arm A: 5 of 5 responders Arm B: 1 of 4 responders	NA
Filippazzi *et al* (2012)	43	Stage II/III melanoma	Arm A: Melan-A (27L), gp100 (210M), NY-ESO-1 (165V), surviving (97M) Arm B: observation	Arm A: Montanide, CY, IL-2 Arm B: observation	ELISPOT. Multimer, ICS, T_{reg} suppression assay	CMV, EBV, Flu	Arm A: 75% of patients IRto at least 1 peptide 65% to at least 2 peptides Arm B: no IR	No difference in DFS or OS in both arms
Goldinger *et al* (2012)	21	Stage III melanoma	Melan-A/MART-1_{16-35} MelQbG10 nanoparticle	Arm A: Montanide Arm B: Montanide + Imiquimod Arm C: Imiquimod Arm D: intranodal injection	tetramer	NA	16 of 21 tet+ 11 of 11 Arm A+B tet + 5 of 10 Arm C+D tet + 21 of 21 patients Melan-A specific IgG +	9 PD, 5 SD No difference between arms
Kono *et al* (2012)	60	Advanced ESCC	TTK, LY6K and IMP3	Montanide	ELISPOT, pentamer	CMV, PMA, ionomycin	Positive CTL response to LY6K (63% of patients), TTK (45% of patients) and IMP3 (60% of patients)	Improved OS in responders to LY6K and TTK (no difference reported)

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Leffers *et al* (2012)	20	Ovarian cancer, stage Ic-IV	P53 SLP combined with chemotherapy	Montanide	ELISPOT	MRM	5/8 + proliferation 2/7 patients + for ELISPOT.	No differences in clinical response rates (60.0% vs 61.5%, p=0.925) to secondary chemotherapy No difference in survivalfic survival (median 44.0 vs 47.4 months, (p=0.601).
Noguchi *et al* (2012)	42	CRPC with or without prior chemotherapy	PPV: Max 4 out of 31 candidate peptides	Montanide	ELISPOT, bead-based multiplex assay	NA	Prior chemotherapy: 9 of 19 + IgG response, 6 of 19 + ELISPOT No prior chemotherapy: 9 of 22 + IgG response, 8 of 22 + ELISPOT	No difference in OS and PFS Presence of T cell response or IgG response not associated with OS Low IL-6 level in pre-vaccine samples associated with OS
Oshita *et al* (2012)	24	Metastatic melanoma	DCs pulsed with gp100, tyrosinase, MAGE-A1/A2/A3 and MART-1 peptides	None	ELISPOT, DTH, ICS	KLH	9 of 22 DTH+ 18 of 24 ELISPOT+ to ≥ 1 peptide 6 of 24 ELISPOT+ to ≥ 3 peptide Th1 profile in 12 of 19 patients	1 PR, 7 SD, 16 PD Improved OS in vaccinated patients and broad ELISPOT responders

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Rahma *et al* (2012)	21	Stage III/IV ovarian cancer	Wt p53 peptide sc (arm A) vs. p53 pulsed DC (arm B)	GM-CSF, Montanide (arm A) CD40L (arm B) IL-2 (both arms)	ELISPOT, tetramer	NA	Arm A: 9 of 13 patients (69%) CD8 response Arm B: 5 out of 6 (83%) CD8 response	No significant difference in OS and PFS
Schaefer *et al* (2012)	37	Melanoma stage IV	$Gp100_{209-217}$, $MART\text{-}1_{27-35}$ and $tyrosinase_{368-376}$	No adjuvant (arm A), GM-CSF (arm B), IFN-α-2B (arm C) or both (arm D)	ELISPOT, tetramer	Flu specific response	Increase in CD8+tet+ Tcells for all peptides.	No significant correlation immune results to OS
Terazaki *et al* (2012)	10	Refractory SCLC	PPV: Max 4 out of 31 candidate peptides	Montanide	ELISPOT CBA ELISA	NA	5 of 6 IgG+ after 1st cycle 4 out of 4 IgG + after 2nd cycle 5 of 6 (83%) ELISPOT+ after 1st cycle 3 out of 3 ELISPOT+ after 2nd cycle	3SD, 8PD, no clear correlation
Vermeij *et al (2012)*	10	Recurrent ovarian cancer	p53 SLP + CY	Montanide	ELISPOT, LST, CBA	MRM	8 of 10 LST + after 2nd vac 5 of 8 LST + after 4thvac 9 of 10 ELISPOT+after 2nd vac 7 of 8 ELISPOT+after 4th vac	2 SD, both responders 8 PD

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Walter *et al* (2012)	68	Advanced RCC	IMA901 with CY or no pretreatment	GM-CSF	ELISPOT multimer	HBV	39 out of 61 response to ≥ 1 peptides	Prolonged survival for pretreated immune responders (HR = 0.38) Prolonged survival for immune responders to multiple peptides
Kotsakis *et al* (2011)	55	solid tumors stage III-IV	Vx-001 ($hTERT_{572Y}$, $hTERT_{572}$)	Montanide	ELISPOT (IFN-y, perforin) ICS	ConA, SEB	NA	Better DCR for immune responders vs non-responders [44% vs 14% (P = 0.047) Better PFS(5.2 vs 2.2 months Better OS (20 vs 10 months
Sarnaik *et al* (2011)	75	Resected stage IIIc/IV melanoma	Ipilimumab 3mg/kg, n=25 Ipilimumab 10mg/kg, n=50 $gp100_{209-217}$, $MART\text{-}1_{26-35}$ and $tyrosinase_{368-376}$ (n=50)	Montanide	ELISPOT, tetramer	HLA-DR staining	10 of 40 ELISPOT + for gp100 or MART-1 Increase in MART1 tet+ (0.30 vs 0.17, p<0.001)	2-year RFS rate 56% (CI: 44-67%) 2-year OS 86% (CI: 75-92%) No correlation with IR

Table 1. Clinical peptide vaccination trials in cancer (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Vetsika *et al* (2011)	55	solid tumors stage III-IV	Vx-001 ($hTERT_{572Y}$, $hTERT_{572}$)	Montanide	ELISPOT (IFN-y, perforin) ICS	ConA, SEB	27 of 53 ELISPOT+ after 2 vaccinations 22 of 32 ELISPOT+ after 6 vaccinations	NA
Wedén *et al* (2011)	23	resected pancreatic adenocarcinoma	4 personalized peptides + Seven ras peptides	GM-CSF	Proliferation assay	NA	17 out of 20 responders 5 memory T cell responders	No correlation
Phase III								
Schwartzentruber *et al* (2011)	185	Melanoma stage III or IV	$gp100_{209-217}$ +IL-2 vs IL-2 alone	Montanide	ELISPOT	NA	IL-2 alone: 0 of 12 ELISPOT+ IL-2 + gp100: 7 of 37 ELISPOT+	Improved CR rate and prolonged PFS in vaccine treated group No relationship between IR and CR

Legend: CBA: cytokine bead array, Con-A: Concanavalin A, CR: complete response, CRPC: Castration resistant prostate cancer, Cyclophospamide: CY, DCIS: ductal carcinoma in situ, DC: dendritic cell, DCR: Disease control rate, DFS: disease free survival , DTH: Delayed type hypersensitivity, ELISPOT: enzyme-linked immunosorbent spot, EMP: estramustine phosphate, GBM: Glioblastoma multiforme, GIS: general immune status, HSIL: high grade cervical squamous intraepithelial lesion, ICS: intracellular staining, IR: Immune response, i.v. : intravenous, KLH: keyhole limpet hemocyanin, LC: Langerhans cell, LST: lymphocyte stimulation test, MHP: mixture of melanoma helper peptides, moDC: monocyte derived DC, MP: melanoma HLA class I restricted peptides, NA: Not assessed, NR: not reported, NSCLC: non-small cell lung cancer, OLP: overlapping long peptide, OS: overall survival, PA: progression arrest, PD: progressive disease, PMA: phorbol-12-myristate-13-acetate, PPV: personalized peptide vaccine, PR: partial response, RFS: relapse free survival , SCCHN: squamous cell carcinoma of head and neck, SCLC: small cell lung cancer, SD: stable disease, SEB: Staphylococcal Enterotoxin B, SEC: Staphylococcal enterotoxin C, SKIL: Skin infiltrating lymphocytes, SLP: synthetic long peptide, TP: tetanus helper peptide.

expressed in most cancer cells [23]. Antigen-specific T-cell responses to at least one of the vaccine peptides were detected in 13 of 41 evaluable patients. Progression arrest (PA) and overall survival (OS) were higher in immune responders (OS: median 19.6 vs. 8.6 months, p = 0.0077) [24]. Another study with the HLA-A24 restricted survivin-$2B_{80-88}$ peptide vaccine showed vaccine-induced survivin-specific T-cells in 6 out of 8 oral cancer patients, one of whom showed a partial clinical response [25]. Subsequently, this research group performed two trials using the same vaccine but adding interferon-α (IFNα) as adjuvant. In the first trial, in 4 out of 6 pancreatic cancer patients a survivin-$2B_{80-88}$-specific T-cell response was observed after antigen-driven expansion of PBMC. Interestingly, these four responders showed stable disease (SD), whereas the non-responders showed progressive disease (PD) [26]. In the second trial, survivin-$2B_{80-88}$ was administered with or without IFNα. In 5 patients who did not receive IFNα, only one patient showed a vaccine-induced T-cell response to survivin as assessed by enzyme-linked immunosorbent spot (ELISPOT), whereas such a response was noted in 5 out of 8 patients treated with IFNα. This suggested that IFNα is able to enhance T-cell reactivity to survivin but it is unclear whether this coincides with a better clinical outcome [27].

One study group performed two trials with a vaccine targeting glypican-3 (GPC3), a cell surface proteoglycan frequently expressed in hepatocellular carcinoma (HCC). In a phase I trial, a peptide vaccine based on the HLA-A2-restricted epitope $GPC3_{144-152}$ was shown to induce GPC3-specific T-cell responses in 12 out of 14 patients (86%) across all dose levels but with the strongest response in the high-dose-treated patients. One of the vaccine-responders in the highest dose group showed a partial clinical response, but there was no clear relation between the vaccine-induced immune response and clinical outcome. Peptide-specific clones isolated from 3 cases were shown *in vitro* to lyse HCC cell lines expressing GPC3 in a HLA class I-restricted manner [28]. A second trial was performed in which the HLA-A24 restricted epitope $GPC3_{298-306}$ was added to the vaccine. Again the response rate was high as vaccine-induced T-cell responses to 1 or both peptides were found in 30 of 33 patients with the strongest T-cell responses in the highest dose level. In 7 patients, a tumour biopsy was obtained which showed an increase of CD8+ T-cell infiltration after vaccination. Interestingly, the frequency of GPC3-specific CD8 T-cells was a prognostic factor for OS (HR 2.71, CI 1.09-6.72). These results warrant a randomized phase II trial with a placebo group [29].

A HLA A2/A3-restricted peptide vaccine derived from HER2/Neu, a surface receptor protein highly expressed in breast cancer[30], was combined with the immunomodulator granulocyte macrophage colony stimulating factor (GM-CSF) in previously treated breast cancer patients considered disease free at enrolment. Non-vaccinated HLA A2/A3-negative patients served as controls. Disease free survival (DFS) was seen in 94.3% of the vaccinated patients and 86.8% in the non-vaccinated patients (p = 0.08). A subgroup analysis indicated that vaccinated patients with low-grade tumours (98.4% vs 86%,

p=0.01) or with low HER2 expression (94.0% vs 79.4%, p=0.04) had better DFS. Unfortunately, no immunological assessment of the vaccine was reported [31].

Vascular endothelial growth factor receptor (VEGFR) has also been considered as a suitable target for peptide vaccination. Patients with metastasized renal cell carcinoma (RCC) received escalating doses of HLA-A2 or HLA-A24-restricted VEGFR-1 peptides. In 15 of 18 patients (across all dose groups) a vaccine-induced T-cell response was measured after *in vitro* pre-stimulation. No relationship between the clinical response and the strength of the VEGFR-1-specific immune response after vaccination was found [32], but this could not be expected as the immunomonitoring required pre-expansion of T-cells before analysis. Another study of 9 patients treated with OTS11101, a VEGFR1 epitope, showed a vaccine-induced T-cell response in only 2 patients after antigen-driven expansion of PBMC *in vitro*. These two immune responders were among 5 patients who showed SD after vaccination. Post-vaccination serum analysis showed significantly increased levels of soluble VEGRF1 [33]. Elpamotide, a HLA-A24-restricted VEGFR2 peptide vaccine, was evaluated in 10 patients. The vaccine was safe and two patients showed SD for at least 2 treatment cycles (56 days). The serum concentration of VEGFR2 declined after vaccination; however no immune monitoring results were reported [34].

A phase I trial was published with data on the use of a 9-mer peptide spanning the SYT-SSX fusion region (wild-type (wt) B peptide) and a modified peptide (K9I) in 21 patients with synovial sarcoma. Patients were allocated to 4 treatment arms: wt B peptide (arm A), modified K9I (arm B), wt B peptide combined with Montanide and IFNα (arm C), and modified K9I with Montanide and IFNα (arm D). DTH testing was negative in all patients, whereas tetramer analysis showed increase in antigen-specific T-cell frequency in 3 of 5 patients in arm A, 1 of 4 in arm B, 0 of 6 in arm C, and 3 of 5 in arm D. The results with the wt B peptide showing no responses when injected with Montanide and IFNa are difficult to interpret in comparison to the increased response to the K9I peptide when Montanide and IFNα were used (arm C versus arm D). Clinical outcomes (7 SD, 14 PD) did not correlate with vaccine-induced immune responses [35].

The reverse transcriptase subunit of human telomerase (hTERT), an antigen frequently expressed in cancer, was also used as a target for vaccination. 53 patients with different types of chemo-resistant tumours were vaccinated with the modified peptide $hTERT_{572Y}$ (YLFFYRKSV) followed by 4 vaccinations with wt $hTERT_{572}$ (RLFFYRKSV). In 27 of 53 patients, IFNg and perforin secretion-associated immune reactivity to the modified and wt form of $hTERT_{572}$ was established after 2 vaccinations and in 22 of 32 evaluable patients after 6 vaccinations [36]. Immune responders (showing reactivity at any time during vaccination) were found to have a better disease control rate (DCR) than non-responders (DCR 44% versus 14% (P = 0.047)). Furthermore, the group of immune responders displayed better progression-free survival (PFS 5.2 vs 2.2 months (P = 0.0001)) and OS (20 vs 10 months (P = 0.041). It is not clear whether immune responsiveness to the vaccine

reflects an overall better immune status and performance of the patient, as no controls for immune status were reported [37].

Wilms' Tumour 1 (WT-1) has been identified as one of the most suitable targets for peptide vaccines [38]. A phase II trial was performed in 40 patients with WT-1 overexpressing gynaecological malignancies. In 27 patients a positive DTH was observed after WT-1 (235-243) vaccination. A correlation was found between immune response and OS, as an adjusted hazard ratio for a positive DTH reaction of 2.73 was found (95% CI 1.04-7.19, p=0.043) [39]. Furthermore, a WT-$1_{235-243}$ peptide vaccine was combined with GM-CSF and the TLR-9 ligand CpG as adjuvants in 28 patients with WT-1 overexpressing tumours. Patients who received both GM-CSF and CpG had better clinical outcomes (6/10 SD) than those with GM-CSF alone (2/8 SD) or no adjuvants (2/10 SD). However, the merits of these seemingly promising clinical data are difficult to assess due to the small sample size and lack of immune-monitoring data [40].

In two studies novel cancer testis (CT)-antigens, identified by genome-wide expression analysis, were targeted. Six patients with advanced bladder cancer were vaccinated with the HLA-A24-restricted epitopes M-phase phosphoprotein 1(MPHOSPH1-278) and DEP domain containing 1 (DEPDC1-294), both of which are involved in bladder carcinogenesis. In 3 out of 4 patients with MPHOSPH1 positive tumours and in 4 of 6 patients with DEPDC1 positive tumours a T-cell response was induced. In 4 patients with a vaccine-induced immune response, SD or partial response was observed whereas in the non-responders only PD was observed. However, clinical responses were not evaluated according to RECIST criteria [41]. A phase II trial was conducted in which esophageal squamous cell carcinoma (ESCS) patients were vaccinated with a multi-peptide vaccine comprising 3 HLA-A24-restricted epitopes TTK, LY6K and IMP3. HLA-A24+ ESCS patients showed CD8+ T-cell responses to LY6K (63% of patients), TTK (45%) and IMP3 (60%) after culture *in vitro* and depletion of CD4 T-cells. Notably, also 3 HLA-A24-negative patients displayed a vaccine-induced T-cell response against any of 3 antigens when analysed by this method. HLA-A24-positive patients who were vaccinated had better PFS than HLA-A24-negative patients (p=0.024; difference not reported). Interestingly, a positive CTL response against LY6K or TTK correlated with a significantly improved OS (p=0.015 and p=0.006 respectively; difference not reported). Patients who mounted CTLs against all 3 antigens tended to have a better OS, but no data on significance was reported. Although the authors describe the use of a CMV-derived peptide epitope as positive control for T-cell responsiveness, these data were not reported which is unfortunate as this information may have supported their observations [42]. LY6K was also used as a target in a phase I study in 6 advanced gastric cancer patients, all responding to vaccination and of whom 3 patients displayed SD [43].

Multi-peptide vaccines have also been used to treat other tumours. A vaccine for melanoma, comprising $gp100_{209-217}$, MART-1_{26-35} and $tyrosinase_{368-376}$ was administered

with the adjuvants CpG 7909 (PF-3512676) and GM-CSF. In 9 of 20 patients a peptide-specific T-cell response was detected during or after vaccination. Among the responders, 6 showed clinical response (PR or SD) and 3 showed PD. To properly investigate the potentiating effect of CpG 7909 and GM-CSF, a phase II trial is warranted in which patients are randomized to treatment with or without the adjuvants [44]. In a previous trial, immunogenicity and clinical efficacy was assessed of a melanoma multi-peptide vaccine (modified $gp100_{209-217}$, MART-1_{26-35} and modified $tyrosinase_{368-376}$) administered with or without adjuvants GM-CSF and/or IFNα-2b. Interestingly, neither IFNα-2b nor GM-CSF significantly increased the immune response rate in this setting. Nevertheless, immune responders in this trial had better OS (median OS, 21.3 versus 10.8 months; $P = 0.033$) [45]. Recently, additional immunological analyses were published. General immune status (by detection of influenza-specific T-cells) was assessed and remained constant during vaccination. Although an increase in CD8 tetramer-positive T-cells was seen for all vaccine peptides, no significant correlation to clinical outcome was established (frequency change 8.35% in clinical responders vs 1.3% in non-responders, p=0.071). An immune score, based on ELISPOT and tetramer assay and calculated to define the breadth of the immune response, did not correlate with clinical response. In addition, the differentiation status of tetramer-positive CD8 T-cells did not correlate with clinical response, leading the authors to conclude that a functional assay (e.g. ELISPOT) is preferable as immune-monitoring assay for this melanoma vaccine [46]. Two studies reported results of peptide vaccination together with the immunomodulating compound IL-2. A phase III randomized controlled trial (RCT) was reported in which 185 patients with advanced melanoma were treated with either high dose IL-2 or with $gp100_{209-217}$ peptide vaccine combined with high dose IL-2. Toxicity was severe (grade 3-5) in both groups, mostly reflecting side-effects of IL-2 treatment. In the peptide treated group, an improved clinical response (partial or complete) rate (16% vs. 6%, p = 0.03), as well as longer PFS (2.2 months vs. 1.6 months; p = 0.008) was observed. Twelve post-treatment samples were available from IL-2 treated patients and none showed reactivity against gp100 peptide. In 7 of the 37 samples from patients treated with IL-2 plus gp100 vaccine an immune response was found. No relationship between vaccine-induced immune responses and objective clinical response was found. The authors observed an increase of $CD4+CD25+Foxp3^{high}$ Tregs in patients who had a clinical response in both treatment groups. This effect was ascribed to IL-2 treatment [47], but what this means in terms of potential anti-tumour immunity is not clear. Another study investigated the immunogenicity of a multi-peptide vaccine ($gp100_{207-217}$, modified MART-1_{27-35} and survivin) combined with GM-CSF (300 or 500μg) with or without low dose IL-2. Although in all 19 patients vaccine-induced responses against at least 1 of the peptides was shown by tetramer staining, low dose IL-2 did not improve immune responses. Again an increase in $CD4+CD25+Foxp3^{high}$ Tregs was observed. Unfortunately, a potential confirmation of

the association between Tregs and clinical outcome could not be assessed as no clinical outcomes were investigated [48].

Recently, several trials have been published in which the precise constitution of the peptide vaccine is determined on the basis of preexisting host immunity (mainly IgG-responses) to a predefined set of peptides [49], turning vaccines into a personalized peptide vaccination (PPV). One study reported use of PPV (a maximum of 4 peptides selected from a pool of 31 peptides) in patients with castration-resistant prostate cancer (CRPC) who either failed prior docetaxel-based chemotherapy (n=20) or did not receive prior chemotherapy (n=22). In the group who received prior therapy, 9 of 19 showed an IgG-response and 6 of 19 a T-cell response against at least one vaccine peptide. In the therapy-naïve group, 9 of 22 showed an IgG-response and 8 of 22 a T-cell response. No difference in OS and PFS was seen between groups or when compared to historical controls. Furthermore, there was no relation between the presence of an immune response and OS [50]. Another PPV study was performed in 6 refractory small cell lung cancer (SCLC) patients where PPV (4 of 31 candidate peptides) was administered simultaneously with chemotherapy and/or radiotherapy. After 1 cycle, 5 of the 6 patients showed augmented IgG-responses and T-cell responses to at least one of the vaccine peptides. Four patients finished a second cycle of PPV after which all displayed a humoral and cellular immune response to one of the selected peptides [51]. PPV was also used in a trial with 12 glioblastoma multiforme (GBM) patients. A maximum of 4 out of 14 candidate HLA-A24-restricted peptides (ITK-1) was administered at 2 dose levels. After 1 cycle of vaccination, T-cell responses were increased in 4 of 6 patients (1mg) and in 5 of 6 patients (3mg). Furthermore, in both dose levels 1 out of 6 patients displayed a peptide-specific IgG-response to at least 1 peptide. In two patients with a partial response, a broad humoral and cellular immune response to 2-4 peptides developed after 2 cycles (12 vaccinations) [52]. Broad and robust immune responses seem to be required to reach clinical efficacy, which is in line with observations of a previous clinical trial of our group [15], in which strong and broad vaccine-prompted HPV16-specific responses were observed in vaccinated patients who had complete regression of premalignant lesions of the vulva [16].

MelQbG10 is a virus-like nano-particle loaded with A-type CpG and coupled to the well-known MART-1_{16-35} peptide. In a phase II trial in stage III/IV melanoma patients, MelQbG10 was administered with either Montanide (n=5), Montanide and topical TLR-7 ligand Imiquimod (n=6), Imiquimod alone (n=5), or injected intranodally (n=5). Of 14 evaluable patients, 9 showed PD and 5 showed SD with no difference in clinical outcomes between arms. In 16 of 21 patients an increase in MART-1-specific tetramer frequency was found. All patients treated with vaccine in Montanide (Arm A and B, n=11) not only were immune responders but also showed higher MART-1-specific T-cell frequencies than patients treated without Montanide (Arm C and D, n=10) of whom only 5 responded to vaccination. This confirms previous findings that Montanide is a good

adjuvant for peptide vaccination. Interestingly, topical application of Imiquimod to the MelQbG10 vaccine in Montanide resulted in a lower MART-1-specific T-cell response when compared to the Montanide only group. MART-1-specific antibody responses developed in all patients but again were significantly higher in patients treated with Montanide. Therefore, using Montanide as adjuvant is pivotal for the immunogenicity of MelQbG10, whereas topical Imiquimod does not have an immune potentiating effect, but may rather decrease immune reactivity [53].

Peptides vaccines aiming to induce tumour-specific CD4 and CD8 T-cell responses

One disadvantage of using exact HLA-class I binding short peptides is the possibility of inducing short-term CD8 T-cell responses that vanish quickly. This can be circumvented by adding a T-helper peptide to the vaccine or by using long peptide vaccines. For a better understanding of the theoretical basis for this as well as for detailed explanations, we refer to two recent reviews by our group [9,19]. Addition of epitopes aiming to induce a CD4 T-helper cell response can help solve this problem in several ways, such as APC activation via the CD40–CD40L axis[54] and secretion of interferon-g (IFNg) and IL-2, which leads to priming and full activation of CD8 T-cells to effector cells [55]. In several trials, peptide vaccines included both CD4 and CD8 T-cell epitopes. In one trial, the CD4 epitope GV1001 ($hTERT_{611-626}$) and the CD8 epitope p540 ($hTERT_{540-548}$) were administered with GM-CSF or tuberculin (PPD23). Only the group injected with the immunomodulator GM-CSF responded to the vaccine, albeit that for the detection of T-cell reactivity pre-stimulation *in vitro* was required. Nevertheless, in 6 of 10 patients a proliferative response to the CD4 T-cell epitope GV-1001 was found and in 1 of 9 patients a proliferative response to the CD8 epitope. The absence of DTH responses to hTERT but not PPD23 indicates that for this vaccine GM-CSF is a more effective adjuvant than tuberculin [56]. Another study investigated the use of IMA901, a vaccine consisting of 9 HLA-A2- and 1 HLA-DR-restricted peptides with known immunogenicity in RCC. It was combined with GM-CSF in 28 HLA-A2+ advanced RCC patients. 12 of 27 evaluable patients showed a T-cell response against 1 peptide, and 8 patients responded to 2 or more peptides. Importantly, the latter group showed a significantly lower frequency of $CD4+CD25+Foxp3^+$ Tregs prior to vaccination than the prior group and the non-responders (p=0.016). After 3 month evaluation, 16 patients showed progression, 11 patients showed SD and 1 patient showed a partial response. Patients who responded against multiple peptides had a significantly better DCR (SD or PR, p=0.019) [57]. Furthermore, data on long-term memory T-cell responses and survival of 20 patients who were vaccinated in two previous trials with long synthetic mutant Ras peptides combined with the adjuvant GM-CSF were reported. Five patients, all immune responders in the initial trials, were tested between 7-9 years post vaccination for memory T-cell responses. Peptide pre-stimulated

PBMC cultures still displayed reactivity to the vaccine peptides in 3 out of 5 patients. Interestingly, T-cell reactivity was not exclusively aimed at the mutant sequence as a weak response against the wt Ras peptide 12G was also observed. However, no clear correlation between immune response and overall survival was seen [58]. Last but not least, patients with metastatic CRPC were allocated to treatment with a 9-mer HLA-A2 restricted NY-ESO-1 peptide (arm A), a 24-mer HLA-DP4 or a 12-mer HLA-DR4- restricted peptide (arm B) or both class I- and II-restricted NY-ESO-1 peptides (arm C). Peptide cultured PBMC showed NY-ESO-1-specific T-cell responses both to the CD8 and/or to the CD4 T-cell epitopes in 6 of 9 patients. No clear association of immune response with PSA doubling time was established. There was a potential effect of pre-treatment with docetaxel as only 2 of 5 pre-treated and 4 of 4 docetaxel-naïve patients responded to vaccination [59], but this clearly needs further investigation.

Instead of adding a T-helper epitope to a minimal HLA-class I-binding peptide, one can also make use of overlapping long peptides (OLP) for vaccination. The OLP correspond to parts or the whole sequence of tumour antigens, thereby including any potential CD4 or CD8 epitope within the amino acid sequences covered [9]. One study reported the use of OLP comprising the sequence of the CT antigen NY-ESO-1. This vaccine was combined with Montanide and TLR3 ligand Hiltonol® (Poly- ICLC) or with Montanide alone in 28 patients. The OLP vaccine boosted NY-ESO-1-specific CD4 and CD8 T-cells together with NY-ESO-1-specific antibody in 1 of 4 patients treated with NY-ESO OLP alone, 4 of 13 patients treated with NY-ESO OLP and Montanide, and 10 of 11 patients treated with NY-ESO OLP with both Montanide and poly-ICLC. The vaccine was safe, however adding both Montanide and Hiltonol® led to an increase of local injection site reactions. As a precautionary measure, vaccination in the last 4 patients was stopped prematurely. Injection site reactions either resolved or were classified as grade 1 at the end of the observation period [60]. A long NY-ESO-1 peptide vaccine (NY-ESO-$1_{91–110}$) was used in a phase I trial in patients with NY-ESO-1 expressing tumours. OK-432 (Picibanil), a Toll-like receptor (TLR)-4 ligand derived from *Streptococcus pyogenes*, was used as adjuvant. This strategy resulted in NY-ESO-1 protein- and peptide-specific IgG-response in 9 of 10 patients. *In vitro* stimulated PBMC also showed NY-ESO-1-specific CD4 T-cell response (9 of 10 patients) and CD8 T-cell response (9 of 10 patients). In 3 of 10 patients (all immune responders) SD was observed after vaccination. Interestingly, in several patients (with different HLA types) neighbouring peptides (NY-ESO-1_{85-102} and NY-ESO-1_{97-114}) were recognized, indicating that the NY-ESO-$1_{91–110}$ peptide comprises multiple HLA class I and II binding epitopes [61]. Clonal analysis identified several new HLA A, B and C restricted epitopes, which were used to construct new peptide/HLA tetramers for future immune monitoring. Furthermore, several clones were shown to recognize HLA-matched tumour cell lines overexpressing the relevant NY-ESO-1 epitope [62]. Our group published several studies in which immunogenicity and clinical efficacy were assessed of synthetic long

peptide (SLP) vaccines targeting human papilloma virus (HPV16) E6/E7 oncogenic proteins and the tumour suppressor protein p53. The SLP vaccine against HPV16 comprised overlapping long peptides covering the entire amino acid sequences of the E6 and E7 oncoproteins. Patients with HPV16+ high grade cervical squamous intraepithelial lesion (HSIL) were randomized for treatment with HPV16 E6/E7 SLP or placebo. General immune status was assessed by stimulating T-cells with a mix of recall antigens (memory recall mix, MRM) and showed a positive response in all but 1 patient. Two of the placebo treated patients showed a weak HPV16-specific T-cell response as measured by IFNg-ELISPOT. Another patient showed a broad proliferative response which was induced by invasive treatment (biopsy). This proliferative response was accompanied by very low IFNg release. All vaccinated patients displayed a strong and broad HPV16-specific proliferative and IFNg-ELISPOT response to at least 2 E6/E7 peptide pools and a mixed Th1/Th2 cytokine profile [63], revealing that vaccine-induced HPV16-specific T-cell responses are much stronger than spontaneously induced T-cell reactivity. In another study, patients with HPV16-induced advanced gynaecological carcinomas were treated with this HPV16 E6/E7 SLP vaccine in a phase II trial. In 9 of 16 evaluable patients, a HPV16-specific proliferative response was induced after 2-4 vaccinations, with a significant increase in strength during vaccination ($p<0.0001$) compared to baseline values. ELISPOT analysis revealed a HPV16-specific response in 12 of 13 evaluable patients with increasing frequency of IFNg-producing T-cells during vaccination compared to baseline values ($p<0.0001$), but these responses were much weaker than previously observed in patients with a high grade lesion of the vulva [15,16]. General immune status was established by stimulating T-cells with MRM. Of 6 patients lacking a MRM-specific response, 3 patients were able to mount a HPV16-specific immune response and 3 were not. However, in all 10 patients with MRM-reactive T-cells at baseline a HPV16-specific immune response was induced ($p=0.04$). No tumour regression was observed (3 SD, 8 PD) and there was no difference in OS when compared to historical controls. Median OS of this cohort ($n=20$) was 12.6 months and patients with longer survival (>12.6 months) displayed significantly stronger immune responses than patients with short survival (<12.6 months), reflected by all immune-monitoring assays [64]. Finally, we reported results of a phase II trial in which 11 advanced colorectal cancer patients were vaccinated with a p53-SLP combined with IFN-α. Here, the p53-SLP vaccine consisted of 9 OLP covering the middle part of the amino acid sequence of p53 as this was found to be the most immunogenic part. In all patients, after vaccination a p53-specific T-cell response was detected by IFNg-ELISPOT. In 5 patients also a response to MRM was detected by IFNg-ELISPOT and in 9 of 11 patients by proliferation. Notably, the vaccine-induced p53-specific response was quite strong as p53-specific T-cell reactivity was detectable in a direct *ex-vivo* intracellular cytokine assay in all patients after vaccination. In a direct head-to-head immune comparison using samples of a previous trial where patients were vaccinated with p53

SLP without IFN-α [11], a higher median frequency of p53-specific IFNg-producing T-cells (p=0.018) were detected in patients vaccinated with p53 and IFNa [65], indicating that immunogenicity of OLP vaccines can effectively be enhanced with IFNα, which is in line with the previously discussed survivin-based vaccination studies.

Trojan-peptide based vaccines are cell penetrating peptides. These vaccines do not require proteasomal processing and transport by TAP (transporter associated with antigen processing). A "penetrin" peptide sequence allows Trojan peptides to translocate through the cell membrane and penetrate directly into the endoplasmic reticulum (ER) and Golgi apparatus where they can form peptide-HLA complexes. Furin-cleavable linkers were used to couple the different T-cell epitopes enabling the release of the individual epitopes within the Golgi apparatus. Results were reported from a phase I trial in which a Trojan vaccine was used in patients with advanced squamous cell carcinoma of head and neck (SCCHN) with overexpression of the involved tumour antigens. The Trojan vaccine comprised HLA-class I and HLA-class II restricted epitopes from either HPV16 or melanoma antigen E (MAGE)-A3 and injected together with GM-CSF. Trojan-specific T-cell responses were detected in 4 of 5 patients, however, the Trojan vaccine failed to induce HLA-class I peptide-specific responses. Trojan vaccine-induced T-cell responses comprised T-cell reactivity to the individual HLA class II epitopes, but the authors noted that the response to the complete Trojan sequence was much stronger than the response to the individual peptides indicating to them that the construct created novel HLA class II epitopes [66].

Peptide pulsed DC vaccines

DCs are professional antigen presenting cells. When pulsed with peptides derived from tumour antigens, they can induce antigen-specific CD4 and CD8 T-cell responses [67]. Results from a phase II trial were reported in which stage III/IV melanoma patients were injected intranodally with a DC vaccine pulsed with gp100 and tyrosinase HLA-class I- and II-restricted epitopes. HLA-DR4-positive patients received DCs pulsed with HLA-class I and II restricted gp100 (154-162, 290-288, 44-59) and tyrosinase (369-377, 448-462) peptides (arm A, n=15). HLA-DR4 negative patients were treated with HLA-class I restricted gp100 (154-162, 290-288) and tyrosinase (369-377) peptide pulsed DCs (arm B, n=14). DCs were also loaded with the control antigen keyhole limpet hemocyanin (KLH) as measure of immunocompetence. Most patients showed KLH-specific proliferation and IgG antibodies upon KLH stimulation. In arm A, tetramer analysis of peripheral blood showed vaccine-induced response of CD8 T-cells in 2 of 12 patients and of CD4+CD25+Foxp3- T-cells in 3 of 13 patients. Skin infiltrating lymphocytes (SKILs) from 7 of 14 patients showed tetramer-positive CD8 T-cells reactive to at least 1 peptide, with 3 of 14 patients being reactive to multiple peptides. In arm B, no tetramer positive CD8 T-cells were found in peripheral blood, however SKILs from 6 of 14 patients in arm B

were responding to at least 1 peptide, with 5 of 14 patients responding to multiple peptides. When the reactivity of PBMC to a melanoma cell line was tested, 4 of 11 patients in arm A and 1 out of 6 patients from arm B were able to produce IFNg, indicating that in these patients a T-cell response with the ability to recognize tumour cells was mounted. When compared to matched controls, patients in arm A (but not in arm B) showed an improved DFS (5.0 vs 2.8 months, p=0.0089) and a trend towards improved OS (15.0 vs 8.3 months, p=0.089) [68]. This group also conducted a clinical trial comparing intradermal with intranodal injection of gp100 (154-167,280-288) and tyrosinase (369-376) pulsed DCs in stage III/IV HLA-A2.1^{+} melanoma patients combined with low-dose IL-2 in half of patients. KLH-specific proliferation and IgG-response was similar in all groups. Tetramer analysis did not show a difference in antigen-specific T-cells induced by route of administration (intradermal 90% vs intranodal 70%, p=0.2). However, T-cells derived from positive DTH sites from intradermally vaccinated patients more frequently recognized tumour cells expressing gp100 and tyrosinase (53% vs 16%, p<0.05) illustrated by IFNg-production or cytolytic activity. Although low dose IL-2 treatment resulted in higher numbers of tetramer-positive CD8 T-cells in intranodally treated patients, these T-cells were not capable of recognizing native antigen or tumours, probably reflecting low affinity T-cell receptors. IL-2 did result in an increase of CD4+CD25+Foxp3^{high} T-cell frequency. No difference in outcome between the treatment groups nor a relationship with vaccine-induced immune response was established. This study indicates that intradermal DC vaccination results in superior antigen specific T-cell response without a clear advantage of IL-2 treatment [69]. A first-in-human study was performed in metastatic melanoma patients treated intranodally with autologous activated plasmacytoid dendritic cells (pDCs) loaded with gp100 (154-162, 290-288) and tyrosinase (369-377) peptides. The inactivated thick-borne encephalitis virus vaccine (FSME) was used as a natural Toll-like receptor (TLR) agonist to activate pDCs and also served as control antigen for vaccine responsiveness. In 9 of 14 patients a T-cell response to FSME was detected, which reflects a functional immune system that can be primed by activated and antigen-loaded pDCs in these patients. *In vitro* peptide cultured PBMC samples of 7 of 15 patients showed a significant increase of $gp100_{154-162}$-specific CD8^{+} T-cell frequency. In 10 of 15 patients, a positive DTH response was found. In 2 patients, tetramer positive CD8 T-cells, cultured from the DTH site, were detected. Furthermore, despite the small sample size, the median OS showed an interesting improvement as compared with matched control patients (22.0 (95% CI 1.8–42.2) vs 7.6 months (95% CI, 5.8–9.4), p= 0.001). It was not clear if the FSME T-cell responders were the same patients responding to the melanoma antigens and as such if improved OS could be contributed to better performance status of the patients. A randomized phase II trial with a placebo-treated group should shed more light on this [70]. A phase II trial was conducted in 24 metastatic melanoma patients who were treated with a DC vaccine pulsed with HLA class I-restricted gp100, tyrosinase,

MAGE-A1/A2/A3 and MART-1 peptides. 64% of evaluated patients showed a DTH response to KLH. 18 of 24 patients showed reactivity to at least one peptide and in 6 cases response to 3 or more peptides was shown. A Th1 cytokine profile was detected in 12 of 19 patients by intracellular cytokine staining. Combined survival data from this study and a previous phase I trial [71] showed an improved OS in the vaccinated patients (13.6 months vs 7.3 months in matched controls, $p<0.05$) and in patients showing a broad ELISPOT response (21.9 months ELISPOT+ ≥ 2 peptides vs 8.1 months ELISPOT+ <2 peptides, $p<0.05$) [72]. One phase I study aimed to compare human Langerhans cells (LC) with monocyte-derived dendritic cells (moDCs) pulsed with heteroclitic tyrosinase (368-376) and gp100 (209-217) peptides in stage III/IV melanoma patients. KLH-specific proliferation was stronger in moDC-treated patients (post 3 DC vaccinations, $p < 0.001$). The CD8+ T-cell response to an influenza-derived epitope remained stable in both treatment groups throughout vaccination. Only tyrosinase-specific CD8+ T-cell reactivity achieved significance in favour of LCs over moDCs ($p=0.04$), but both treatment groups were otherwise comparable in terms of immunogenicity [73]. Patients with newly diagnosed or recurrent GBM were vaccinated in a phase I study with DCs pulsed with a mix of HLA class I-restricted peptide-epitopes (EHR2, TRP2, gp100, MAGE-1, IL13Rα2 and AIM-2) which have been identified as targets in brain tumours. Peptide-specific CD8+ T-cells producing IFNg (generally accompanied by TNFα production) were found in 5 of 15 patients by ICS of *in vitro* stimulated PBMC. No relation with clinical parameters was established [74]. Subcutaneous injection of the HLA-0201 restricted $p53_{264-272}$ peptide in Montanide and GM-CSF was compared with injection of CD40-matured DC pulsed with this peptide in ovarian cancer patients. Low dose IL-2 was given to both arms as adjuvant. Peptide-specific T-cells were detected in both groups and were comparable. No significant difference in OS and PFS was observed between both arms. Both vaccines were safe; however grade 3 or 4 toxicities occurred in 11 patients during IL-2 cycles. IL-2 administration led to increase of $CD4+CD25+Foxp3^{high}$ Tregs in both arms [75]. DCs have also been used to promote immune reactivity against HER2/neu. Intranodal injection of IFNg- and LPS-activated DCs, pulsed with 6 HLA class II and 2 HLA class I HER2/neu peptides, was performed in 27 patients with HER2/neu overexpressing ductal carcinoma in situ (DCIS). This resulted in a clinical response in 18 patients, 5 of which showed no residual DCIS at all. Complete loss of detectable HER-2 expression was found in 11 patients. A control group consisting of 11 non-vaccinated DCIS patients displayed no clinical response [76]. After antigen driven *in vitro* expansion, 11 of 13 tested HLA-A2 positive patients showed IFNg-associated CD8 T-cell reactivity against one or both peptides. Furthermore, in 22 of 25 patients an IFNg-associated CD4 T-cell response against at least 1 of 6 peptides was detected post vaccination. Moreover, this CD4 T-cell response was paralleled in directly *ex-vivo* examined tumour draining lymph nodes. Long-lasting immunity, ranging from 6 to 52 months post vaccination, was observed in 13 of 14 evalu-

able patients. Unfortunately, the authors did not elaborate on the correlation of vaccine-induced T-cell responses and observed clinical responses [77]. Safety and immunogenicity were assessed of 20 amino acid long MUC-1 peptide-pulsed DCs in patients with MUC-1 expressing ductal pancreatic adenocarcinoma. The generated DCs had good functional characteristics, as measured by flow cytometry (CD209, HLA-DR and CD86 staining) and mixed leucocyte reaction (MLR) and were capable of inducing MUC-1-specific IFNg- and granzyme B-release associated T-cell immunity in 2 of 7 patients. All patients showed PD [78]. A comprehensive study on the immunogenicity of quickly matured DCs (via the use of OK432, prostanoid and IFNα), pulsed with an HLA-A24-carcinoembryonic antigen CD8 T-cell epitope was performed in colorectal cancer patients. An increase in antigen-specific CD8 T-cell frequency measured by MHC-peptide-pentamer staining was reported for several patients, however, these responses could not be confirmed by functional assays [79]. In another trial, DCs were matured with a type I polarizing cytokine cocktail (IFNα, IFNg, IL1β, TNFα and poly-I:C) and pulsed with 4 HLA-A2-restricted glioma-associated peptides (GAA) peptides derived from the sequences of EphA2, IL-13 receptor-α2, YKL-40 and gp100 as well as the pan-DR T-helper epitope PADRE. These DCs were then injected intranodally in patients with GBM together with a concomitant intramuscular injection with poly-ICLC. The function of DC as measured by their IL-12 production was correlated with time to progression. Reactivity to at least one of the GAA peptides was found in more than half of the patients, independent of dose DCs given. Nine patients were progression free for at least 12 months [80]. Type I polarized DCs were also used in 9 patients with recurrent high grade glioma. Patients were treated with DCs loaded with HLA class 1 restricted WT-1 (126-134, 235-243), HER-2 (63-71, 369-377), MAGE-A1 (135-143), MAGE-A3 (195-203,271-279) and gp100 (209-217) peptides and KLH. In 6 of 8 evaluable patients, ELISPOT responses to at least 1 vaccine peptide were detected, and in 3 cases reactivity to 3 or more peptides was established. DTH response to KLH was detected in 4 patients, of which one was strongly and the others weakly positive. The patient with a strong KLH-specific DTH also showed a broad ELISPOT response (reactive to 4 vaccine peptides) and this was accompanied by stable disease for more than 2 years. None of the 5 patients failing to react to KLH demonstrated that capacity to mount a T-cell response to the tumour antigens suggesting that the immunogenicity of this DC vaccine could very well be influenced by the adaptive immunity status of patients [81]. Overall, DC pulsed with defined T-cell peptide epitopes are highly immunogenic vaccines that may induce favourable clinical outcomes when injected intradermally or intranodally.

Peptide vaccines combined with chemotherapy

Chemotherapy can have a synergistic effect when combined with vaccines. It can promote anti-tumour immune responses in several ways, e.g. stimulation of cross-pre-

sentation or by elimination of immune suppressive cells [82,83]. Chemotherapy has been shown in mouse models to skew the tumour microenvironment into a permissive state for peptide vaccination through an accumulation of dendritic cells, which stimulated CD8+ T-cells and the type I IFN pathway [84]. Here we report several clinical trials in which peptide vaccines were combined with chemotherapeutic compounds.

Gemcitabine is a chemotherapeutic compound with significant immunomodulatory activity and the ability to mediate anti-tumour T-cell responses [85]. A HLA-A2-restricted WT-$1_{126-134}$ vaccine was combined with gemcitabine in 25 patients with pancreatic or biliary tract cancer. In 13 of 20 patients, CD8 T-cells stained positive with WT1-tetramers, however, these results could not be confirmed by analysis of antigen-specific IFNg production. Clinically, 16 patients showed SD, 9 of whom showed a vaccine-induced immune response [86]. The orally given alkylating chemotherapeutic agent Temozolomide was combined with GV1001 ($hTERT_{611-626}$) vaccination in 25 patients with stage IV melanoma. hTERT-specific T-cell proliferation was found in 18 of 23 evaluable subjects (78%) and these responses persisted in 10 out of 12 evaluable immune responders. Interestingly, all 5 patients with partial response were immune responders [87]. Also the earlier mentioned PPV were used in combination with chemotherapy. The orally given chemotherapeutic agent estramustine phosphate (EMP) was combined with PPV in CRPC patients. Vaccine-induced T-cell response against at least 1 peptide was shown in 10 of 15 patients and peptide-specific IgG-response in 7 of 15 patients. A clinical response, determined by PSA levels, was shown in 2 patients (1 CR and 1 PR) [88]. In a phase II trial, 20 ovarian cancer patients were vaccinated with a p53-SLP vaccine but this did not result in a clinical response. Thereafter these patients received secondary chemotherapy (either monotherapy with carboplatin or doxorubicin, or doublet therapy with carboplatin/paclitaxel or carboplatin/docetaxel). The chemotherapeutic treatment did not markedly decrease or augment p53-specific immunity. Clinical response rates and disease-specific survival did not differ between vaccinated patients and historical controls. Thus, this study could not establish synergy between p53 SLP vaccination and secondary chemotherapy [89].

Low-dose cyclophosphamide treatment schemes have been proposed in order to reduce frequency and/or activity of Tregs without impairing the effector mechanisms of other immune cells [90]. In a phase II trial with 43 stage II/III melanoma patients, half of the patients were vaccinated in a protocol where they were pre-treated with low-dose cyclophosphamide and 2 rounds of vaccination with modified Melan-A/MART-1 (27L), gp100 (210M), NY-ESO-1 (165V) and survivin (97M). Subsequently, a tumour-positive draining lymph node (LN) was surgically removed and followed by low-dose cyclophosphamide (twice), vaccination (four times) and low dose IL-2 (twice). In the control group, patients received no other treatment than surgery. Significantly lower frequencies of CD4+CD25+$^{+}$Foxp3^{high} T-cells were found in LN of the pre-treated group.

These CD4+CD25+$^{+}$Foxp3^{high} T-cells also produced significantly lower levels of IL-10 and TGF-β. This decrease was also seen in PBMC but only transiently as the levels of circulating Tregs returned quickly to pre-treatment levels and then increased as a result of IL-2 treatment. Functional assays confirmed the suppressive activity of these IL-2 induced CD4+CD25+$^{+}$Foxp3^{high} T-cells. Interestingly, this increase in Tregs did not overtly impact the vaccine-induced T-cell response as this response significantly and progressively increased soon after immunization and remained stable or increased after IL-2 treatment throughout the treatment period (12 months). In the end 75% of patients reacted to at least 1 and 65% to at least 2 peptides. In the control group, baseline values remained stable throughout. Clinical evaluation showed no significant difference in DFS and OS between both groups [91]. In depth analysis showed that vaccine-induced modified-peptide-specific T-cells displayed only weak reactivity to the respective wild-type sequence, resulting also in low responses to melanoma cells expressing the cognate antigen. These type of observations have been reported before and warn the field against the use of modified peptides since the inability of vaccine-induced T-cells to recognize the wild-type epitopes presented at the tumour cell surface will result in a failure to kill tumour cells, which might (as suggested by the authors of the above study) explain the lack of clinical efficacy despite a clear vaccine-induced T-cell response [92].

A multicenter randomized trial was conducted to test whether addition of 6 melanoma-associated helper peptides (MHP) to a multi-peptide vaccine composed of 12 HLA class I-restricted melanoma peptides (MP) could boost T-cell response compared to a tumour non-specific tetanus helper peptide (THP). Again the effect of pre-treatment with cyclophosphamide to enhance the T-cell reactivity to vaccination was assessed. No effect of cyclophosphamide pre-treatment was found with respect to vaccine responsiveness. Unexpectedly, frequency and strength of vaccine-induced immune response was less in patients who received 12 MP combined with 6 MHP than in patients who were treated with 12 MP combined with THP (74% vs 24 %, $p<0.001$). This may be, in part, be explained by the stronger and more frequently induced CD4 T-cell responses to THP (91% Arm A + B) as opposed to the 6 MHP (18%). No difference in OS or PFS was found between all 4 study arms [93]. Pre-treatment with a single dose of cyclophosphamide was also used to augment the T-cell response to p53-SLP vaccination in patients with recurrent ovarian cancer. There was a clear vaccine-induced proliferative and IFNg-associated T-cell response to multiple peptide-epitopes in the great majority of patients. Vaccine site infiltrating p53-specific T-cells were detected in 4 of 9 biopsies after the 2nd vaccination and in 6 of 7 biopsies after the 4th vaccination. Treatment with a single dose of cyclophosphamide neither resulted in a change in frequency of CD4+CD25+Foxp3^{high} T-cells nor in a change of suppressive function throughout vaccination [94]. However, comparison of the data to the results of a previous p53 SLP trial in a comparable group of patients [12] conducted by the same group suggested that cyclophosphamide pre-

treatment may result in stronger and longer lasting ELISPOT responses. In another phase II trial, 68 HLA-A2+ advanced RCC patients were randomized to receive IMA901 combined with pre-treatment with a single dose of cyclophosphamide or no pre-treatment. More than half of the 61 evaluable patients showed an immune response to at least one peptide but there was no difference between both groups indicating that there was no effect on the induction of T-cell responses. Subgroup analysis showed that among the immune responders those who were pre-treated with cyclophosphamide had a prolonged survival. Interestingly, a broad response to multiple peptides was associated with prolonged survival. Pre-treatment with cyclophosphamide showed a 20% reduction in the absolute frequency of Tregs and that of the proliferating Tregs [57]. Pre-treatment with a single dose of cyclophosphamide was also investigated in a study where 10 advanced ovarian cancer patients previously treated and in remission when enrolled, were randomized to treatment with heteroclitic Her2/neu, hTERT and PADRE peptide pulsed DCs with or without pre-treatment. Measurements of circulating Tregs did not show a pre-treatment induced reduction. Modest T-cell responses to at least 1 vaccine peptide were detected in 3 of 5 patients without pre-treatment and in 4 of 5 patients with pre-treatment. Importantly, patients were also vaccinated with a pneumococcal vaccine (CRM197). Overall, only 4 patients mounted a weak response which was transient in 2 patients. Since this is well below the rate observed in healthy controls and even in myeloma control patients, the results of this study might reflect an unexpectedly weak general immune status in these patients [95].

Peptide vaccines combined with immunomodulating antibodies

CTLA-4 inhibition by ipilimumab leads to an increase in T-cell proliferation due to the blockage of the inhibiting response on the B7-CTLA-4 interaction. A phase II study was conducted in patients with resected melanoma who were treated with escalating doses of ipilimumab. Antibody infusions were accompanied in HLA-A2-positive patients (n=50) by 3 separate vaccinations with $gp100_{209-217}$, $MART\text{-}1_{26-35}$ and $tyrosinase_{368-376}$. Two-year relapse-free survival (RFS) rate was 56% (CI: 44-67%) and 2-year OS was 86% (CI: 75-92%). 10 of 40 evaluable patients showed IFNg-associated T-cell reactivity to MART-1 or gp100, whereas no patient responded to tyrosinase. Tetramer analysis showed a significant increase in MART-1-specific CD8+ T cells after vaccination (median expression 0.30% vs 0.15 % at baseline, $p<0.0001$). No correlation was found between reactivity to vaccine peptides and relapse rate or survival. There were no consistent effects on antigen-specific T cells or $CD4+CD25^{high}$ Tregs. However, ipilimumab treatment resulted in increased frequency of CD4+ and CD8+ T cells expressing HLA-DR, which is considered as a late marker for T-cell activation. In addition, there was an increase in the frequency of T-cells producing IL-17 upon stimulation. Small but significant changes in this frequency were positively associated with freedom of relapse [96].

PEPTIDE VACCINATION IN NSCLC

Stage I/II non-small cell lung cancer (NSCLC) patients are generally treated by surgical resection with curative intent. However, the majority of NSCLC patients present with advanced disease (stage III/IV) which is treated with combined chemo-radiotherapy (stage III) or (palliative) chemotherapy (stage IV) [97-99]. Current therapies provide limited benefit. However, NSCLC displays a high number of genetic alterations. Mutations in certain signalling pathways, such as anaplastic lymphoma kinase (ALK) gene rearrangements or epidermal growth factor receptor (EGFR) mutations provide a rationale for the use of targeted therapies. Small-molecule kinase inhibitors have shown impressive clinical benefit and improved prognosis in NSCLC patients harbouring these mutations [100]. On the other hand, these mutations may also function as targets for T-cells and benefit personalized immunotherapeutic approaches.

Most studies on the use of immunotherapy in NSCLC have focused on identifying new lung cancer antigens as T-cell targets or have studied the NSCLC tumour microenvironment. This is characterized by a dense infiltrate with several immune cells, such as lymphocytes (mainly T-cells), macrophages and mast cells. The role of these immune cells and its association with clinical parameters has been extensively discussed in a recent review [101]. Briefly, tumour associated M2-macrophages are present in high frequencies. Co-infiltration of M1-macrophages is frequently found and this is positively correlated with extended survival, despite the fact that they can be outnumbered by M2-macrophages [102,103]. The infiltration with CD4, CD8 T-cells or both is also positively associated with clinical outcome [104,105]. In contrast, the presence of tumour-infiltrating Tregs is associated with higher risk of recurrence [106,107]. Thus similar to many of the immunogenic tumour types known, NSCLC also presents itself as an immunogenic tumour infiltrated by immune cells which can either promote or suppress tumour growth. Clearly, new immunotherapeutic approaches should take this immunological background into account when designing a strategy to treat NSCLC. Below we discuss recent progress in the field of peptide vaccination in NSCLC, summarized in **Table 2**.

In a phase I trial, 15 patients with advanced or recurrent NSCLC were treated with a HLA-A24 restricted multi-peptide vaccine. Results of two dose escalating studies were reported, in which patients were treated with two peptides derived from VEGFR1 and VEGFR2 in combination with 2 peptides from the CT antigens URLC10 and TTK (n=9) or 2 peptides from URLC10 and CDCA1 (n=6). After antigen driven expansion of PBMC samples, reactivity to at least one of the vaccine peptides was observed in 13 of 15 patients. Furthermore, 4 of 6 patients in the high dose (3 mg) group showed an immune response which was both strong and broad (reactive to 3 or more peptides). There was a clear relationship with a good performance status of the patient and the chance to detect a strong CTL response. The absence of a strong CTL response was also

Table 2. Clinical peptide vaccine trials in NSCLC

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Phase I								
Suzuki et al (2013)	15	Advanced/ recurrent NSCLC	Multipeptide vaccine: URLC10, CDCA1, LY6K,VEGFR1, VEGFR2	Montanide	ELISPOT, tetramer	PMA, ionomycin	13 of 15 ELISPOT + 7 of 15 ELISPOT+ to 3 or more vaccine peptides	7 SD, of which 6 had strong IR 8 PD 2-year survival rate 32.8%
Ohyanagi et al (2011)	6	NSCLC stage III	L-BLP25 (MUC-1)	None	NA	NA	NA	NA
Perroud et al (2011)	5	Stage III/IV NSCLC	DCs pulsed with 1of 4 peptides (WT1, CEA, MAGE-1, HER-2)	None	CFSE labeled proliferation, ICS	NA	All patients showed peptide specific proliferation	2 PR, 3 SD
Phase II								
Takahashi et al (2013)	62	Advanced NSCLC	DCs pulsed with either tumor lysate, WT1 or MUC1 peptides	OK-342, prostaglandin E2	NA	NA	NA	DCR 32% at 6 months Improved survival in WT1 subgroup
Yoshiyama et al (2012)	41	Advanced/ recurrent NSCLC	PPV: Max 4 out of 31 candidate peptides	Montanide	ELISPOT, bead-based multiplex assay	NA	17 of 35 IgG + to PPV after 1st cycle 18 of 18 IgG + to PPV after 2ndcycle 11 of 32 ELISPOT+ after 1st cycle 5 of 9 ELISPOT+ after 2ndcycle	14 SD, 11 PD 1-yr survival 42% IR not correlated with OS

Table 2. Clinical peptide vaccine trials in NSCLC (continued)

Study (year)	N=	Cancer type	Treatment (nr of vaccinations)	Adjuvant	Immune monitoring	GIS	Immune response	Clinical response
Brunsvig et al (2011)	20	NSCLC stage III	GV1001+chemoradiotherapy (docetaxel)	GM-CSF	Proliferation	SEC	16 out 20 + for Ag specific proliferation Durable response in 13 out of 15 responders 1 out of 20 + for DTH	5 immune responders among 6 SD cases
Butts et al (2011)	171	NSCLC stage IIIb/IV	L-BLP25 (MUC-1)	None	NA	NA	NA	no difference in OS

Legend: CFSE: carboxyfluorescein succinimidyl ester , CR: complete response, DC: dendritic cell, DCR: disease control rate, DFS: disease free survival, DTH: delayed type hypersensitivity, ELISPOT: enzyme-linked immunosorbent spot, ICS: intracellular staining, IR: Immune response, NA: Not assessed, NSCLC: non-small cell lung cancer, OS: overall survival, PD: progressive disease, PMA: phorbol-12-myristate-13-acetate, PPV: personalized peptide vaccine, PR: partial response, SD: stable disease, SEC: Staphylococcal enterotoxin C

related to rapid progression whereas the presence was related to SD. Furthermore, an improvement in 1-year survival was observed in patients who showed T-cell response to multiple peptides (85.7% vs 33.3 %, p=0.0176). The apparent favourable clinical effect of vaccination is however mostly observed in patients with good performance status, therefore a subsequent study should be randomized to avoid this bias [108]. PPV vaccination was performed in 41 NSCLC patients. In 19 of 41 patients, PPV (selected from a pool of 31 HLA class I restricted peptides) was administered simultaneously with other therapy (e.g. chemotherapy). Antibody response to at least one PPV peptide was induced in 17 of 35 (49%) patients after the first vaccination cycle and in all 18 evaluable patients after the second cycle. PBMC samples were pre-sensitized in culture with peptide and subsequently tested for reactivity to PPV peptides. 11 of 32 patients showed a vaccine-induced T-cell response after the first cycle and 5 of 9 evaluable patients after the second cycle. Induction of an immune response was not associated with OS. However, a low frequency of activated $CD3^+CD26^+$ T-cells after vaccination was predictive of an unfavourable clinical outcome in a multivariate Cox regression analysis [109]. In a phase I/II trial, NSCLC patients with advanced disease were injected intradermally with a DC vaccine pulsed with either autologous tumour lysates (if available) or MUC-1 long peptide, WT1 (mutant or wt) or CEA peptide based on patient's HLA type, histological subtype of tumour and the serum level of CEA. At 6 months after vaccination, 20 patients (DCR: 32%) showed either clinical response (1 CR, 2 PR) or stable disease (17 SD). Although there was a good suggestion that the use of DCs pulsed with WT-1 peptides could improve the median survival, the immune response was not assessed. Due to a number of clinical factors associated with survival, the current setup makes assessment of the relation between the immune system and clinical data difficult [110]. A small study in 5 HLA-A2 expressing advanced, pretreated stage III/IV NSCLC patients tested the feasibility and immunogenicity of DCs pulsed with HLA-A2 restricted peptide epitopes from WT1, CEA, MAGE-1, HER-2. One patient experienced grade 2 and 3 adverse events following vaccinations, requiring hospital admission but resolving after 5 days. Peptide-specific proliferative responses to one or more peptides were detected in all patients after the first and second dose of vaccine [111].

A phase II trial was reported in which 20 stage III NSCLC patients were vaccinated with GV1001 ($hTERT_{611-626}$) after combined chemoradiotherapy that included docetaxel. *In vitro* stimulated PBMCs of 16 out of 20 patients showed GV1001-specific T-cell proliferation and this response was sustained in time after vaccination. Immune responders showed a trend for improved PFS (median 371 days vs 182 days; p=0.20) [112]. Long-term immunological and clinical follow-up were also reported of a previous phase I/II trial in which mostly stage IV NSCLC were vaccinated [113]. Interestingly, also here an improved OS for immune responders was observed (median 19 months vs 3.5 months, p<0.001),

with surviving subjects clearly showing strong and durable GV-1001-specific immune responses [112]. These data warrant randomized trials to establish the effect of vaccination.

Overexpression of MUC-1 is often observed in epithelial cancers like colorectal cancer, pancreatic cancer and lung cancer. L-BLP25 is a MUC-1 based vaccine composed of a lyophilized preparation of the 25-amino-acid BLP25 lipopeptide, an immunoadjuvant (monophosphoryl lipid A) and three lipids (cholesterol, dimyristoylphosphatidylglycerol and dipalmitoylphosphatidylcholine). Two studies were conducted with L-BLP25 vaccine and pretreatment with cyclophosphamide. Due to the limited immunological and clinical data reported, a clear judgement on the value of MUC-1 vaccination and pretreatment with cyclophosphamide is not possible. In the first study, stage IIIb/IV NSCLC patients were randomized for vaccination with L-BLP25 in combination with best supportive care (BSC) versus BSC alone. No vaccine-induced immunological responses were reported. However, there was also no difference in OS between both arms [114]. In another study, 6 stage III NSCLC patients, previously treated with chemoradiotherapy, received 4 or more L-BLP25 vaccinations after pre-treatment. However, the primary objective was safety, thus no immunological or clinical data were reported [115]. Very recently, results from the START trial were presented at the annual meeting of the American Society of Oncology. In this phase III trial, 1513 patients with stage III NSCLC that did not progress after chemoradiotherapy treatment were randomized to either L-BLP25 (pre-treated with cyclophosphamide) or placebo. Unfortunately, OS (which was the primary endpoint of this study) was not improved (25.6months for L-BLP25 vs. 22.3 months for placebo, p=0.123). In a subgroup of patients (n=806) previously treated with concurrent chemoradiotherapy, prolongation of OS was observed (30.8 months (L-BLP25) vs. 20.6 months (placebo), HR 0.78, 95% CI 0.64-0.95, p=0.016) [116]. One multicenter, phase II, double blind placebo-controlled L-BLP25 vaccination trial is currently being conducted [117].

EXPERT COMMENTARY

In several trials, clinical outcomes after peptide vaccination were promising and justify the extensive research efforts of recent years in the field of peptide vaccination in cancer. However, the majority of studies fail to find convincing correlations between vaccine-induced immunity and clinical outcome. This can, in part, be explained by the fact that the majority of trials were phase I/II. These are designed to assess safety, dose-finding and immunogenicity, but not to investigate clinical outcomes. A second explanation might be that most assessments of T-cell reactivity are performed after a period of *in vitro* stimulation and as such will not properly reflect the actual response size *in vivo*, albeit that it may reveal the breadth of the T-cell response which is one of the more recent parameters potentially associated with clinical reactivity [16,57]. Furthermore, when

correlations between vaccine-induced immunity and survival are reported one should be cautious. Whereas there is little doubt that regressions of lesions after vaccination will be due to a strong immune response, the observation of only longer survival may not. A number of studies report that a good vaccine-induced immune response as well as survival is associated with a better performance status of the patient at study entry. This suggests that these patients, due to a better clinical condition, would live longer anyway but also may display a better immune status allowing these patients to respond more vigorously to vaccination [108,109]. One way to control for this is to measure general parameters of adaptive immune status, for instance by analysis of so-called recall responses to bacterial and viral antigens [63-65]. It is our observation that various study reports mention the use of such control antigens but do not present the data nor use it for a better interpretation of immunological data in relation to clinical outcome. Future exploratory trials should incorporate these types of measurements as it will help to decide how to move a product forward.

Another main stumbling block for successful peptide vaccination is insufficient T-cell activation [118]. It is well known that antigen presentation by immature APCs is detrimental to naive T-cells since this will lead to tolerance [119]. In most discussed trials, the oil-in-water emulsion Montanide was used as a vehicle and non-specific adjuvant. By forming a depot and slowly releasing peptide, it works as an adjuvant and is able to enhance immune responses [120]. However, responses are generally not robust or well polarized towards IFNg production and CTL activity and clinical effects are rare. Therefore, vaccines need to be supplemented with immunomodulating compounds. In order to enhance the magnitude of the vaccine-induced T-cell response, studies have been performed with the immunomodulator IL-2 [47,48,75,92]. Immunomonitoring revealed that the use of IL-2 generally resulted in an increase in the frequency of CD4+CD25+Foxp3^{high} T-cells. Isolation of these T-cells showed that this population contained immune suppressive cells. Does this forbid the use of IL-2? So far no evidence has been provided that the increased Treg population bears a direct impact on the immune and clinical response [92]. In parallel, efforts to improve vaccine-induced immunity have focused on pre-treatment with cyclophosphamide to decrease Tregs before vaccination. However, despite the lack of overt differences in cyclophosphamide pre-treatment, two trials failed to detect a reduction in the number of Tregs whereas two other trials do. Notably, the analysis of Tregs was based on the expression of CD4 and CD25 or based on CD4, CD25 and Foxp3 or on a broader marker set including also CD45, CD127 and Ki67 [59,91-95]. Differences in the reduction of Tregs by low dose cyclophosphamide have also been found in the more recent past and because Tregs are measured in many different ways it seems imperative to harmonize these types of measurements in order to get a better grip on the effects of cyclophosphamide on Tregs in patients with cancer. Such a harmonization effort is currently undertaken by the CIMT Immunoguiding Program (http://cimt.eu/workgroups/

cip/). Interestingly, a reduction in Tregs did not translate into a stronger vaccine-induced T-cell response [59,91].

Finally, clinical benefit from peptide vaccination in cancer patients may also depend on the tumour burden of the patient, stage of the disease and a functional immune system. As yet, successful peptide vaccination is rare in late-stage patients with a high tumour burden and distant metastases. Reasons for this can be related to the presence of an immune suppressive tumour micro-environment and/or a dysfunctional immune repertoire in these patients. Indeed, the majority of studies discussed in this review were phase I trials conducted in advanced cancer patients with limited information on immunocompetence, stressing the point made above by us. One could envisage that peptide vaccination is applied in patients with minimal residual disease after standard treatment (e.g. surgery or chemo-radiotherapy) in whom an immune suppressive tumour micro-environment is less likely expected to be present and vaccination may lead to a more effective immune response. As a result one may achieve clearance of residual cancer cells and an increase in disease-free survival. In a recent review, the question of how to apply therapeutic cancer vaccines in order to achieve the most optimal clinical benefit is discussed in more detail [121].

Frequently used immunomodulators aiming not only to enhance the immune response but also to polarize it towards a Th1 profile are IFNα and GM-CSF, cytokines that are involved in DC activation [122,123]. In several recent trials a beneficial effect of IFNa on the vaccine-induced T-cell response was established. This warrants the use of IFNa as stimulating agent in larger studies. Another way of improving DC activation and subsequent T-cell priming is by use of TLR ligands [124]. Robust vaccine primed responses were induced in several studies [60,61] and a clinical effect was observed in a few cases [40,80]. A recent review by our group elaborates on another promising application of TLR ligands by conjugation to tumour antigens [125].

Since the last update on lung cancer vaccines [126], a number of trials using peptide vaccines to stimulate tumour immunity in NSCLC have been reported, but their numbers are still not overwhelming. Recent studies in which patients with NSCLC were treated with antibodies against immunological checkpoints molecules with the aim to reinforce the spontaneously induced NSCLC-specific T-cell response showed great clinical benefit [3,5,127], indicating that T-cell based immunotherapy of NSCLC can be successful. The parameters that influence the outcome of peptide vaccines have been systemically examined in mouse models and confirmed in patients in the last decade [19] and this knowledge is waiting to be translated into well-designed therapeutic vaccination trials to boost tumour-specific T-cell immunity for the treatment of NSCLC.

FIVE YEAR VIEW

We anticipate that within the next 5 years, therapeutic peptide vaccination will evolve in three directions. First of all the use of personalized peptide vaccines (PPV) will increase. These PPV can be created on the basis of antigen expression by the patient's tumour, the patient's HLA type and the presence of suitable peptides in a warehouse comprising already identified peptide-epitopes, such as described already in this review. Alternatively, PPV will be made on the basis of integrated techniques that assess the tumour-specific mutated peptide repertoire presented at the cell surface HLA of primary tumour tissue, assessment of antigen expression in tumours and their capacity to activate the patient's T-cells, after which peptides are manufactured or taken from a warehouse when previously identified. The feasibility of such an approach is currently tested in a European Community project called GAPVAC for the treatment of glioma [128].

Furthermore, after the first immunogenicity trials showing that overlapping long peptides (OLP) vaccines are highly immunogenic, inducing strong directly *ex-vivo* detectable CD4 and CD8 T-cell responses in patients with cancer that can be potentiated by immunomodulators such as IFNa and poly-ICLC, we expect new trials to show their clinical efficacy. Moreover, we expect OLP vaccines to be tested as treatment in various types of cancer. Potential target antigens will be formed by CT antigens other than the above discussed trials with NY-ESO-1. For this matter, XAGE-1b (GAGED2a) has recently been identified as a CT antigen mainly overexpressed in lung adenocarcinoma. XAGE-1b overexpression is observed in 35-50% of lung adenocarcinomas [129] and spontaneously induced XAGE-1b-specific CD4 and CD8 T-cell responses have been reported in lung adenocarcinoma patients [130]. We aim to start a phase I trial with XAGE-1b OLP in Montanide using poly-ICLC as immune modulator in patients with stage III/IV lung adenocarcinoma within this year.

Finally, there will be a strong effort made to focus on synergy of peptide vaccination with other therapies, such as chemotherapy and/or antibodies blocking inhibitory receptors. This will require larger studies providing an opportunity to determine relevant biomarkers.

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Chapter 5

The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell lung carcinoma

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ABSTRACT

Introduction

Tumor-infiltrating CD8+ T cells are associated with improved clinical outcomes in non-small cell lung cancer (NSCLC). Here we studied their prognostic effect in the context of the expression of HLA molecules that are key in tumor recognition (HLA-A, B and C) or suppression of immunity (HLA-E) as this is still unknown.

Methods

Tumor tissue of 197 patients with resected pulmonary adenocarcinoma was analyzed for the presence of CD8+ T cells and the expression of β2-microglobulin, HLA-A, HLA-B/C and HLA-E. The relation of these parameters with overall survival (OS) was assessed.

Results

Loss and low expression of HLA-A or HLA-B/C was found in 44% and 75% of cases respectively. A high CD8+ tumor infiltration was strongly associated with clinical benefit only when the tumors retained good expression of HLA-A and HLA-B/C (p=0.004). In addition, more than 70% of the tumors were found to display a high expression of HLA-E. The expression of HLA-E by tumor cells was an independent negative prognostic factor for OS (p=0.031). Importantly, a dense stromal CD8+ T cell infiltration was strongly associated with improved OS only in HLA-E negative tumors (p=0.005) and its prognostic effect was completely abolished when tumors highly expressed HLA-E (p=0.989).

Conclusions

CD8+ T cell infiltration strongly contributes to a better prognosis in NSCLC when the tumor cells retain the expression of classical HLA class I and do not express HLA-E. Therefore, analysis of HLA-A, -B/C and HLA-E expression should be included as biomarkers to predict the response to immunotherapy.

INTRODUCTION

Non-small cell lung cancer (NSCLC) is a leading cause of death globally[1-3]. The reported overall 5-year survival is 17%[2,4], indicating the need for therapies that extend survival and provide a better quality of life. T-cell based immunotherapies hold great promise as a powerful new approach to treat NSCLC as treatment with antibodies interrupting immune checkpoint PD-1/PD-L1 has shown great clinical benefit in NSCLC[5-7]. The programmed death 1 (PD-1) receptor blocking antibody nivolumab was recently approved by the U.S. Food and Drug Administration to treat metastasized squamous NSCLC[8].

T-cell based immunotherapy of cancer is highly dependent on the presentation of tumor-specific antigens in the context of human leukocyte antigen (HLA) class I or class II molecules to tumor-infiltrating T cells (TILs)[9]. In NSCLC, the density of TILs, in particular the number of stromal CD8+ T cells, have strong prognostic value[10-15]. The expression of the classical HLA class I molecules A, B and C in NSCLC, however, frequently is down regulated[16,17] and was found to affect overall survival (OS)[18]. Remarkably, studies on the interaction between CD8+ T cell infiltration and the expression of classical HLA class I are limited to one study showing that loss of HLA class I is associated with a sparser T-cell infiltrate[19].

The non-classical HLA class I molecules E, F and G can also be expressed by cancer cells. HLA-G expression was associated with limited lymphoid infiltration and poor prognosis in NSCLC[20], potentially via increased regulatory T-cell activity[21]. The expression of HLA-F, acting via the immune inhibitory receptors ILT-2 and ILT-4 [22], also had a negative impact on the prognosis of NSCLC patients[23]. HLA-E, which binds to the inhibitory CD94/NKG2A receptor expressed by activated NK cells and CD8 T cells, can directly suppress innate and adaptive immunity when expressed by cancer cells[24,25]. We have studied the expression of HLA-E in different cancers[26,27] and found that the beneficial prognostic effect of infiltrating CTLs in ovarian cancer was thwarted by high expression of HLA-E[27]. However, the expression and prognostic effect of HLA-E in NSCLC has not been studied.

To investigate the prognostic value of CD8+ tumor infiltrating T cells in the context of HLA-A, B and C as well as HLA-E and its association with OS, we retrospectively studied a group of 197 patients with NSCLC. We exclusively focused on pulmonary adenocarcinoma not only because this is the main histological subtype in NSCLC[1,28] but also because HLA loss has been reported to be less frequent than in squamous cell carcinoma, the other major subtype of NSCLC[16-19,29] and therefore is expected to benefit the most from active T-cell-mediated immunotherapy. Our study revealed that the expression of HLA-E by tumor cells was an independent prognostic factor for OS. High expression of HLA-E neutralized the positive prognostic value of high stromal CD8+ T cell infiltration in NSCLC.

RESULTS

Stromal CD8 T-cell infiltration correlates best with overall survival.

A cohort of 197 patients with pulmonary adenocarcinoma was evaluated. The grade of differentiation by the tumor was classified as either poor (50%), moderate (33%) or well differentiated (17%). In 31% of cases, patients had advanced disease (stage III/IV) despite being classified as stage I/II based on pre-operative diagnostic modalities (**Table 1**). Mean age was 66 years (range 37- 90 years) and the number of males (n=99) and females (n=98) was evenly distributed.

Table 1. Overview of stage, differentiation and immunohistochemical expression patterns in pulmonary adenocarcinoma.

Surgical-pathological staging (number, %)		
I	62	(31 %)
II	74	(38 %)
III	35	(18 %)
IV	26	(13 %)
Differentiation (number, %)		
Poor	98	(50 %)
Moderate	66	(33 %)
Well	33	(17 %)
β2-M (number, %)		
Low	47	(24 %)
High	150	(76 %)
HLA-A (number, %)		
Low	87	(44 %)
High	110	(56 %)
HLA-B/C (number, %)		
Low	148	(75 %)
High	49	(25 %)
HLA-E (number, %)		
Low	55	(28 %)
High	142	(72 %)
Total CD8+ (number, %)		
Low	96	(59 %)
High	68	(41 %)
CD8+ in tumor (number, %)		
Low	104	(64 %)
High	59	(36 %)
CD8+ in stroma (number, %)		
Low	92	(56 %)
High	71	(44 %)

The extent of CD8+ T-cell infiltration was studied by enumeration of intraepithelial and stromal CD8+ T cells in tumor sections. Examples of representative immunohistochemical stainings of CD8+ T cells are displayed in **Figure 1**. Overall intraepithelial CD8+ T-cell infiltration ranged from 7 to 1460 cells/ mm^2 tumor (mean 194; median 150), stromal CD8+ T cells from 35 to 1332 cells/ mm^2 tumor (mean 348; median 320) and total CD8+ T cells from 32 to 1008 cells/ mm^2 tumor (mean 271; median 246). There were no differences in total CD8+ T-cell tumor infiltration between males and females (chi square test, p=0.267). Patients were divided in two groups with low or high CD8+ T cell infiltration, based on the mean CD8+ T-cell count for all patients, and the association with OS was plotted. A relatively strong stromal CD8+ T-cell infiltration displayed the best association with a beneficial clinical outcome (log-rank test, p=0.068; **Figure 2 A-C**). The negative effect of low stromal CD8+ T-cell infiltration was magnified when the patients were divided on the basis of tertiles, with patients in the lower tertile defined as having low CD8+ stromal T cell infiltration and the other patients as having high stromal CD8+ T cell infiltration (p=0.046, **Supplementary Figure 1**), similar to what was reported before[10-14].

Interaction between classical HLA class I expression and CD8+ T cells.

Assessment of the expression of classical HLA class I molecules was performed using antibodies against β2-M, HLA-A and HLA-B/C (**Figure 1**). β2-M was expressed in 76% of cases, but HLA-A and HLA-B/C were expressed in only 56% and 25% of the cases, respectively (**Table 1**).

Subsequently, the association between tumor stage, HLA class I molecules and CD8+ T cell infiltration was assessed (**Supplementary Table 1**). High expression of HLA-A strongly correlated with high expression of HLA-B/C (p=0.0001). A clear correlation existed between the presence or absence of functional HLA class I expression and the total number of tumor-infiltrating CD8+ T cells. Tumors with downregulation of HLA-A (p=0.012) or HLA-B/C (p=0.018) displayed on average lower numbers of total tumor-infiltrating T cells (**Supplementary Table 1 and Supplementary Figure 2)**.

When patients were grouped according to a low or high expression of HLA-A or HLA-B/C, Kaplan Meier curves did not reveal any direct impact of classical HLA class I expression on clinical outcome (**Figure 2D and 2E**). However, an interaction analysis between classical HLA expression and total CD8+ T cell infiltration in tumor tissue revealed a clear beneficial effect of a dense CD8+ T cell infiltration in HLA-B/C positive tumors (HR 0.212, 95% CI 0.074-0.606, p=0.004) or HLA-A and HLA-B/C-positive tumors (HR 0.215, 95% CI 0.069-0.673, p=0.008) with respect to OS (**Table 2 and Figure 3**). This was not the case when CD8+ T-cell infiltration was analyzed in the context of HLA-A expression only. These results indicate that the presence of high numbers of CD8+ T cells is correlated with a favorable prognosis when classical HLA class I expression by the primary tumor is retained.

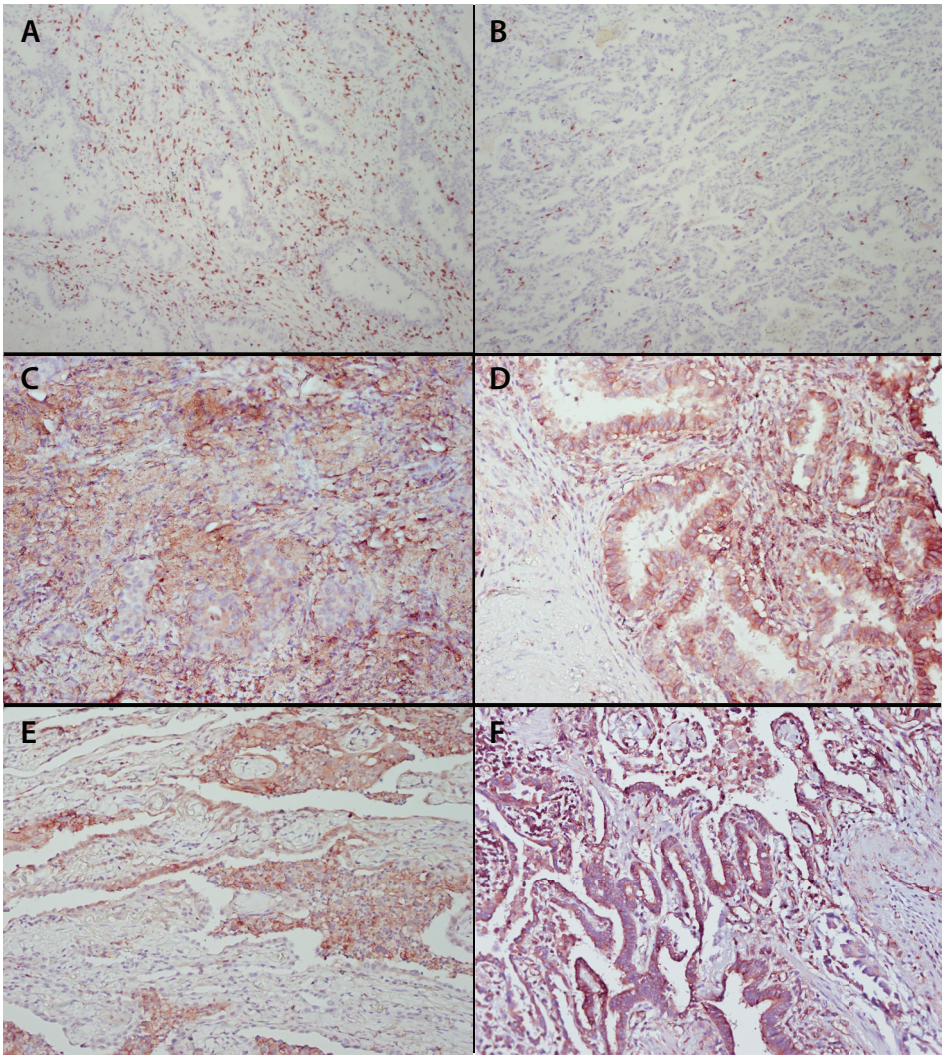

Figure 1: Staining of tumor infiltrating CD8+ T cells, β2-microglobulin, HLA-A, HLA-B/C and HLA-E in pulmonary adenocarcinoma.

Formalin-fixed, paraffin embedded tumor specimens of 197 non-small cell lung cancer patients were cut in 4 µm sections and immunohistochemically stained for CD8, β2-microglobulin, classical HLA-A and HLA-B/C, as well as non-classical HLA-E. According to the Ruiter scoring system [46] both the intensity and percentage of cells stained were assessed and expression was categorized as low (score 1-4) and high (score 5-9). Examples are shown of high (**A**) and low (**B**) stromal and intraepithelial CD8+ T cell infiltration; tumor with high β2-microglobulin expression **(C)**; examples of HLA-A **(D)**, HLA-B/C **(E)** and HLA-E **(F)** staining. Original magnification x200.

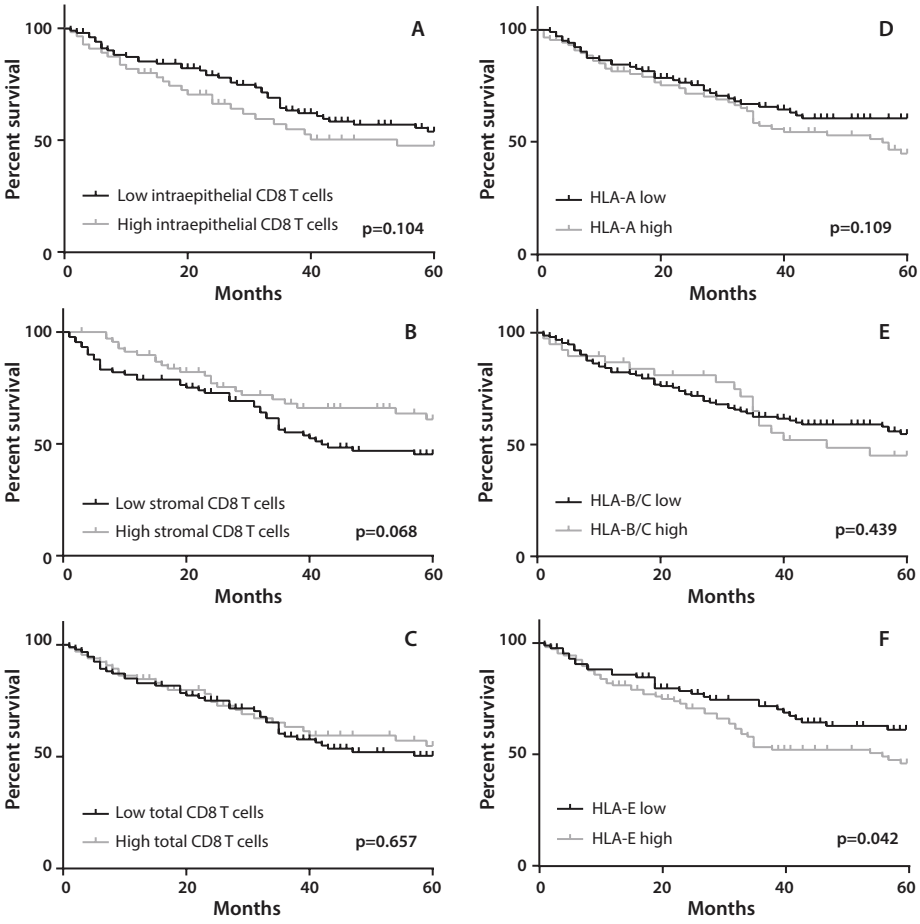

Figure 2. Association of CD8+ T cell infiltration and HLA expression with overall survival (OS).
Patients were divided in two groups with low or high CD8+ T cell infiltration, based on the mean CD8+ T-cell count for all patients or on the basis of HLA expression. OS was defined as date of surgery until date of death due to any cause, or date of last follow-up with a maximum follow-up time of 5 years. Kaplan-Meier curves were used to estimate OS of the two groups whereas the log-rank test was used to compare the difference between the two curves.
Survival curves are presented for **A)** patients with low (n=104) or high (n=59) intraepithelial CD8+ T cells (mean CD8+ cell count 194 cells/ mm^2 tumor) ; **B**) low (n=92) or high (n=71) stromal CD8+ T cells (mean CD8+ cell count 348 cells/ mm^2 tumor) ; **C**) low (n=95) or high (n=68) total CD8+ T cells (mean CD8+ cell count 271 cells/ mm^2 tumor). Furthermore, survival curves are presented for functional (i.e. positive staining for both HLA and β2-M) expression of **D)** HLA-A low (n=106) and high (n=91) ; **E**) HLA-B/C low (n=156) and high (n=41) ; **F**) HLA-E low (n=87) and high (n=110). A significant correlation (p=0.042) was observed between low HLA-E expression and improved survival (**F**).

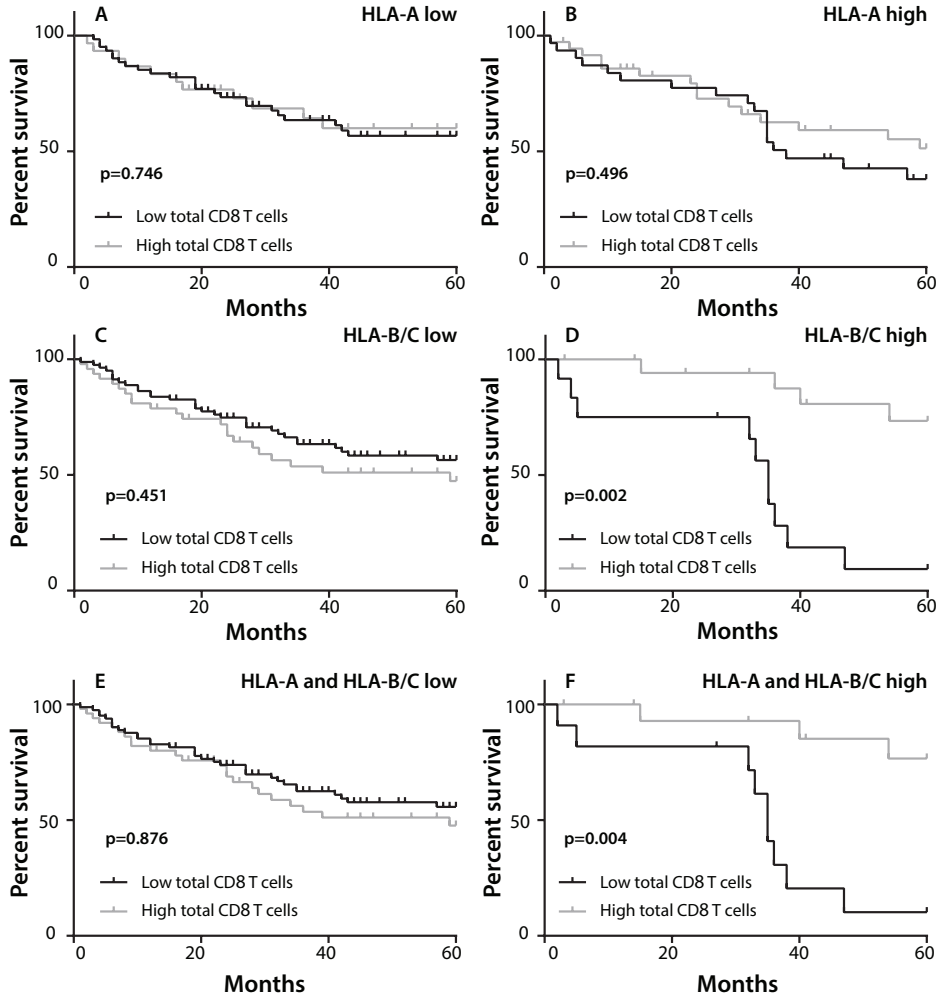

Figure 3. Effect of classical HLA class I expression and CD8+ T cell infiltration on overall survival (OS). Patients were divided in two groups with low or high CD8+ T cell infiltration, based on the mean CD8+ T-cell count for all patients and on the expression of classical HLA class I. Kaplan-Meier curves were constructed to estimate OS of the two groups whereas the log-rank test was used to compare the difference between the two curves.

(**A,B**) Comparison of OS between patients with low or high total CD8+ T cell infiltration in the context of low (**A**; n=63 vs 30, respectively) or high (**B**; n=32 vs 38, respectively) HLA-A expression.

(**C,D**) Comparison of OS between patients with low (n=83) or high (n=47) CD8+ T cell infiltration of whom the tumors displayed low HLA-B/C expression (**C**). Comparison of OS between patients with HLA-B/C positive tumors and high (n=21) or low (n=12) total CD8+ T cell infiltration (**D**).

(**E,F**) Comparison of OS between patients with low (n=62) or high (n=27) CD8+ T cell infiltration in tumors with low expression of both HLA-A and HLA-B/C (**E**). Comparison of OS between patients with low (n=11) or high (n=18) total CD8+ T cell infiltration in the context of tumors with high expression of both HLA-A and HLA-B/C. (**F**)

Table 2. Univariate and multivariate Cox proportional hazard analysis. Significant differences (p<0.05) are indicated in bold.

Variable		Univariate analysis HR (95% CI)	p value	Multivariate analysis HR (95% CI)	p value
Stage	I/II vs III/IV	0.619 (0.399 - 0.961)	**0.033**	0.587 (0.377-0.913)	**0.018**
Sex	Male vs Female	1.834 (1.184 - 2.839)	**0.007**	1.785 (1.152-2.765)	**0.009**
Differentiation	poor vs medium/well	1.423 (0.928 - 2.182)	0.106		
β2-microglobulin	low vs high	0.762 (0.442 - 1.314)	0.328		
HLA-A	low vs high	0.703 (0.462 - 1.084)	0.112		
HLA-B/C	low vs high	0.822 (0.498 - 1.358)	0.443		
HLA-E	low vs high	0.632 (0.406 - 0.984)	**0.042**	0.612 (0.392-0.956)	**0.031**
Intraepithelial CD8	low vs high	0.682 (0.427 - 1.087)	0.108		
Stromal CD8	low vs high	1.560 (0.962 - 2.530)	0.072	1.613 (0.993-2.620)	0.054
Total CD8	low vs high	1.130 (0.705 - 1.812)	0.659		
HLA-E low	high vs low stromal CD8	0.303 (0.124 - 0.741)	**0.009**		
HLA-E high	high vs low stromal CD8	1.004 (0.550 - 1.835)	0.989		
Stromal CD8 high	high vs low HLA-E	3.282 (1.308 - 8.232)	**0.011**		
Stromal CD8 low	high vs low HLA-E	1.032 (0.585 - 1.818)	0.914		
HLA-B/C high	high vs low total CD8	0.212 (0.074 - 0.606)	**0.004**		
HLA-A and B/C high	high vs low total CD8	0.215 (0.069 - 0.673)	**0.008**		

HLA-E expression is a strong negative determinant for OS.

In more than 70% of pulmonary adenocarcinoma cases a high expression of HLA-E was observed (**Figure 1F and Table 1**). In 20.3% of these tumors displaying a functional HLA-E molecule, no expression of HLA-A and HLA-B/C was observed. The high expression of HLA-E was associated with worse OS (HR 0.632, 95% CI 0.406-0.984, p= 0.042; **Table 2 and Figure 2F)**. Since both stromal CD8+ T-cell infiltration and the expression of HLA-E displayed the strongest effects on overall survival as a single determinant (**Figure 2B and 2F, Supplementary Figure 1**), a subsequent analysis was performed to study the interaction between these two factors. Clearly, a dense stromal CD8+ T cell infiltration showed a strong positive prognostic value in HLA-E negative tumors (HR 0.303, 95% CI 0.124-0.741, p=0.009; **Figure 4A and 4B**). However, this beneficial effect of a dense stromal CD8+ T cell infiltration disappears in patients with high expression of HLA-E (HR 1.004, 95% CI 0.550-1.835, p=0.989; **Figure 4C and 4D**). In conclusion, the beneficial effect displayed by tumor-infiltrating stromal CD8+ T cells is impeded when HLA-E is highly expressed by tumors.

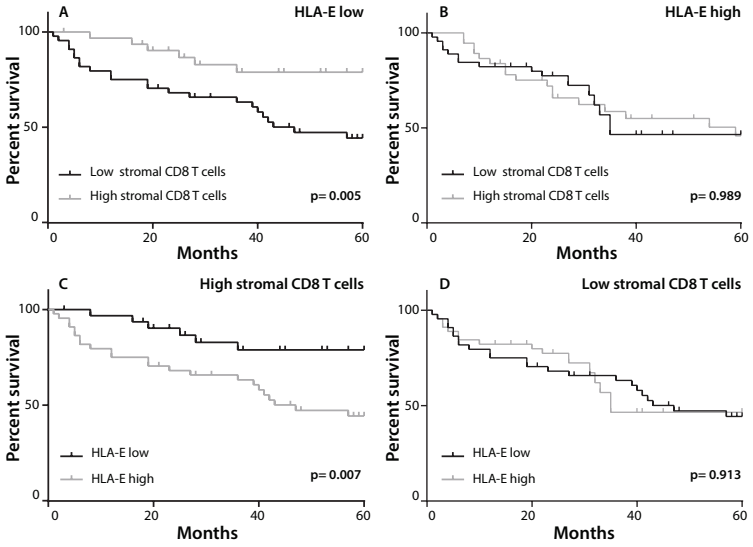

Figure 4. Prognostic benefit in HLA-E negative tumors with high CD8+ T cell infiltration.
Patients were divided in two groups with low or high stromal CD8+ T cell infiltration, based on the mean CD8+ T-cell count for all patients and on the expression of HLA-E. Kaplan-Meier curves were constructed to estimate OS of the two groups whereas the log-rank test was used to compare the difference between the two curves.
(**A)** The effect of CD8+ T cell infiltration in patients with low HLA-E expression (n=77), showing that a high stromal CD8+ T cell infiltration was strongly associated with a better OS.
(**B**) The effect of a high stromal CD8+ T cell infiltrate was neutralized in tumors with high HLA-E expression (n=86).
(**C,D**) Conversely, in patients with high stromal CD8+ T cell influx (n=71), a high HLA-E expression is associated with a worse OS (**C**). In patients with low presence of stromal CD8+ T cells (n=92), HLA-E expression had no effect on OS (**D**).

HLA-E expression is an independent determinant of OS in pulmonary adenocarcinoma.

In order to assess the effect of each single variable on the relative risk of death, univariate and multivariate Cox proportional hazards analysis were performed to quantify survival differences (**Table 2**). Tumor stage and male gender have been reported before as negative risk factors for OS in pulmonary adenocarcinoma[30] and indeed in our cohort high stage tumors (stage I/II vs stage III/IV, HR 0.619, 95% CI 0.399-0.961, p=0.033) as well as male gender (HR 1.834, 95% CI 1.184-2.839, p=0.007) were associated with worse OS. In the univariate analysis, a low expression of non-classical HLA-E by tumor cells was associated with a strong reduced risk of death in this cohort (HR 0.632, 95% CI 0.406-0.984, p=0.042). Presence of high stromal CD8+ T cells correlated with improved OS and reached near-significance (HR 1.560, 95% CI 0.962-2.530, p=0.072) and hence

was included in the multivariate analysis together with tumor stage, gender and HLA-E expression.

Similar to the univariate analysis the positive effect of stromal CD8+ T cells on OS approached statistical significance (HR 1.613, 95% CI 0.993-2.620, p=0.054) in the multivariate analysis. In addition to tumor stage and gender, the increased expression of HLA-E was significantly associated with OS (HR 0.612, 95% CI 0.392-0.956, p= 0.031) indicating that low HLA-E expression is an independent positive prognostic factor for OS in pulmonary adenocarcinoma.

DISCUSSION

The infiltration of NSCLC by CD8+ T cells is positively associated with a longer OS irrespective of tumor stage[10-15] and our study confirms this association of CD8+ T-cell infiltration with better OS. It is important to identify the most important factors governing a successful attack of NSCLC by CD8+ T cells as illustrated by the facts that a) more than 40% of NSCLC patients respond to checkpoint inhibitor therapy [5-7]; and b) especially those patients are likely to respond in whom the tumor has generated neo antigens for CD8+ T cells [31]. One of the key molecules in this process is the expression of HLA molecules required to present tumor-specific peptides to T cells. When measured with a pan-HLA class I antibody, the loss of HLA is observed in almost half of the patients with pulmonary adenocarcinoma[16-19,32]. We used antibodies to distinct the expression of HLA-A and HLA-B/C in order to chart the HLA loss in more detail (**Table 1**). We found that HLA-A was decreased in about 40% of the patients while the decrease in HLA-B/C expression was even as high as 75% which is in line with only one other study that reports specifically on loss of HLA-B/C in NSCLC[33]. In addition, interaction analyses of HLA expression and CD8+ T-cell infiltration led to the novel observation that the prognostic effect of a dense CD8+ T-cell tumor infiltration is only retained when tumors display a high expression of classical HLA class I, in particular HLA-B/C (**Figure 3**).

Other key molecules governing a successful attack of T cells in NSCLC are the so-called checkpoints[34]. The non-classical HLA-E molecule is the ligand for the inhibition receptor CD94/NKG2A and represents an important immunologic checkpoint[24,25]. This study is the first to show that a high expression of the non-classical HLA-E molecule affects overall survival in NSCLC. Both univariate and multivariate analysis revealed high HLA-E expression by tumor cells as an independent predictor of poor prognosis (**Table 2 and Figure 2**). The expression of HLA-E can inhibit the function of T lymphocytes and natural killer (NK) cells when it engages with CD94/NKG2A[24,25,35], as well as activate these cells when HLA-E engages with CD94/NKG2C[36]. A few studies in breast cancer and cervical adenocarcinoma have reported survival benefit for HLA-E expressing tumors[37,38] while

others, similar to us, reported a negative effect of HLA-E on OS in ovarian cancer, colorectal cancer and gastric cancer[27,39-41]. Potentially, the type of receptor for HLA-E expressed by CD8 T cells is at the basis of this difference. In ovarian cancer and colorectal cancer the T cells were shown to express the inhibitory receptor CD94/NKG2A[27,39]. In line with previous studies in NSCLC, a dense stromal CD8+ T-cell tumor-infiltrate was associated with longer OS (**Figure 2 and Supplementary Figure 1**)[10-15]. In our study, a high expression of HLA-E by tumor cells clearly had a negative effect on CD8+ T cells. The positive prognostic effect of stromal CD8+ T cells on OS was only apparent in patients with low expression of HLA-E on their tumor cells. A high tumor expression of HLA-E completely abolished the prognostic effect of CD8+ T-cell infiltrate (**Table 2 and Figure 4**).

Our study on the interaction between tumor expressed HLA and CD8+ T-cell infiltration in relation to survival raises two important issues. Currently, CD8+ T-cell infiltration and expression of PD-L1 are being considered as biomarkers to select the population of NSCLC patients that are most likely to respond to checkpoint antibody therapy[42]. Based on our data showing that the prognostic effect of CD8+ T cells is most pronounced in those tumors still expressing HLA-B/C, one could consider including the analysis of classical HLA class I expression in this selection procedure. The second issue concerns the improvement of current PD-1/PD-L1 therapy or alternative checkpoint treatment strategies in NSCLC. Our results showed that about 70% of the pulmonary adenocarcinomas displayed a high expression of HLA-E (**Table 1**). In view of its effect on both T cells and NK cells, blocking HLA-E and/or its CD94-NKG2A inhibitory receptor may form a valuable target for the immunotherapy of NSCLC. Treatment with anti-NKG2A monoclonocal antibody was shown to overcome HLA-E mediated suppression of anti-tumor cellular cytotoxicity *in vitro*[43,44] and this has resulted in a currently ongoing phase I/II trial in which patients with advanced head and neck cancer are treated with an anti-NKG2A monoclonocal antibody (ClinicalTrials.gov, Identifier: NCT02331875). Potentially, a combination of antibodies to these two different checkpoints may even have a synergistic effect.

In conclusion, our results confirm the pivotal protective role of tumor infiltrating CD8+ T cells in NSCLC and in addition show that their effect is particularly apparent when the tumor cells retain the expression of classical HLA class I and do not express the non-classical molecule HLA-E. These results warrant the inclusion of HLA-A, -B/C and HLA-E as biomarkers to predict the response to immunotherapy and the use of HLA-E or NKG2A blocking antibodies for the treatment of NSCLC.

MATERIALS AND METHODS

Study population

We retrospectively identified 197 patients diagnosed with non-small cell lung cancer (NSCLC), subtype adenocarcinoma, in the Leiden University Medical Center (LUMC) between 2000 and 2013. All patients underwent preoperative staging and were classified as stage I/II NSCLC and subsequently underwent surgical resection of the primary tumor with systematic lymph node dissection. After surgical removal of the tumor and its draining lymph nodes, patients were considered disease free. Tumor tissue, clinical data and follow-up data were collected from all patients. Staging of NSCLC was determined according to the TNM (Tumor, Node, Metastasis) classification using the updated guidelines of the International Association for the Study of Lung Cancer (IASLC)[45]. The use of archival tumor blocks was in accordance with guidelines from the Dutch Federation of Medical Research Association. Since this retrospective study does not fall under the scope of the Medical Research Involving Human Subjects Act (WMO), it was not subject to a prior review by a Medical Ethical Committee and written informed consent was not obtained. However, patient data were anonymized.

Antibodies

Mouse monoclonal antibodies HCA-2 (anti HLA-A, 1:1000) and HC-10 (anti HLA B/C, 1:500) were used to detect expression of the free heavy chain of the HLA class I molecule. Rabbit anti-human β2-microglobulin (anti-β2M; clone A-072, DAKO, 1:2000) and mouse anti-human HLA-E (clone MEM-E/02; Serotec, Germany [1:200]) antibodies were used in order to detect the light chain and non-classical HLA-E heavy chain respectively. Mouse monoclonal CD8 antibody (clone IA5, Leica Biosystems, Germany [1:500]) was used for the detection of the CD8+ T-cells.

Immunochemistry

Formalin-fixed, paraffin embedded tumor blocks were cut in 4 µm sections using a microtome and deparaffinized in xylene. The endogenous peroxidase activity was blocked for 20 minutes using 0.3% hydrogen peroxide/methanol. The samples were subsequently rehydrated in 70% and 50% ethanol and antigen retrieval was performed by heating the samples to 97 °C for 10 minutes in citrate buffer (either pH 9.0 or pH 6.0, DAKO, Glostrup, Denmark). Antibodies were diluted in phosphate buffered saline (PBS, Fresenius Kabi Bad Homburg, Germany) with 1% bovine serum albumin (BSA) and incubated overnight at room temperature. The slides were stained immunohistochemically with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (DAKO envision) for 30 minutes at room temperature. NovaRed (Vector, Burlingame, USA) was applied as a chromagen followed

by counterstaining with Mayer's hematoxylin (Klinipath). All washing steps were done with PBS. All slides were mounted with Pertex mounting medium (HistoLab, Sweden).

The microscopic evaluation and analysis of the HCA2, HC10, β2M and HLA-E staining was performed by two independent observers without prior knowledge of clinical or histopathological parameters (observer one 100% of the cohort, observer two 20% of the cohort). The inter-observer agreement was assessed by calculating Cohen's kappa coefficient resulting in a coefficient of >0.70 for all stainings which indicates a substantial inter-observer agreement.

The grade of tumor differentiation was determined and classified as either poorly differentiated, moderately differentiated or well differentiated based on the immunohistochemically stained slides. Expression patterns of the previously mentioned antibodies were assessed according to the scoring system proposed by the Ruiter *et al*[46]. Using this method the entire slide is screened and the percentage of positive tumor cells was classified as: absent 0%, sporadic 1-5%, local 6-25%, occasional 25-50%, majority 51-75% and large majority 76-100% (1-6). Furthermore, this score includes intensity of the staining which is classified as negative, low, medium and high (0-3). The intensity was noted for all antibodies with the exception of CD8 since high intensity was always observed. The final score was based on both intensity and percentage and was categorized as 1-4 (low expression) and 5-9 (high expression).

Quantification of infiltrating CD8+ T-cells

CD8+ T-cell infiltration was assessed by screening five randomly captured high resolution (200X) images of each slide. The area of the tumor nests and stromal areas were marked and calculated using NIH-ImageJ software (v1.48). CD8+ T cells were counted by area and represented as the number of cells per mm2 of tumor area with a distinction between intraepithelial and stromal CD8+ T cells. The mean number of intraepithelial, stromal and total number of tumor-infiltrating CD8+ T cells were calculated and patients were dichotomized for high or low CD8+ T cell infiltration based on the mean CD8+ T cell infiltration for all patients.

Statistical analysis

Nonparametric Mann–Whitney *U* test was used to compare continuous variables between patient groups and group comparisons of categorical data were performed by two-tailed χ^2 test. Overall survival (OS) was defined as date of surgery until date of death due to any cause, or date of last follow-up with a maximum follow-up time of 5 years. When assessing survival based on HLA expression, low and high expression of HLA indicates the presence of a functional HLA molecule, i.e high expression of both β2M and the HLA heavy chain of HLA-A, HLA-B/C and HLA-E respectively. Survival was estimated by using Kaplan–Meier methodology and the log-rank test was used to compare the

two curves. Univariate Cox proportional hazards model was used to study the effect of single determinants on OS. Multivariate Cox regression analysis was performed with variables that reached statistical significance in univariate analysis. Stepwise regression was employed to estimate the final model. Two-sided P values of <0.05 were considered statistically significant. Bonferroni correction was applied for multiple testing. Statistical software package SPSS 20.0 (SPSS, Chicago, IL) was used for data analysis. GraphPad Prism 6.02 (Graphpad Software, LA Jolla, CA) was used to estimate survival curves.

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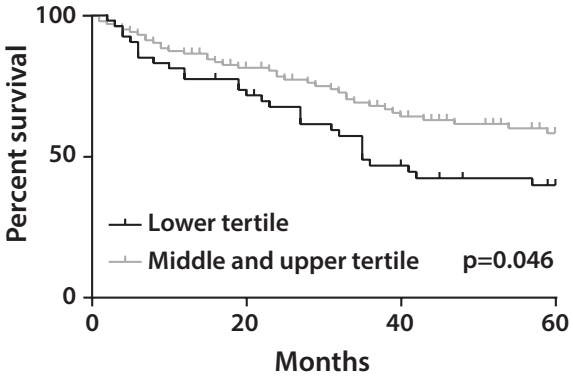
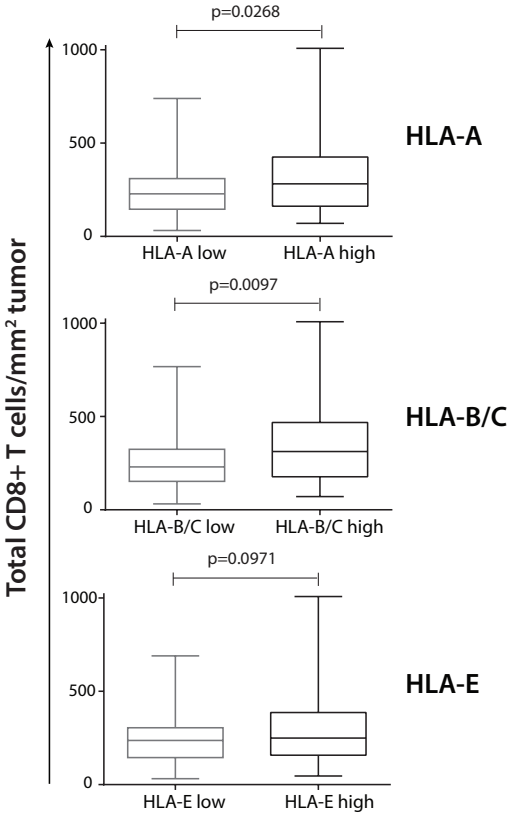
Supplementary Table 1. Relationship of tumor characteristics with HLA expression and CD8+ T cell expression in pulmonary adenocarcinoma. Significant results (p<0.050) are indicated in bold.

	HLA-A		P value	HLA-B/C		P value	HLA-E		P value
	High	Low		High	Low		High	Low	
Stage									
I	36	26	0.621	17	45	0.872	44	18	0.777
II	43	31		16	58		56	18	
III	16	19		9	26		25	10	
IV	15	11		7	19		17	9	
β2-M									
Low	18	29	**0.007**	8	39	0.179	32	15	0.576
High	92	58		41	109		110	40	
HLA-A									
Low				6	81	**0.0001**	60	27	0.426
High				43	67		82	28	
HLA-B/C									
Low							106	42	0.856
High							36	13	
Total CD8+									
Low	41	55	**0.012***	16*	80	**0.018***	64	32	0.480*
High	23			25	43		53	15	
CD8+ in stroma									
Low	45	47	0.819*	19	73	0.444*	66	26	0.990*
High	41	30		22	49		51	20	
CD8+ in tumor									
Low	50	54	0.426*	21	83	0.186*	68	36	0.057*
High	36	23		20	39		49	10	

* Bonferroni corrected p value



Supplementary Figure 1. Tertile based grouping of stromal CD8+ T cells and influence on OS.
The patients were grouped based on the CD8+ T cell counts/mm^2 tumor. Patients with CD8 T cell counts belonging to the lower tertile were categorized as low (n=54) whereas patients with counts in the middle and upper tertile were categorized as high (n=109). Kaplan-Meier curves were used to estimate OS whereas the log-rank test was used to compare the differences between the two curves (p=0.046).



Supplementary Figure 2. HLA expression and its relation with total CD8+ T cell infiltration in the primary tumor.

The association between the total CD8+ T cell count per mm^2 tumor and expression of HLA-A (low vs high, n=106 vs n=91), HLA-B/C (low vs high, n=156 vs n=41) and HLA-E (low vs high, n=87 vs n=110) was assessed. Tumors expressing classical HLA are infiltrated with higher numbers of CD8+ T cells (Mann–Whitney *U* test, $p<0.05$).

Chapter 6

Standard radiotherapy but not chemotherapy impairs systemic immunity in non-small cell lung cancer

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ABSTRACT

Introduction

Advanced non-small cell lung cancer (NSCLC) is traditionally treated with platinum-based chemotherapy and radiotherapy. Since immunotherapy holds promise for treating advanced NSCLC, we assessed the systemic effects of the traditional therapies for NSCLC on immune cell composition and function.

Methods

Eighty-four pulmonary adenocarcinoma patients, treated either with chemotherapy or radiotherapy, were studied. A prospective study of 23 patients was conducted in which the myeloid and lymphoid cell compartments of peripheral blood were analyzed. Changes in cell populations were validated in a retrospective cohort of 61 adenocarcinoma patients using automated differential counts collected throughout therapy. Furthermore, the functional capacity of circulating T cells and antigen-presenting cells (APC) was studied. Blood samples of healthy individuals were used as controls.

Results

In comparison to healthy controls, untreated adenocarcinoma patients display an elevated frequency of myeloid cells coinciding with relative lower frequencies of lymphocytes and dendritic cells. Standard chemotherapy had no overt effects on myeloid and lymphoid cell composition nor on T-cell and APC-function. In contrast, patients treated with radiotherapy displayed a decrease in lymphoid cells and a relative increase in monocytes/macrophages. Importantly, these changes were associated with a reduced APC function and an impaired response of T cells to recall antigens.

Conclusions

Platinum-based standard of care chemotherapy for NSCLC has no profound negative effect on the immune cell composition and function. The negative effect of prolonged low-dose radiotherapy on the immune system warrants future studies on the optimal dose and fraction of radiotherapy when combined with immunotherapy.

INTRODUCTION

Conventional therapies for advanced non-small cell lung cancer (NSCLC) consist of platinum-based chemotherapy combined with a third generation cytotoxic drug (stage IV) or concurrent chemoradiotherapy (stage III) [1,2]. The traditional view that these standard of care therapies cause immunosuppression and therefore cannot be combined with new and promising forms of immunotherapy for advanced NSCLC, such as immune checkpoint antibodies targeting the programmed death 1 (PD-1) receptor and its ligand PD-L1[3], has been challenged in recent years[4-7]. Next to their direct cytotoxic effect, platinum-based compounds can also promote anti-cancer immune responses, for example by enhancing T-cell activation by dendritic cells (DCs) as part of immunogenic cell death. Furthermore, platinum chemotherapy manipulates the tumor microenvironment by sensitizing tumor cells to cytotoxic T cell (CTL)-mediated attack or by upregulation of MHC class I[8,9]. Radiotherapy has various immunomodulatory effects such as promoting DC maturation[10], local production of type-I interferon[11] as well as upregulation of MHC class I and tumor-specific antigens[12], all of which support tumor-specific effector T cells. These insights on the immune-potentiating effects of both chemotherapy and radiotherapy have led to the rationale that combining these conventional anti-cancer therapies with new forms of T cell-based immunotherapies can have synergistic effects and, possibly, result in improved clinical outcomes[4-7].

However, the beneficial effects of chemotherapy and radiotherapy on anti-tumor immunity are mainly documented in animal models and *in vitro* studies. Information on how these therapies affect the human immune system is lacking, in particular in NSCLC patients. To address this, patients with advanced pulmonary adenocarcinoma, the most prevalent subtype of NSCLC[13], treated with platinum-based chemotherapy or chemoradiotherapy were analyzed for the effect of therapy on myeloid and lymphoid cell composition in the circulation. Furthermore, the functionality of circulating T cells and antigen-presenting cells (APC) was investigated upon standard of care therapy. Our study reveals that, while standard platinum-based chemotherapy does not affect immune cell composition and function, radiotherapy has a negative effect on the number and function of circulating T cells and APCs.

MATERIALS AND METHODS

Patients and tissue collection

Between March 2011 and April 2014, a prospective study was conducted in patients with histologically proven pulmonary adenocarcinoma, who were treated at the outpatient clinic of the Department of Pulmonology from three hospitals (Leiden University Medical

Center, Alrijne Ziekenhuis and Groene Hart Ziekenhuis). Patients were grouped according to their received therapy. Patients in the first group (carboplatin-vinorelbine) were treated with a 21-day chemotherapy cycle in which they were administered carboplatin (AUC 5 regimen, depending on renal function) and vinorelbine (25 mg/m^2) on day 1, as well as vinorelbine (25 mg/m^2) monotherapy on day 8. The second group (carboplatin-pemetrexed) was composed of patients treated with carboplatin (AUC 5 regimen) and pemetrexed (500mg/m^2) on day 1. In both groups, at least three cycles of chemotherapy were administered every 3 weeks. Patients in the last group (radiotherapy) received a 5-week cycle of concurrent chemoradiotherapy in which a daily radiation dose of 2.75 Gy (delivered in 24 fractions) was preceded by low-dose cisplatin (6 mg/m^2) as a radiosensitizer. From all patients, venous blood samples were collected prior to treatment with chemotherapy or radiotherapy (baseline) and at least 14 days after cessation of therapy (post therapy). From all blood samples, pperipheral blood mononuclear cells (PBMC) were isolated by Ficoll-density centrifugation according to standard operating procedures (SOP) [14] and used for analysis of recall antigen T cell response, mixed lymphocyte reaction (MLR) and flow-cytometric phenotyping.

A retrospective cohort of pulmonary adenocarcinoma patients, treated between 2008 and 2014 with at least three chemotherapy cycles of 21 days (carboplatin-vinorelbine and carboplatin-pemetrexed), was analyzed by collecting automated differential counts from blood samples before start of therapy (baseline), at two time points during the 21-day chemotherapy cycle at week 2 and week 3, and at least 14 days after cessation of therapy (post therapy). From patients treated with a 5-week cycle of radiotherapy, we retrieved automated differential counts at baseline, after each week during the 5-week cycle and at least 14 days after radiotherapy was completed (post therapy). Based on the analysis of this historical cohort, we included an additional five adenocarcinoma patients from whom blood samples were taken at baseline, at week 2 and week 3 of the 21-day chemotherapy cycle, for phenotyping and functional analyses.

Analysis of recall antigen T cell response

The capacity of T cells to proliferate upon stimulation with recall antigens was assessed in a 3-day proliferation assay with memory response mix (MRM), composed of tetanus toxoid (Netherlands Vaccine Institute), tuberculin purified protein derivative (Netherlands Vaccine Institute), and Candida (HAL Allergenen Lab), as previously published by our research group [15]. Briefly, cryopreserved PBMCs were thawed and in triplicate wells (1.0 x 10^5 cells/well) exposed to three conditions: medium control (90% IMDM supplemented with 10% human AB serum (PAA laboratories, Pasching, Austria)), MRM and Phytohaemagglutinin (PHA, 0.5 µg/mL), which was used as a positive control. Proliferation was measured by ^{3}H-thymidine incorporation during the last 18 hours of the assay. A positive response was defined as equal or above a stimulation index (SI) of 3.

This SI was calculated by dividing the mean counts of stimulated wells by that of the medium control wells.

Mixed lymphocyte reaction (MLR)

To assess the antigen presenting capacity of patient PBMCs, a mixed lymphocyte reaction (MLR) was performed according to SOP[15]. Thawed patient PBMCs were irradiated at 3000 rad to prevent proliferation of cells. Next, 1 x 10^5 patient cells/well were seeded in medium in four replicate wells and co-cultured for 7 days with HLA-mismatched third party non-irradiated lymphocytes (1 x 10^5 cells/well) isolated from buffy coats obtained from healthy donors. Proliferation was measured by ^{3}H-thymidine incorporation during the last 18 hours of the assay. As negative controls, irradiated patient PBMCs alone and third party non-irradiated PBMCs alone were used. A positive response was defined as a SI index of at least 3, which was calculated as the mean counts in wells containing irradiated patient PBMCs co-cultured with third party lymphocytes divided by the mean counts of unstimulated third party lymphocytes.

Flow cytometric phenotyping of PBMCs

Flow cytometry was used to assess the composition of patient PBMCs before, during and after treatment. PBMCs of eight healthy donors were analyzed for comparison. PBMCs were thawed, washed and stained according to SOPs[14,16] for the T-cell markers by using antibodies against CD3 (Pacific Blue; DAKO, Glostrup, Denmark), CD4 (PE-CF594; BD Biosciences, San Jose, CA, USA) CD8 (APC-Cy7; BD) and inhibitory T cell markers PD-1 (BV-605, BioLegend, San Diego, CA, USA), NKG2A (AF700, BD), TIM3 (PE, BioLegend) and CTLA-4 (PE-Cy7; BD). Furthermore, in parallel PBMCs were stained with a set of monocyte/macrophage and dendritic cell (DC) markers. This set was comprised of antibodies against CD1a (FITC; BD), CD3 (Pacific Blue; DAKO), CD11b (PE, BD), CD11c (Alexa Fluor 700; BD), CD14 (PE-Cy7; BD), CD16 (PE-CF594; BD), CD19 (Brilliant Violet 605; BD), CD45 (PerCP-Cy5.5; BD), CD163 (APC; R&D Systems, Minneapolis, MN, USA), CD206 (APC-Cy7; BioLegend) and HLA-DR V500 (BD;). Finally, in a separate experiment PBMCs were stained for regulatory T cell (Treg) markers CD3 (V500, BD), CD4 (AF700, BD), CD25 (PE-Cy7, BD), CD127 (BV650, BD), Foxp3 (PE-CF594, BD), Ki67 (FITC, eBiosciences), Helios (APC, Biolegend) and CD45RA (APC-H7, BD). For each sample 1,000,000 events were acquired by flow cytometry (BD LSR Fortessa). Data analysis was performed with BD FACSDiva software (version 6.2) using a gating strategy that was recently published by our research group[15,16]. This strategy is shown in **Supplementary Figure 1**.

Tregs were classified into three subtypes according to a recent paper by Santegoets et al.[17]: Def 1: CD4+ CD25+ CD127low Foxp3+; Def 1 (activated Tregs): CD4+ CD25+ CD127low Foxp3+Ki67+; Def 2: CD4+ CD25+ CD127low Foxp3+ Helios+; Def 3a: Foxp3high CD45RA- (activated Treg) ; Def 3b: Foxp3int CD45RA+ (naïve Treg). Myeloid and

lymphoid cells were defined using the myeloid and lymphoid cell gate within the CD45+ gate. Monocytes/macrophages were defined as CD45+ HLA-DR+ CD1a- CD14+ CD11b+, with subsets of M1 (CD206-CD163-), M2a (CD206+CD163-) and M2c (CD206-CD163+) monocytes/macrophages. DCs were defined as CD45+ HLA-DR+ CD1a- CD11b-CD14-CD11c+CD206-CD163. Myeloid derived suppressor cells (MDSCs) were characterized in the myeloid cell population according to recent literature describing ten separate MDSC subtypes[18].

Statistical analysis

The Mann-Whitney U test, a non-parametric test for independent samples, was used for statistical comparison of untreated pulmonary adenocarcinoma patients and healthy control PBMCs with regards to lymphoid and myeloid cell populations. For the patients who underwent chemotherapy or radiotherapy, the Wilcoxon signed-rank test (non-parametric test for paired samples) was used to compare recall antigen T-cell response and MLR at baseline and post therapy. This test was also used for analysis of T cell, monocyte/macrophage and DC markers throughout therapy. Percentages within the CD45+ gate (monocytes/macrophage and DC markers) or the lymphoid gate (T cell markers) were used in non-parametric testing. A p-value < 0.05 was considered statistically significant. Statistical software package SPSS 20.0 (SPSS, Chicago, IL, USA) was used for data analysis.

Ethical approval

The study protocol was submitted to and approved by the Medical Ethical Committee of the Leiden University Medical Center (P10.187). All patients gave written informed consent prior to participation.

RESULTS

Twenty-three pulmonary adenocarcinoma patients (14 males, 9 females) with a median age of 61 years (range 48-79 years) were prospectively analyzed. Ten patients were treated with carboplatin-vinorelbine, seven patients received carboplatin-pemetrexed treatment and six patients underwent radiotherapy. The group of 8 healthy controls, who supplied donor PBMCs consisted of 7 females and 1 male with a median age of 55 years (range 38-67 years). A summary of patient characteristics is displayed in **Table 1**.

Table 1. Summary of patient characteristics

Number of patients with pulmonary adenocarcinoma	n=23
Age, median (range)	61 years (48-79 years)
Gender, Male/Female	14/9
Stage	
I/II	n=2
III	n=11
IV	n=10
Treatment	
Carboplatin-vinorelbine	n=10
Carboplatin-pemetrexed	n=7
Radiotherapy	n=6

Pulmonary adenocarcinoma patients display cancer-driven changes in lymphoid and myeloid cell frequency

First, baseline differences in circulating myeloid and lymphoid cell populations between untreated pulmonary adenocarcinoma patients and healthy controls were analyzed (**Figure 1**). Patients showed a significantly elevated level of myeloid cells coinciding with a lower frequency of circulating lymphoid cells (**Figure 1A**) independent of their gender. Although no difference was observed between patients and healthy controls with respect to CD4+ and CD8+ T cell frequency gated as percentage of the lymphoid gate (**Figure 1B**), a lower frequency of CD4+ T cells was noted in adenocarcinoma patients when gated as percentage of the CD45+ gate, and a similar trend was seen for CD8+ T cells, fitting with the observation that the percentage of lymphoid cells is decreased while the percentage of myeloid cells is increased (**Supplementary Figure 2**). Subset analysis of the myeloid cell populations, demonstrated a higher percentage of CD11b+/CD14+ monocytes/macrophages (**Figure 1C**) and a lower percentage of CD11b-CD14-CD11c+CD206-CD163- dendritic cells (DCs) (**Figure 1D**) in the PBMC of the patients when compared to that of healthy donors. No difference was observed with respect to MDSC frequency and expression of inhibitory T cell markers between adenocarcinoma patients and healthy donors (**Supplementary Figure 3**). Thus, adenocarcinoma patients show a tumor-associated abnormal level of myeloid cells which is mainly caused by increased numbers of monocytes/macrophages while in parallel the frequency of circulating lymphoid cells and dendritic cells is lower.

Changes in immune cell composition after standard of care treatment for NSCLC

Next, the effect of standard of care NSCLC treatment modalities on immune cell composition was assessed. In the patient group treated with carboplatin-vinorelbine (n=5),

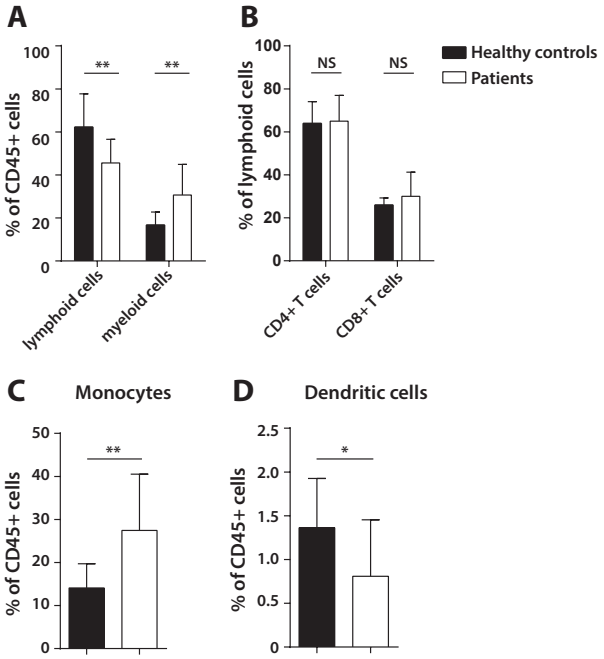

Figure 1. Baseline differences in lymphoid and myeloid cells in healthy controls and pulmonary adenocarcinoma patients

Flow-cytometric phenotyping of peripheral blood mononuclear cells (PBMCs) of 23 untreated pulmonary adenocarcinoma patients and 8 healthy donors was performed to analyze myeloid cells, lymphoid cells, CD4+ and CD8+ T cells, monocytes/macrophages (CD45+HLA-DR+ CD1a-CD11b+/CD14+) and dendritic cells (CD11b-/CD14- CD11c+/CD206-/CD163-) using a gating strategy presented in **Supplementary Figure 1**. Shown are lymphoid and myeloid cells (**A**), CD4+ and CD8+ T cells as percentage of the lymphoid gate (**B**), monocytes/macrophages (**C**) and dendritic cells (**D**) as percentages of the CD45+ gate. Data is shown as mean with standard deviation (SD) and Mann-Whitney U test was used for statistical analysis (** $p < 0.01$, * $p < 0.05$, NS = non-significant).

treatment did not result in overt changes in the percentage of lymphoid and myeloid cells. However, subset analysis of the myeloid cell population showed that carboplatin-vinorelbine treatment caused an increase in DCs and a decrease in monocytes/macrophages (**Figure 2A**). Treatment with carboplatin-pemetrexed (n=7) similarly had no profound effect on the composition of the myeloid and lymphoid cell compartments. Here, a treatment-related increase was observed for both the percentage of monocytes/macrophages and the percentage of DCs (**Figure 2B**). The treatment of patients with radiotherapy (n=6) resulted in a slight decrease in the percentage of lymphoid cells and a concomitant increase in myeloid cells, which was not likely to be associated with more extensive irradiation as there is no relation between the irradiated tumor volume and extent of lymphopenia in this small group of patients (**Supplementary Figure 4)**. The lower frequency of lymphocytes as compared with healthy donors remained after

cessation of radiotherapy. An increase of the percentage of monocytes/macrophages was observed (**Figure 2C**). All three treatment groups did not display changes in the frequency of T cells expressing inhibitory markers (**Supplementary Figure 3**) or Tregs (**Supplementary Figure 5**). Patients treated with radiotherapy displayed a significant increase in the frequency of MDSC type 4 (CD14+ HLA-DR^{low}) and MDSC type 7 (CD14+ CD33+ HLA-DR^{low}) cells (**Supplementary Figure 3**).

Standard of care NSCLC therapies cause transient and permanent changes in absolute lymphocyte and monocyte counts during therapy

To validate these observations and to obtain more detailed insight into the changes in immune cell composition during treatment, we analyzed the lymphocyte and monocyte counts in a historical cohort of pulmonary adenocarcinoma patients for whom automated leucocyte differential counts were retrieved (**Figure 3**). In 22 patients, who were treated with carboplatin-vinorelbine (**Figure 3A**), a small drop in absolute lymphocyte cell counts from baseline (mean 1.87 x 10^9 cells) was observed in week 2 of the first cycle (cycle 1.1, mean 1.41 x 10^9 cells, $p<0.05$), which normalized at week 3 within the same cycle (cycle 1.2, mean 1.63 x 10^9 cells, $p<0.01$). Lymphocyte counts remained stable during therapy, and there was no effect on absolute lymphocytes counts post therapy when compared to baseline (mean 1.61 x 10^9 cells, $p=0.98$). On the other hand, carboplatin-vinorelbine treatment caused a strong decline in absolute monocyte counts from baseline (mean 0.81 x 10^9 cells), which is evident in week 2 of the first cycle (cycle 1.1, mean 0.22 x 10^9 cells, $p<0.001$), but almost recovers a week later during the same 21-day cycle (cycle 1.2, mean 0.57 x 10^9 cells, $p<0.001$), only to drop again sharply in the next chemotherapy cycle (cycle 2.1, mean 0.09 x 10^9 cells, $p<0.05$). After therapy cessation, absolute monocyte cell counts show a quick recovery nearly to the baseline level (mean 0.75 x 10^9 cells, $p<0.05$), explaining the results of our prospective patient group (**Figure 2A**). The analysis of 16 patients treated with carboplatin-pemetrexed showed no changes in absolute numbers of lymphocytes and monocytes during or after the course of chemotherapy (**Supplementary Figure 6**).

Finally, 23 adenocarcinoma patients treated with radiotherapy were analyzed (**Figure 3B**). The baseline absolute lymphocyte numbers (mean 1.88 x 10^9 cells) showed a significant decline during the course of radiotherapy, which persisted in the last week of the 5-week treatment cycle (Cycle 5, mean 0.49 x 10^9 cells, $p<0.001$) and despite a recovery after completion of treatment, the total lymphocyte counts remained lower than baseline (post therapy, mean 0.96 x 10^9 cells, $p<0.01$). Baseline monocyte counts (mean 0.68 x 10^9 cells) generally remained stable during radiotherapy treatment, with only a small drop observed after week 5 (Cycle 5, mean 0.44 x 10^9 cells, $p<0.05$). This modest effect disappeared after treatment (post therapy, mean 0.67 x 10^9 cells, $p=0.90$). The absolute loss in lymphocytes and stable number of monocytes explains the treatment-

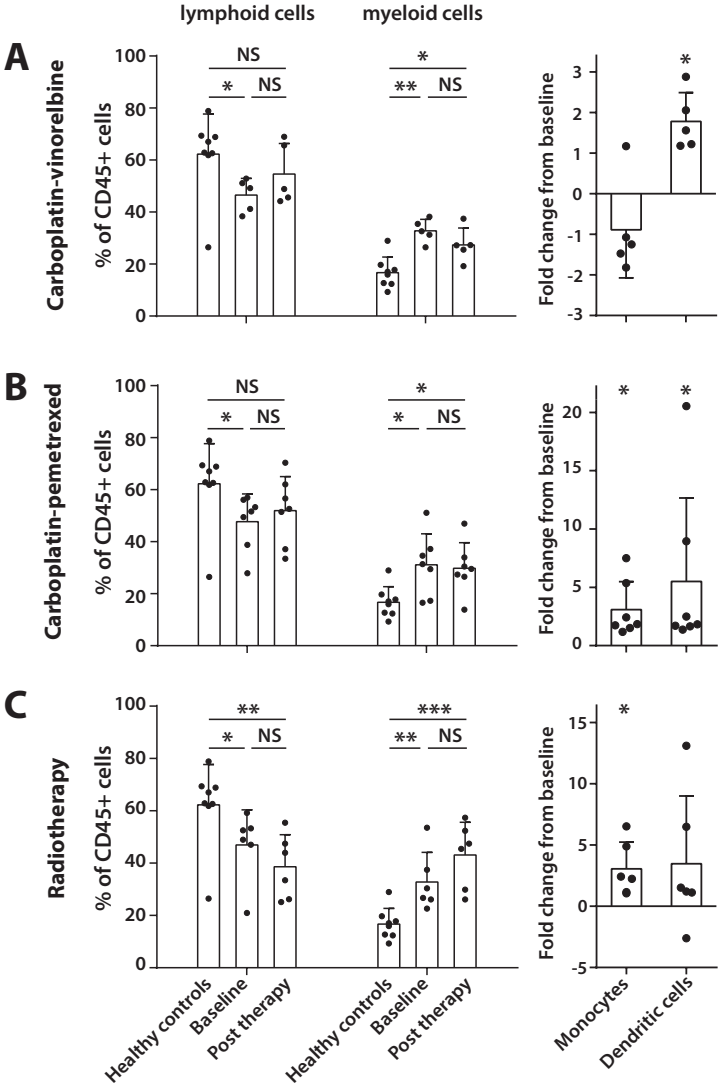

Figure 2. Effect of standard of care therapies for advance pulmonary adenocarcinoma on lymphoid and myeloid cell populations

Peripheral blood of 18 pulmonary adenocarcinoma patients was analyzed for lymphoid and myeloid cell composition (depicted as percentages of the CD45+ gate) as well as changes in monocyte/macrophage (CD14+ CD11b+) and dendritic cell (CD11b-CD14-CD11c+CD206-CD163-) frequency. This was done prior to treatment (baseline) and at least 14 days after cessation of therapy (post therapy). Three treatment groups were assessed: carboplatin-vinorelbine (**A**, n=5), carboplatin-pemetrexed (**B**, n=7) and radiotherapy (**C**, n=6). Healthy donor PBMC were taken along for comparison. The fold change of post therapy from baseline is given for the patients concerning the monocyte/macrophage and dendritic cell frequencies. Data is shown as mean with SD and Mann-Whitney U test (healthy controls compared to patients at baseline) and Wilcoxon signed rank test (patients at baseline compared to post therapy) were used for statistical analysis (*** $p<0.001$, ** $p< 0.01$, * $p< 0.05$, NS non-significant).

related relative drop in the lymphocyte to monocyte ratio observed in our prospective patient group (**Figure 2C**).

Radiotherapy impairs recall T-cell responses and APC function in patients with pulmonary adenocarcinoma

Subsequently, we tested the impact of these therapies on the stimulatory capacity of APCs and on the responsiveness of T cells to antigenic stimulation (**Figure 4**). The APCs isolated from patients, who were treated with either carboplatin-vinorelbine or carboplatin-pemetrexed, displayed a good capacity to stimulate allogeneic lymphocytes both at baseline and after chemotherapy in a mixed lymphocyte reaction (MLR) assay. Moreover, none of the chemotherapy schedules influenced the T-cell response to a mix of recall antigens (MRM). In addition, T-cell reactivity to mitogenic stimulation with PHA was strong at baseline and not affected by chemotherapy (**Figure 4A and 4B**). Based on the marked decrease in absolute monocyte counts within the 21-day carboplatin-vinorelbine treatment (**Figure 3A**), we prospectively collected PBMCs from five additional patients at three time points (baseline, week 2 and week 3) during the course of their chemotherapy cycle (**Supplementary Figure 7**). In these five patients, the absolute decrease in monocytes observed in their leucocyte differential counts (**Supplementary Figure 7A**) was reflected by a relative decrease in myeloid cells ($p<0.05$) and a concomitant increase in lymphoid cells ($p=0.06$) (**Supplementary Figure 7C**). The sharp decline in myeloid cells was mirrored by a drop in monocytes/macrophages. Analysis of M1/M2a/M2c monocytes/macrophages showed a similar decline in all subsets (**Supplementary Figure 7C)**. The observed changes in myeloid cells did not lead to overt changes in the response of T cells to recall antigens or that of APC to stimulate allogeneic T cell reactivity (**Supplementary Figure 7B**).

In contrast, the T cells of patients, who were treated with radiotherapy, demonstrated a significant decline in their reactivity to recall antigens (**Figure 4C**, mean baseline MRM response SI index 4.4, post therapy mean MRM response 1.5, $p<0.05$). This decline in T cell reactivity coincided with a weaker MLR response post radiotherapy (mean SI index 7.9) compared to baseline (mean SI index 16.0, $p<0.05$). Since the PHA-stimulated T cells showed a good proliferative response before (mean SI index 106.4) and after radiotherapy (mean SI index 128.2), reflecting an intact intrinsic capacity of T cells to proliferate, our data suggest that the negative effect of radiotherapy upon the proliferative capacity of PBMCs to stimulation with recall antigens might be related to a hampered APC function.

Taken together, standard of care chemotherapy schedules for pulmonary adenocarcinoma have no profound effect on both the levels and function of myeloid and lymphoid cells in the peripheral blood. The negative effect of radiotherapy on the recall T-cell response can not be explained by the absolute decrease in lymphoid cells only but is also

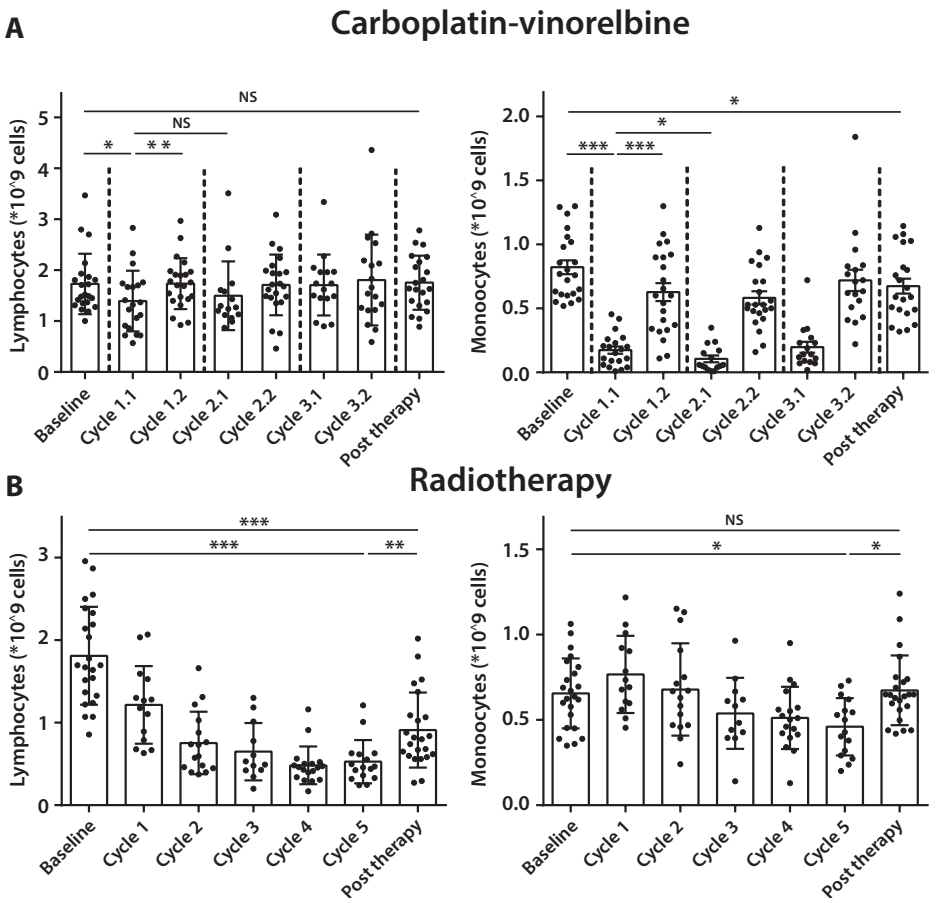

Figure 3. Standard of care therapies for pulmonary adenocarcinoma cause transient and permanent changes in absolute lymphocyte and monocytes counts during therapy.

From 22 pulmonary adenocarcinoma patients treated with at least three cycles of carboplatin-vinorelbine (**A**), automated leucocyte differential counts were retrospectively collected at two time points (at week 2 and week 3) during a 21-day cycle of chemotherapy. Weekly automated leucocyte differential counts were also collected from 23 patients treated with a 5-week cycle of radiotherapy (**B**). From all patients, counts were retrieved at baseline and post therapy. Results are presented as mean lymphocyte and monocyte counts (per 10^9 cells) with SD. Wilcoxon signed rank test was used for statistical analysis (*** $p<0.001$, ** $p< 0.01$, * $p< 0.05$, NS non-significant)

related to the impaired APC function which might be fostered by the relative increase in monocytes/macrophages.

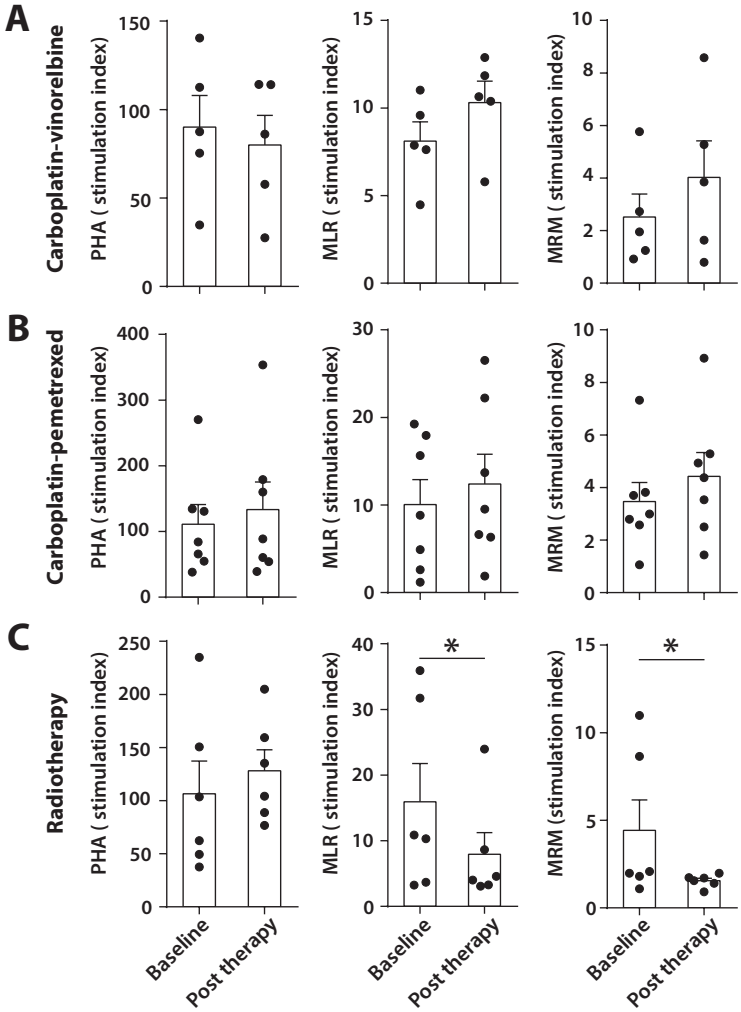

Figure 4. Effect of standard of care pulmonary adenocarcinoma therapies on T cell and APC function
PBMCs of 18 patients with pulmonary adenocarcinoma were tested for the capacity of T cells to proliferate upon stimulation with phytohaemagglutinin (PHA) or with recall antigens (memory response mix, MRM). Antigen-presenting cell (APC) function was tested by mixed leucocyte reaction (MLR). Three treatment groups were investigated: carboplatin-vinorelbine (**A**, n=5), carboplatin-pemetrexed (**B**, n=7) and radiotherapy (**C**, n=6). Data is shown as mean stimulation index (SI) at baseline and at least 14 days after cessation of therapy (post therapy). Wilcoxon signed rank test was used for statistical analysis (*** p<0.001, ** p< 0.01, * p< 0.05, NS non-significant)

DISCUSSION

In the present study, we demonstrate that treatment of advanced pulmonary adenocarcinoma patients with radiotherapy has several negative effects on circulating immune

cells. The most striking observation is a persistent drop in lymphocyte counts during treatment, which coincides with a decline in reactivity of T cells to recall antigens after completion of radiotherapy. Previously, lymphocytopenia has been observed as a side-effect of radiotherapy [19,20] and a negative association of lymphocytopenia with OS has been reported, both for NSCLC and other cancer types[21,22]. Spearman correlation analysis failed to show a relation between the tumor volume irradiated and the observed lymphocytopenia (Supplementary Figure 4), however this might be due to a small sample size (n=6). The fact that radiotherapy aimed at smaller target volumes can reduce lymphocytopenia in cancer patients is well known and discussed in more detail elsewhere[23].

Whether radiotherapy also affects lymphocyte function has been less well described in cancer patients. Radiotherapy has been shown to negatively affect T-cell responses to recall antigens in testis carcinoma patients who received adjuvant localized radiotherapy[24] and in patients with Hodgkin's disease treated with mantle field irradiation[25]. This study is the first to report a negative functional effect of radiotherapy with respect to circulating T cells in pulmonary adenocarcinoma patients. This effect is unlikely to be caused by infusion with low-dose cisplatin (6 mg/m^2) that preceded radiotherapy fractions, since cisplatin has been shown to stimulate anti-tumor immunity, in particular by the induction of tumor-specific CD8+ T cells, by promoting infiltration of inflammatory APCs harbouring T-cell costimulatory ligands into the tumor, and by improving cure rates when combined with long peptide vaccines[26,27]. The detrimental effect on APC function by radiotherapy before and after therapy (**Figure 4**) could in part explain this decline in recall-directed T-cell function. Studies on the association of radiotherapy with antigen-presenting capacity of PBMC in NSCLC patients are scarce. Only one study has reported a decline in MLR response and a concomitant decline in PHA-stimulated T-cell proliferation in NSCLC patients who underwent radiotherapy[28]. Our finding that radiotherapy hampers APC stimulatory capacity, potentially explained by decreased APC function or by the suppressive effect of MDSC subtypes which are present at higher frequencies after radiation, contradicts data from mouse studies reporting a beneficial role of radiotherapy with respect to DC maturation and activation[4,6]. For example, one study in C57BL/6 mice bearing B16gp melanoma tumors demonstrated that local irradiation with a single high dose of 10 Gy resulted in upregulation of CD70 and CD86 on local DCs, both of which are costimulatory molecules involved in T-cell priming[10]. In contrast, NSCLC patients in our study received a daily dose of 2.75 Gy with a total of 24 fractions, amounting to a total dose of 66 Gy during the course of 5 weeks. One possible explanation for the discrepancy between our results and findings from mouse studies is that a single high dose or a few fractions of high-dose radiotherapy are needed to recruit circulating immune cells to the tumor site, whereas continuous low dose irradiation over the course of five weeks may finally kill these tumor-infiltrating cells before they can

even exert their tumoricidal function. In this light, future studies should address how the immune system is influenced by stereotactic ablative body radiotherapy, a form of high precision radiotherapy with high radiation doses in a few fractions which has achieved excellent tumor control rates in early stage NSCLC[29]. Regardless, our results indicate that prolonged low-dose radiotherapy negatively affects T-cell and APC function in pulmonary adenocarcinoma patients, a matter which needs to be addressed when considering combinatorial approaches with T cell based immunotherapies.

Another important observation from this study is the strikingly high frequency of myeloid cells in the peripheral blood of pulmonary adenocarcinoma compared to healthy subjects (**Figure 1**). Elevated levels of circulating myeloid-derived suppressor cells (MDSC) have been previously reported in advanced NSCLC and their presence was negatively associated with the frequency of CD8+ T lymphocytes[30,31], which is in line with our observation of decreased lymphoid cells in the PBMC of advanced pulmonary adenocarcinoma patients compared to healthy donors. Recently, our research group demonstrated that abnormal levels of circulating myeloid cells in cervical carcinoma patients were shown to normalize after treatment with carboplatin and paclitaxel. Importantly, this was associated with higher T-cell reactivity against common microbial recall antigens[15]. In the present study, advanced pulmonary adenocarcinoma patients, who were treated with carboplatin and vinorelbine also demonstrated a sharp decline in circulating myeloid cell populations (**Figure 3**), but this did not result in changes in recall antigen response to MRM or changes in ability of APCs to stimulate allogeneic T-cell proliferation (MLR response) (**Supplementary Figure 7**). Several factors might explain this apparent discrepancy between our pulmonary adenocarcinoma patients and the cervical cancer patients, such as the use of a different third generation cytotoxic drug in this study cohort. Another important contributing factor might be the impaired APC function of the adenocarcinoma patients at baseline. This is not only reflected by the relative low frequency of DCs in the treatment-naïve patients (**Figure 1**), but moreover by the relatively weak MLR response at baseline (**Figure 4**, mean MLR SI index of 11.5) when compared to cervical cancer patients (mean MLR SI index of >50)[15]. This difference in APC function of advanced NSCLC patients might be related to smoking, which is the main risk factor for development of NSCLC[13]. *In vitro* studies have shown that nicotine diminishes DC activation and maturation thereby promoting T-cell polarization to the T-helper 2 phenotype[32,33]. Nevertheless, the effect of carboplatin-vinorelbine therapy on reducing levels of immunosuppressive myeloid cell populations in pulmonary adenocarcinoma patients is remarkable and further studies into its effect on anti-tumor T-cell immunity are warranted.

In conclusion, this study shows that standard platinum-based chemotherapy used in advanced pulmonary adenocarcinoma patients has no profound adverse effects with respect to immune cell composition and function. Hence, no theoretical objection

exists to combining these chemotherapy schedules with novel T-cell based immunotherapies. However, the decline in function of circulating T cells and APCs caused by prolonged low-dose radiotherapy impairs systemic immunity in these patients, which warrants further debate and studies on how radiation can be optimally applied (e.g. by hypofractionated treatment targeting smaller volumes) in order to sustain anti-tumor immunity and perhaps to act synergistically when combined with new forms of T-cell based immunotherapy in NSCLC patients.

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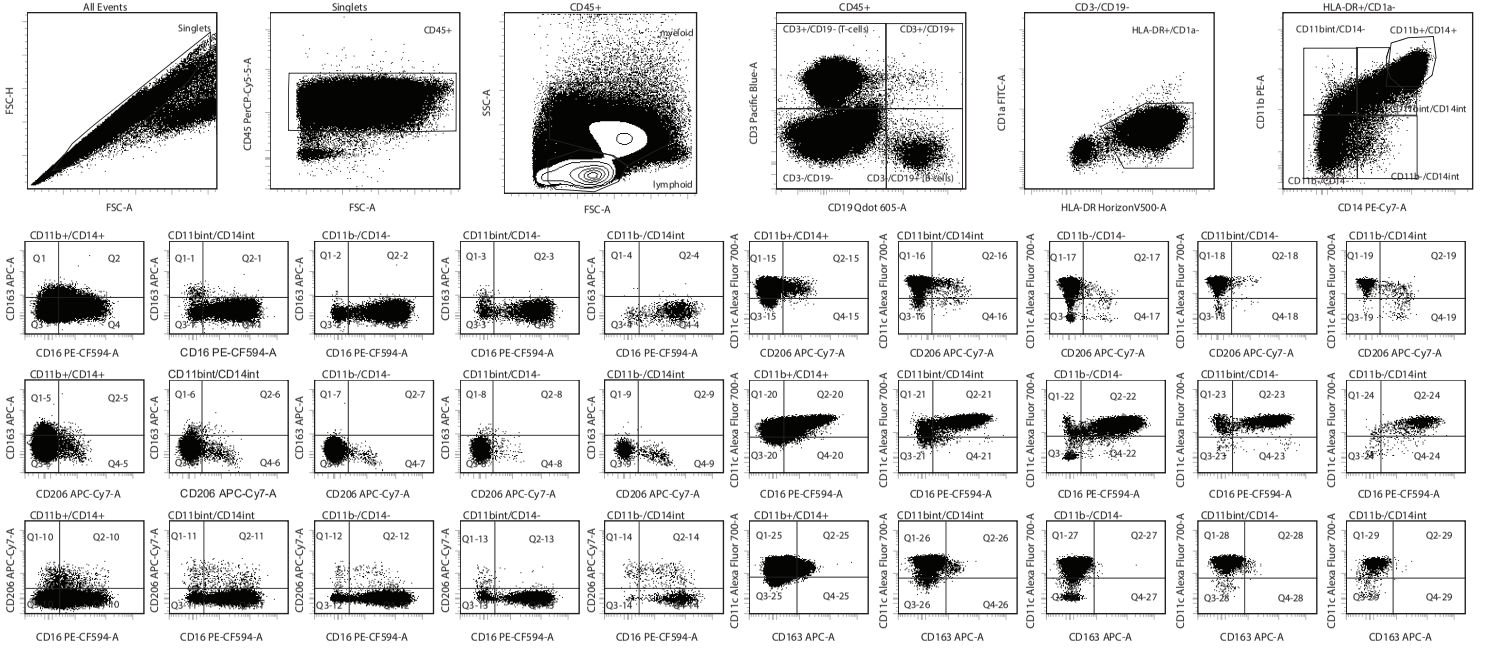
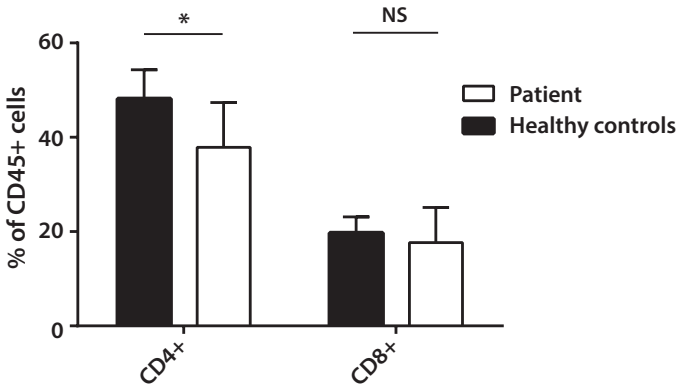
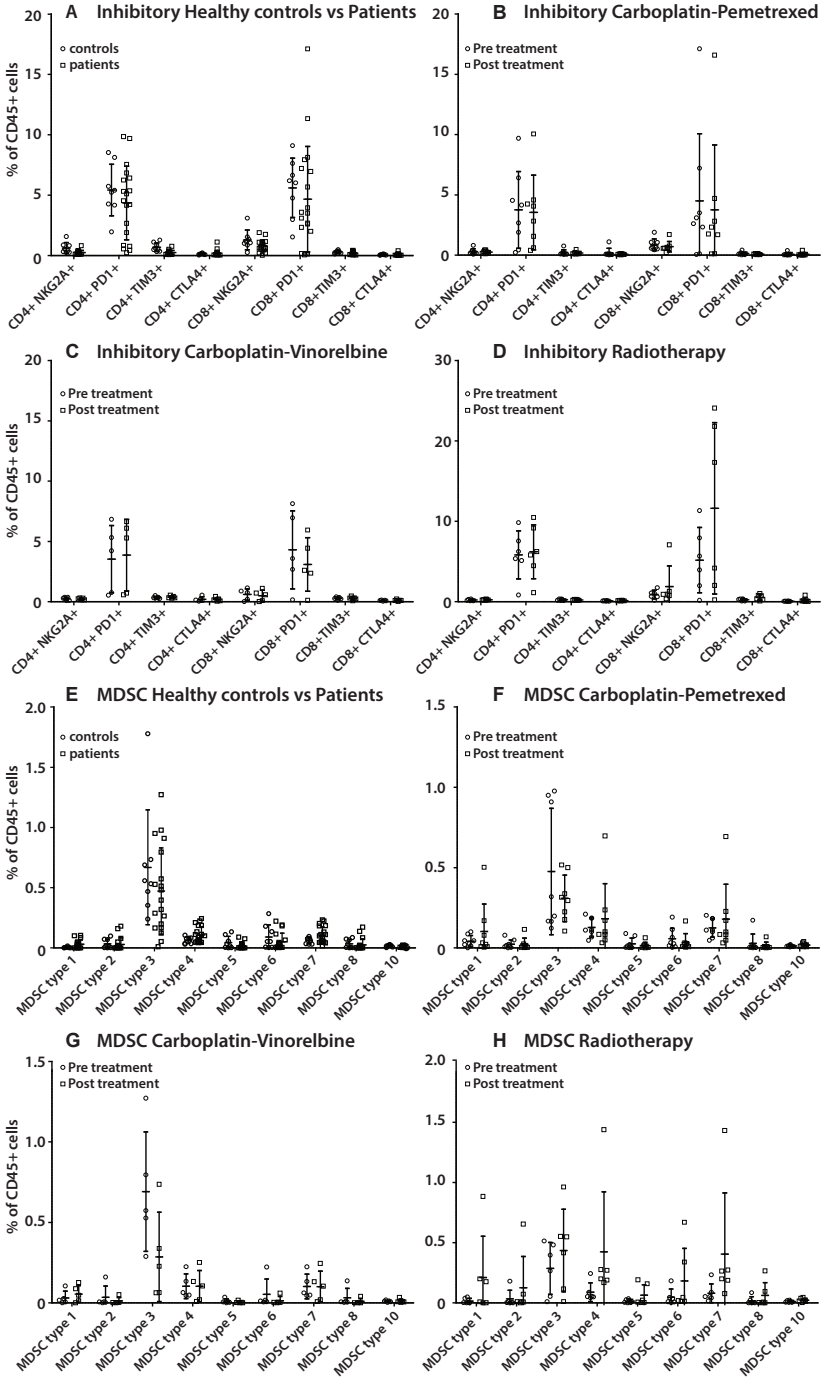


Supplementary Figure 1. Gating strategy for myeloid and lymphoid cell phenotyping

Singlets were gated based on FCS-A and FCS-H to exclude dead cells. Then, leucocytes were selected based on high expression of CD45. The CD45+ cells were divided into myeloid cells and lymphoid cells with use of the myeloid cell gate and lymphoid cell gate within the forward-side scatter plots. Also, CD45+ cells were plotted for the expression of CD3 and CD19. T cells (CD3+CD19-) were subdivided in CD4+ and CD8+ T cells. Monocytes/macrophages (CD3-CD19- cells) were gated for HLA-DR+ / CD1a- cells and then plotted for CD14 and CD11b. Based on differential expression of CD163, CD16, CD206 and CD11c, monocytes/macrophages were subdivided into M1/M2a/M2c and DC subsets.

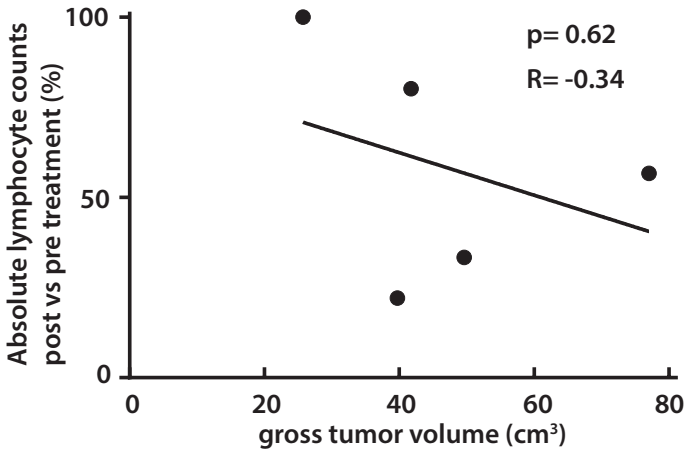


Supplementary Figure 2. Flow-cytometric phenotyping of peripheral blood mononuclear cells (PBMCs) of 23 untreated pulmonary adenocarcinoma patients and 8 healthy donors revealed that CD4+ T cells, displayed as percentage of the CD45+ gate, were significantly lower in patients than in healthy controls ($p<0.05$) whereas the same trend was observed for CD8+ T cells (albeit not significant). Data is shown as mean with SD and statistical analysis was performed using the Mann-Whitney U test (* $p< 0.05$, NS = non-significant).

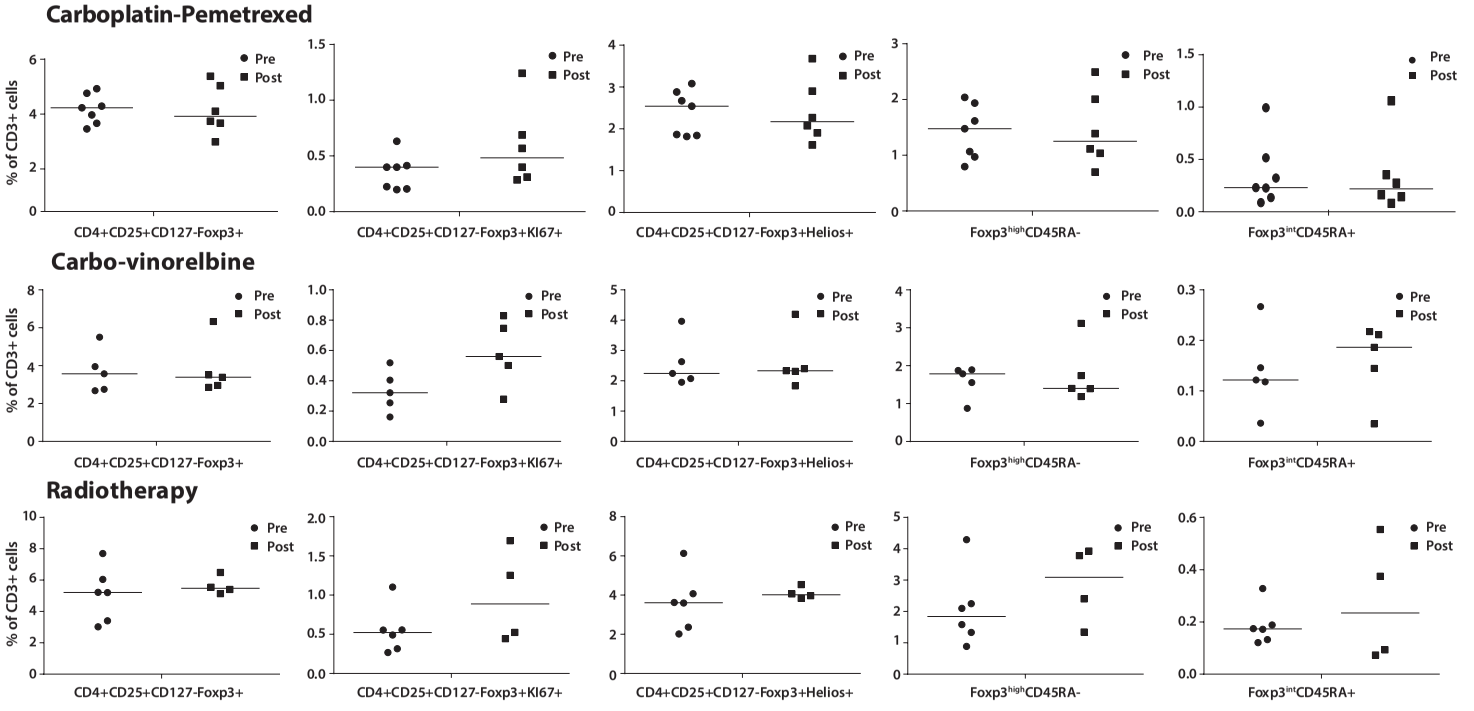


Supplementary Figure 3. Effect of standard of care NSCLC treatment on inhibitory T cell markers and myeloid derived suppressor cells (MDSC).

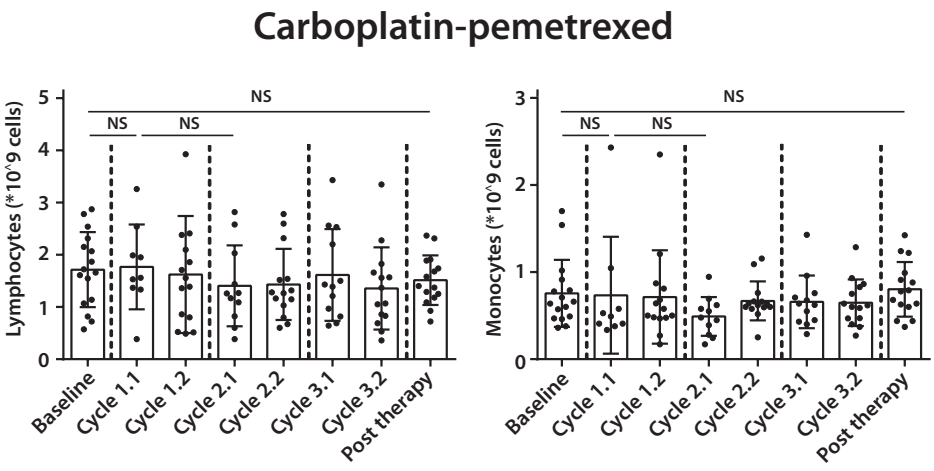
PBMCs of pulmonary adenocarcinoma patients were used for analysis of inhibitory T cell markers (PD-1, NKG2A, TIM3 and CTLA-4 on CD4+/CD8+ T cells) and MDSCs at baseline (pre treatment) and at least 14 days after cessation of therapy (post treatment). Healthy donor PBMCs were taken along for comparison. Three treatment groups were assessed: carboplatin-pemetrexed (n=7), carboplatin-vinorelbine (n=5) and radiotherapy (n=6). All three treatments did not induce changes in T cell inhibitory markers (**B-D**, $p > 0.05$). Comparison of healthy controls and patients at baseline also did not show a difference in these markers (**A**, $p>0.05$). Treatment with doublet chemotherapy (carboplatin-pemetrexed and carboplatin-vinorelbine) was not associated with changes in MDSCs (**F-G**, $p > 0.05$), whereas a significant increase in MDSC type 4 (CD14+ HLA-DRlow) and MDSC type 7 (CD14+ CD33+ HLA-DRlow) was observed in patients treated with radiotherapy (**H**, $p<0.05$). Frequency of MDSCs was comparable between healthy controls and patients at baseline (**E**, $p>0.05$). Data is shown as mean with SD and Wilcoxon signed rank test (patients at baseline compared to post therapy) and Mann-Whitney U test (healthy controls compared to patients at baseline) were used for statistical analysis.



Supplementary Figure 4. Effect of irradiated tumor volume on extent of lymphopenia
To investigate to which extent irradiated tumor volume, expressed as gross tumor volume, had an effect on the decrease in lymphocytes in the blood of pulmonary adenocarcinoma patients, lymphocyte counts pre- and post-radiotherapy were collected from five out of six patients who underwent radiotherapy.
Spearman correlation analysis failed to show a relation between the tumor volume irradiated and the observed lymphopenia (R=-0.34, p=0.62).

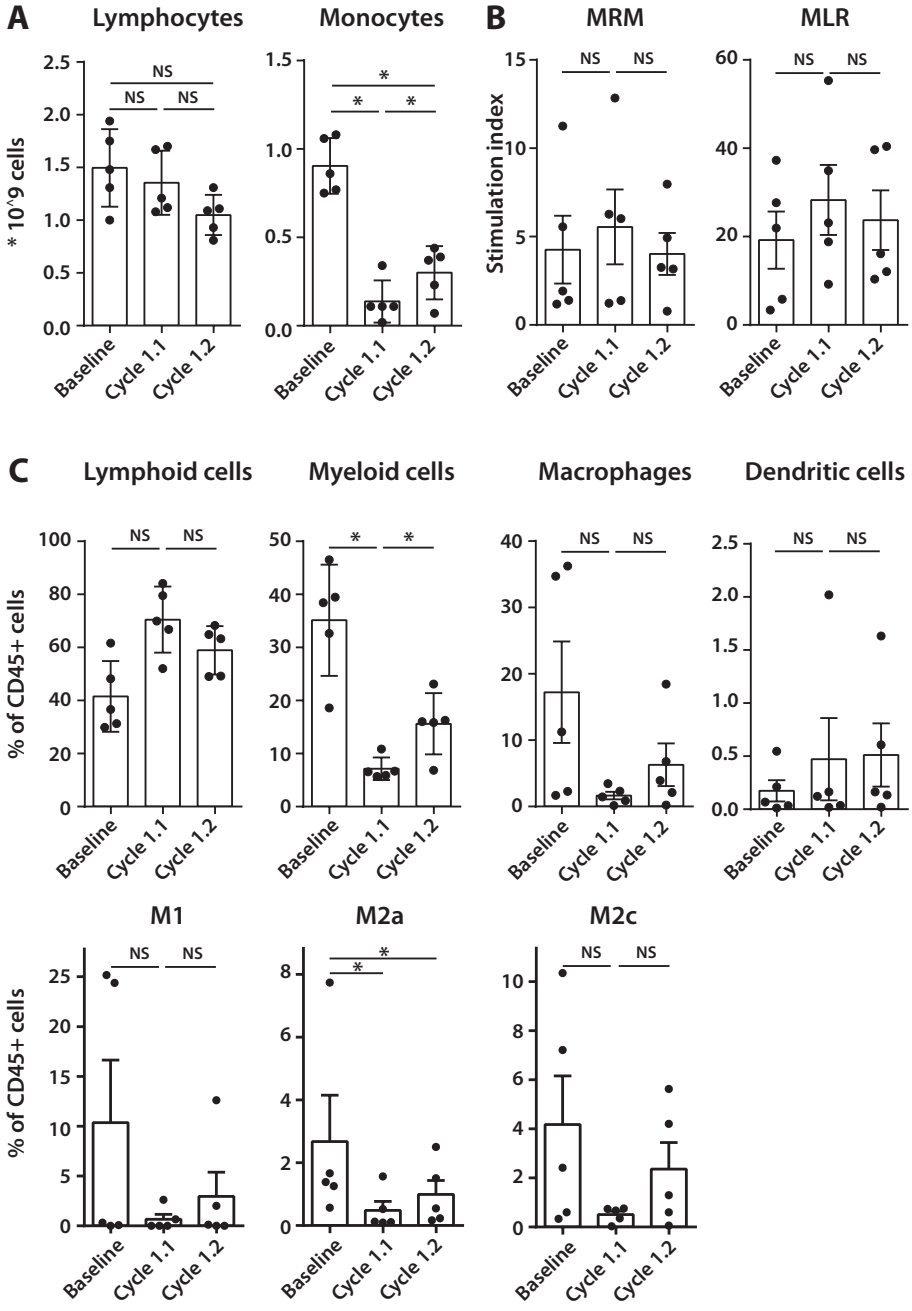


Supplementary Figure 5. Effect of standard of care NSCLC treatment on regulatory T cells (Tregs)
PBMCs of pulmonary adenocarcinoma patients were used to assess changes in Treg frequency. Three definitions of Tregs were used:
Def. 1: CD4+CD25+CD127^{low}Foxp3+ and CD4+CD25+CD127^{low}Foxp3+Ki67+ (activated Tregs); Def. 2: CD4+CD25+CD127^{low}Foxp3+Helios+
Def. 3a: Foxp3^{high}CD45RA- (activated Treg); Def. 3b: Foxp3^{int}CD45RA+ (naïve Treg). Three treatment groups were assessed: carboplatin-pemetrexed (n=7), carboplatin-vinorelbine (n=5) and radiotherapy (n=6). All three treatments did not induce changes with respect to the different Treg subtypes ($p>0.05$). Data is shown as mean with SD and Wilcoxon signed rank test was used for statistical analysis.



Supplementary Figure 6. Carboplatin-pemetrexed treatment does not alter absolute lymphocyte and monocytes counts during therapy.

Automated differential leucocyte counts were retrieved from a historical cohort of 16 pulmonary adenocarcinoma patients treated with at least three cycles of carboplatin-pemetrexed. Automated leucocyte differential counts were collected at baseline, post therapy and at two time points (at week 2 and week 3) during the 21-day cycle of chemotherapy. Results are shown as mean lymphocyte and monocyte counts (per 10^9 cells) with SD. Wilcoxon signed rank test was used for statistical analysis (NS = non-significant).



Supplementary Figure 7. Lymphoid and myeloid cells change during carboplatin-vinorelbine treatment without an effect on T-cell and APC function

At three time points (baseline, at week 2 and week 3) during a 21-day cycle of carboplatin-vinorelbine, PBMCs of 5 patients were prospectively collected for flow-cytometric phenotyping, testing of recall antigen

response (MRM) and ability of APCs to stimulate allogeneic T cell proliferation (MLR). Shown are leucocyte differential counts (per 10^9 cells) (**A**), Stimulation index (SI) of MRM and MLR response (**B**), and frequency of lymphoid and myeloid cells, monocytes/macrophages and dendritic cells as percentages of the CD45+ gate. Subset analysis is shown for M1 (CD206-CD163-), M2a (CD206+CD163-) and M2c (CD206-CD163+) monocytes/macrophages (**C**). Results are presented as mean with SD and Wilcoxon signed rank test was used for statistical analysis. (* $p < 0.05$, NS = non-significant)

Chapter 7

Local and systemic XAGE-1b specific immunity in patients with lung adenocarcinoma

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ABSTRACT

XAGE-1b is a cancer-testis antigen aberrantly expressed in pulmonary adenocarcinoma. Systemic antibody and T-cell responses have been demonstrated in adenocarcinoma patients, but so far local antigen-specific immunity has not been reported. In this study, XAGE-1b expression by tumor cells as well as the presence of systemic and/or local XAGE-1b specific immunity was assessed in peripheral blood, tumor tissue and tumor-draining lymph nodes of Caucasian patients with pulmonary adenocarcinoma. XAGE-1b protein expression was detected in 43.6% (17 of 39) of patients when at least two different parts of a resected tumor were assessed. In 20 patients, analysis of T cells isolated and expanded from the primary tumor and its draining lymph node demonstrated XAGE-1b specific responses in 2 patients. XAGE-1b-specific immunoglobulin G antibodies were found in 3 of 40 patients. These 3 antibody positive patients had also mounted a systemic T-cell response to XAGE-1b, measured by proliferation, cytokine production and expression of T-cell activation markers on peripheral blood mononuclear cells (PBMCs). The population of XAGE-1b-specific T cells comprised both CD4+ and CD8+ T cells secreting both type I and II cytokines. Epitope mapping showed that T cells predominantly targeted the N-terminal part of the XAGE-1b protein while the B-cell response was directed against the C-terminal domain. Our study for the first time provides evidence for the presence of XAGE-1b-specific T cells within adenocarcinoma tissue, which supports the concept that XAGE-1b acts as a genuine tumor antigen and, therefore, might form an attractive target for a vaccine-based approach of immunotherapy.

INTRODUCTION

Lung cancer is the most common cause of cancer mortality in men in the developed world and one of the leading causes in women [1]. Non-small cell lung cancer (NSCLC) comprises about 80 % of all lung cancers [2]. The 5-year survival rates rapidly drops with increased stage at diagnosis [3]. The current treatment modalities include surgery, radiotherapy combined with chemotherapy or palliative chemotherapy [4]. Active immunotherapy, focusing on the reinforcement of the tumor-specific T cell response, has emerged as a new modality to treat cancer [5]. NSCLC is characterized by infiltration of different types of immune cells. Infiltration with M1-macrophages and T cells is positively associated with clinical outcomes, suggesting a protective role for the immune system in NSCLC [6]. This is supported by the recent finding that infusion of antibodies blocking programmed cell death protein 1 (PD1) on T cells has clinical impact in advanced NSCLC [7]. Peptide-based therapeutic vaccines aim at the induction of tumor-specific T-cell responses [5]. This approach is highly dependent on the identification of suitable tumor antigens [8]. An important group of tumor antigens is encoded by the cancer-testis (CT) genes. These CT antigens are present in a significant subset of tumors, including NSCLC[9], and comprise XAGE-1. The XAGE-1 protein has 4 transcripts (a, b, c and d), of which XAGE-1b (81 amino acids) is the mainly expressed isoform [10,11]. Nuclear staining has been observed in 53% of pulmonary adenocarcinomas, a subtype that accounts for 40% of NSCLC, but not in adjacent normal tissues indicating its preferential expression by cancer cells [12]. A positive association between the expression of XAGE-1b and HLA class I with prolonged survival was reported [10], although no link with XAGE-1b specific immunity was made. A recent study revealed the presence of XAGE-1b-specific antibodies in 10% of all NSCLC patients and in 19% of stage IIIb/IV adenocarcinoma patients. More than half of the patients with a XAGE-1b antibody response displayed a concomitant systemic CD4+ and CD8+ T-cell response [13].

To date, studies on XAGE-1b have been performed in Asian populations but not in Caucasian subjects. Furthermore, no data exist on the presence of XAGE-1b specific T cells within the tumor or its draining lymph node. To this end, we have conducted an explorative study in which a European cohort of patients with pulmonary adenocarcinoma was studied with respect to XAGE-1b expression and the presence of systemic and local XAGE-1b mediated immunity.

MATERIALS AND METHODS

Patients and tissue collection

Forty patients with histologically proven primary NSCLC, subtype adenocarcinoma, were included from 2011 to 2014. Patients either underwent surgical resection (stage I/II), stereotactic radiotherapy (stage I), combined chemo-radiotherapy (stage III) or chemotherapy alone (stage IV). Stage IV patients with epidermal growth factor receptor (EGFR) mutations were treated with tyrosine kinase inhibitors. The available tissue blocks of formalin-fixed paraffin embedded tumor were collected. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-density centrifugation and subsequently cryopreserved in liquid nitrogen [14,15]. In case of surgical resection, fresh tissue from the primary tumor and its draining lymph node was obtained.

XAGE-1b immunohistochemistry (IHC)

Tumor blocks were cut in 4 µm sections and deparaffinized in xylene. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol for 10 minutes at room temperature (RT). Antigen retrieval was performed by heating the samples to 97°C for 30 minutes in citrate buffer (pH 6.0, DAKO, Glostrup, Denmark), cooled on ice and incubated at RT for 1 h with 2 µg/ml XAGE-1 mouse monoclonal antibody LX199#5 (kindly provided by the Ludwig Institute for Cancer Research, LICR) in phosphate buffered saline (PBS, Fresenius Kabi Bad Homburg, Germany) with 1% bovine serum albumin (BSA). After washing, the slides were incubated with horseradish peroxidase-conjugated anti-mouse IgG (DAKO envision) for 30 minutes at RT. NovaRed (Vector, Burlingame, USA) was applied as a chromagen and sections were counterstained with Mayer's hematoxylin (Klinipath). All slides were mounted with Pertex mounting medium (HistoLab, Sweden). All washing steps were done with PBS.

XAGE-1b expression was scored according to a previously described method [11] as negative (<5% cancer cells positive), focal (5-10% positive), intermediate (11-50% positive) or diffuse (>50% positive). From each slide, 10 random tumor fields (magnification 20x, approximately the size of a biopsy) were scored for XAGE-1b expression. When available, tissue blocks from multiple tumor sections were assessed.

XAGE-1b protein and overlapping peptides

E. coli produced recombinant XAGE-1b protein (81 amino acids) was obtained using a XAGE-1 plasmid DNA, kindly provided by the LICR. Five synthetic overlapping peptides covering the entire sequence of the XAGE-1b protein were synthesized at the LUMC by solid-phase strategies on an automated peptide synthesizer (Abimed AMS 422, Germany) using Fmoc-chemistry. Peptides were analyzed by reverse-phase HPLC; dissolved

in DMSO at 50 mg/ml, aliquoted and stored at -80°C until use. The amino acid sequences of the 5 peptides are:

p1, amino acid 1-32, MESPKKKNQQLKVGILHLGSRQKKIRIQLRSQ;
p2 amino acid 18-42, LGSRQKKIRIQL-RSQCATWKVICKS;
p3, amino acid 34-59, ATWKVICKSCISQTPGINLDLGSGVK;
p4, amino acid 37-68, KVICKSCISQTPGINLDLGSGVKVKIIPKEEH;
p5, amino acid 55-81, GSGVKVKIIPKEEHCKMPE-AGEEQPQV.
Working solutions were prepared at a concentration of 2.5 mg/ml and stored at -20°C.

Detection of XAGE-1b-specific IgG antibodies

Serum samples were analyzed for XAGE-1b peptide-specific immunoglobulin G (IgG) by ELISA. Serum samples with known high IgG titers for XAGE-1b (KLU 187, kindly provided by Dr. E. Nakayama, Okayama University, Japan) were used to develop the ELISA. One serum sample (X-14) with proven high XAGE-1b IgG titers was included in each experiment as a positive control. All washing steps were done with PBS. A 96-well EIA/RIA plate (Costar 3590) was coated overnight at 4°C with 50 µl of the individual XAGE-1b peptides and a mix of all peptides (5 µg/ml of each peptide diluted in 0.1 M carbonate/bicarbonate coating buffer; Merck, Darmstadt, Germany). The next day, non-specific binding sites of the plate were blocked with 5% FCS/PBS (100 µl/well, Fetal Calf Serum, PAA laboratories, Austria) for 1 hour at RT. Subsequently, serially diluted serum samples (1:100, 1:500, 1:1000, 1:2000, 1:4000 in blocking buffer) were added in triplicate wells (50 µl/well) and incubated at RT for 2 hours. Next, goat anti-human IgG-horseradish peroxidase (HRP, Southern Biotechnology, Birmingham, AL) was added (diluted 1:3000 in blocking buffer) and incubated for 1 hour at RT. Finally, tetramethylbenzidine liquid substrate (Sigma Aldrich, 50 µl/well) was added for the colorimetric enzymatic reaction, which was stopped by adding 50 µl/well of 2 M H_2SO_4 (Merck) and the plate was read in an ELISA reader at 450 nm. The average OD-value of the triplicate uncoated wells (background value) was calculated and an outlier per triplicate was discarded when the value exceeded the average plus 2x standard deviation (SD). A positive XAGE-1b peptide specific IgG response was defined as an average of the triplicate wells which was at least 2-fold above background value.

Cell culture

The tumor-infiltrating lymphocytes (TILs) culture method has been published previously [14,15]. Briefly, TIL were isolated by mincing fresh tumor tissue into pieces followed by a 2-3 week homeostatic *in vitro* culture in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% human AB (hAB) serum (PAA laboratories) and a mix of homeostatic cytokines: 10% T-Cell Growth Factor (TCGF, Zeptometrix, USA), 5 ng/ml of interleukin-15 (IL-15; Peprotech) and (only on day 1) 5 ng/ml of IL-7 (Peprotech).

Lymph node (LN) mononuclear cells were isolated from tumor-draining lymph nodes and cultured for a week in medium alone (LN neg) or supplemented with a mix of 5 XAGE-1b overlapping peptides (LN XAGE, 2.5 µg/ml/per peptide), after which the T-cells were expanded with recombinant human IL-2 (150 IU/ml, refreshed 3 times per week) for 2-3 weeks. After harvesting, the T cells were evaluated for presence of XAGE-1b-specific CD4+CD25+ Foxp3^{high} T cells, XAGE-1b-specific proliferation and cytokine production.

Detection of XAGE-1b-specific CD4CD25+ Foxp3^{high} T cells

PBMC, TIL and LN cells were stained for CD4, CD8, Foxp3 and CD25 as previously reported [16,17].

Analysis of XAGE-1b-specific T cells by proliferation assay and cytokine profile

T cells (50,000/well) were stimulated with autologous monocytes pulsed with XAGE-1b peptides and/or protein (5 µg/ml) in triplicate wells in a three day proliferation assay [14] with phytohaemagglutinin (PHA) (Remel, Germany) used as positive control. Supernatants collected after 2 days were analyzed for Th1/Th2 cytokines (IFNγ, TNF-α, IL-10, IL-5,IL-4, IL-2) by cytometric bead array (CBA, BD Biosciences). Proliferation was measured by ^{3}H-thymidine incorporation (0.5 µCi/well) during the last 18 hours of the assay [18]. The stimulation index (SI) was calculated by taking mean counts of stimulated wells divided by mean counts of the medium control wells. A XAGE-1b-specific T cell response was defined by either a SI index of >3 or by XAGE-1b-specific cytokine production, which was defined as a cytokine concentration above the cut-off value (20 pg/ml, except for IFNγ for which the cut-off was 100 pg/ml) and more than twice the concentration of medium control [19].

ELISPOT

Details of the 4-days IFNγ ELISPOT assay have been reported previously [17]. Spots were counted with a fully automated computer-assisted-video-imaging analysis system (BioSys 5000). Specific spots were calculated by subtracting the mean number of spots in quadruplicate wells + 2xSD of the medium only control from the mean number of spots in test wells. Antigen-specific T-cell frequencies were considered to be increased compared to medium control when specific T-cell frequencies were ≥ 1/10,000 [17].

Analysis of XAGE-1b-specific T cells by multiparameter flow cytometry

T cells were stimulated overnight with autologous monocytes pulsed with individual XAGE-1b peptides (5 µg/mL), a XAGE-1b peptide mix (5 µg per peptide/mL) and XAGE-1b protein (10 µg/mL). E7 protein of the human papillomavirus type 16 (HPV16 E7) was used as negative control. The percentage and polarization of XAGE-1b-specific T-cells was measured by simultaneous staining for T-cell markers (CD3, CD4 and CD8), T cell

activation markers (CD137 and CD154) and cytokines (IFNγ, IL-2) according to standard operating procedures (SOPs) [14,16,20]. XAGE-1b-specific T cells were detected when the percentage of XAGE-1b-specific CD4+CD154+CD137+, CD8+CD137+ or cytokine producing T cells was at least twice the percentage detected in the medium control. The responding cells were visible as a clearly distinguishable population in the flow cytometry contour plot. An example of a gating strategy is provided in **Supplementary Figure 1**.

Isolation of XAGE-1b-specific T-cell clones

T-cell clones were isolated from PBMC using limiting dilution as described earlier [15]. Specificity of T-cell clones for XAGE-1b was tested by proliferation assay on peptide or protein loaded irradiated autologous Epstein Bar virus (EBV) transformed B cell lines (B-LCL) or autologous monocytes. Furthermore, clones were tested by flow cytometry for phenotype (CD4/CD8) and T cell receptor Vβ (TCRVβ) expression using a TCRVβ-kit (Beckman Coulter) comprising eight sets of antibodies, each consisting of three differently labeled antibodies specific for three different TCRVβ families, ultimately covering about 70% of the normal human TCRVβ repertoire. A TCRVβ was considered dominant (>10%), subdominant (3–10%), or minor (<3%) based on the percentage of T cells using the same TCRVβ [14,21].

RESULTS

An overview of patient characteristics is presented in **Table 1**. The mean age was 65.9 years (range 45-82 years) and the male/female ratio was 20/20.

XAGE-1b expression in lung adenocarcinoma

In our patient cohort the whole primary tumor was available for XAGE-1b staining from 28 patients who underwent surgical resection (stage I/II). A biopsy of the primary tumor was available in 9 cases (2 stage I/II patients and 7 stage III/IV patients). In 3 cases, XAGE-1b expression was assessed on metastasized tissue only. In one patient (X-4) the biopsy of the primary tumor could not be retrieved and hence XAGE-1b status was not assessed. Overall, in 17 of 39 evaluable cases (43.6%), XAGE-1b expression was observed: focal (n=5), intermediate (n=6) and diffuse (n=6). Positive staining was found in the primary tumor (n=15), metastatic tissue (n=1) or both (n=1) (**Table 1**). The XAGE-1b staining pattern was always nuclear and occasionally, also cytoplasmic staining was observed. An example of XAGE-1b staining is presented in **Supplementary Figure 2**. While 54% (15 of 28) of the resected tumor specimens stained positive for XAGE-1b, this was only the case for 1 out of 8 biopsies evaluated. To assess whether the XAGE-1b status in biopsies truly reflects the XAGE-1b status of the primary tumor, 10 random tumor fields

Table 1. Overview of patient characteristics, XAGE-1b immunohistochemistry and IgG response. A blank field indicates that the primary or metastic tumor specimen was not tested.

ID	Stage	Treatment[1]	Tumor Material	XAGE-1b IHC[2]	Nr. of + fields[3]	Nr. of + sections[4]	Metastasis	XAGE-1b IgG[5]
X-1	I	RT	Biopsy	Negative				-
X-2	IV	CT	Biopsy	Negative				-
X-3	IIIa	CT-RT	Biopsy	Negative				-
X-4	IIIb	CT	Biopsy					+
X-5	I/II	Surgery	Resection	Focal	2	1 of 3		-
X-6	IV	CT	Biopsy	Negative				-
X-7	I/II	Surgery	Resection	Focal	4	1 of 1		-
X-8	IIIa	CT-RT	Biopsy	Negative				-
X-9	I/II	Surgery	Resection	Intermediate	10	1 of 1		-
X-10	IIIb	CT-RT	Biopsy	Negative				-
X-11	IV	CT	Metastasis				Negative	-
X-12	I/II	Deceased	Biopsy	Focal				-
X-13	I/II	Surgery	Resection	Negative	0			-
X-14	I/II	Surgery	Resection	Diffuse	10	2 of 3	Diffuse	+
X-15	I/II	Surgery	Resection	Focal	4	1 of 1		-
X-16	IIIa	CT-RT	Metastasis				Negative	-
X-17	I/II	Surgery	Resection/ Metastasis	Diffuse	6	2 of 2	Negative	-
X-18	I/II	Surgery	Resection/ Metastasis	Negative	0	0 of 2	Negative	-
X-19	I/II	Surgery	Resection	Focal	3	1 of 3		-
X-20	I/II	Surgery	Resection	Negative	0	0 of 3		-
X-21	I/II	Surgery	Resection	Intermediate	4	2 of 2		-
X-22	I/II	Surgery	Resection	Intermediate	3	2 of 2		-
X-23	I/II	Surgery	Resection	Negative	0	0 of 2		-
X-24	I/II	Surgery	Resection	Intermediate	6	2 of 2		-
X-25	I/II	Surgery	Resection	Negative	0	0 of 2		-
X-26	I/II	Surgery	Resection	Intermediate	4	1 of 2		
X-27	I/II	Surgery	Resection	Negative	0	0 of 6		+
X-28	I/II	Surgery	Resection	Negative	0	0 of 4		-
X-29	I/II	Surgery	Resection	Negative	0	0 of 2		-
X-30	I/II	Surgery	Resection	Intermediate	3	1 of 3		-
X-31	I/II	Surgery	Resection	Negative	0	0 of 1		-
X-32	I/II	Surgery	Resection	Negative	0	0 of 3		-
X-33	I/II	Surgery	Resection	Negative	0	0 of 5		-
X-34	IV	TKI	Metastasis				Diffuse	-
X-35	I/II	Surgery	Resection	Diffuse	6	1 of 1		-

Table 1. Overview of patient characteristics, XAGE-1b immunohistochemistry and IgG response. A blank field indicates that the primary or metastic tumor specimen was not tested. (continued)

ID	Stage	Treatment[1]	Tumor Material	XAGE-1b IHC[2]	Nr. of + fields[3]	Nr. of + sections[4]	Metastasis	XAGE-1b IgG[5]
X-36	I/II	Surgery	Resection	Diffuse	7	4 of 4		-
X-37	I/II	Surgery	Resection	Diffuse	6	3 of 3		-
X-38	I/II	Surgery/CT	Resection/ Metastasis	Negative	0	0 of 2	Negative	-
X-39	IV	CT	Biopsy	Negative				-
X-40	I/II	Surgery	Resection	Negative	0	0 of 4		-

[1]CT chemotherapy ; CT-RT combined chemo-radiotherapy ; RT radiotherapy ; TKI Tyrosine kinase inhibitors
[2] XAGE-1b immunohistochemistry (IHC) scored as negative (< 5% positive), focal (5-10%), intermediate (11-50%) and diffuse (>50%).
[3] 10 random tumor fields (magnification 20x) from each slide were scored. The number (Nr.) of positively scored fields are given.
[4] When available, XAGE-1b overexpression was assessed in multiple tumor sections.
[5] XAGE-1b-specific IgG antibody response is shown.

of the resected tumor specimens (n=28; magnification 20x, approximately the size of a biopsy) were scored for XAGE-1b overexpression. While none of the 13 previously scored negative tumors showed XAGE-1b staining in the 10 random fields, the 15 XAGE-1b positive resected tumors displayed XAGE-1b staining in (on average) 5.2 out of 10 fields (range 2-10). In addition, multiple tissue blocks of the same tumor (average 3.0 blocks, range 2-6) were studied for 22 of 28 operated patients. In 17 of 22 cases, staining score (positive or negative) was identical in all blocks from the same case (**Table 1**). Our data demonstrate that XAGE-1b overexpression is found in about 40% of all tumors, however positive tumors do not show overexpression in all randomly selected tumor fields.

XAGE-1b-specific T cells are present in the primary lung tumor and its draining lymph nodes

Fresh samples of tumor and lymph node tissue were collected from 24 of the 28 stage I/II adenocarcinoma patients. In 20 cases, we successfully expanded TILs. Tumor-draining lymph node (LN) mononuclear cells were expanded *in vitro* in the presence (LN XAGE) or absence (LN neg) of exogenous XAGE-1b peptides. The phenotype (CD4, CD8, Foxp3) and activation status (i.e. CD25 expression) of these T cells is presented in **Supplementary Figure 3.**

Next, the presence of local (TIL/LN neg/LN XAGE) as well as circulating XAGE-1b-specific T cells (PBMC) was investigated by analysis of XAGE-1b-specific proliferation and cytokine secretion in these 20 patients. In one patient (X-14) with diffuse (>50%) XAGE-1b staining in the primary tumor, a T helper 1 (Th1) response to XAGE-1b was detected (**Figure 1**). XAGE-1b-specific secretion of IFNγ and TNFα was detected in both TIL and LN

XAGE cells after co-culture with autologous monocytes pulsed with a mix of XAGE-1b overlapping peptides. In addition, the T helper type 2 (Th2) cytokines IL-5 and IL-10 were secreted (**Supplementary Figure 4**). Despite the XAGE-1b-specific high production of cytokines, the proliferative response was low in TIL (SI index 0.7) and LN XAGE (SI 2.1), indicating that these T cells had poor proliferative capacity after their initial expansion (data not shown).

Another patient (X-20), without XAGE-1b expression in the primary tumor, showed XAGE-1b-specific T cell reactivity in its draining LN. XAGE-1b-specific IFNγ and TNFα production was detected in the LN cells only expanded with IL-2 (LN neg) **(Figure 1)**, and these T cells also produced IL-5 but not IL-10 (**Supplementary Figure 4).** A XAGE-1b-specific response in the LN cells cultured with XAGE-1b peptides (LN XAGE) was not

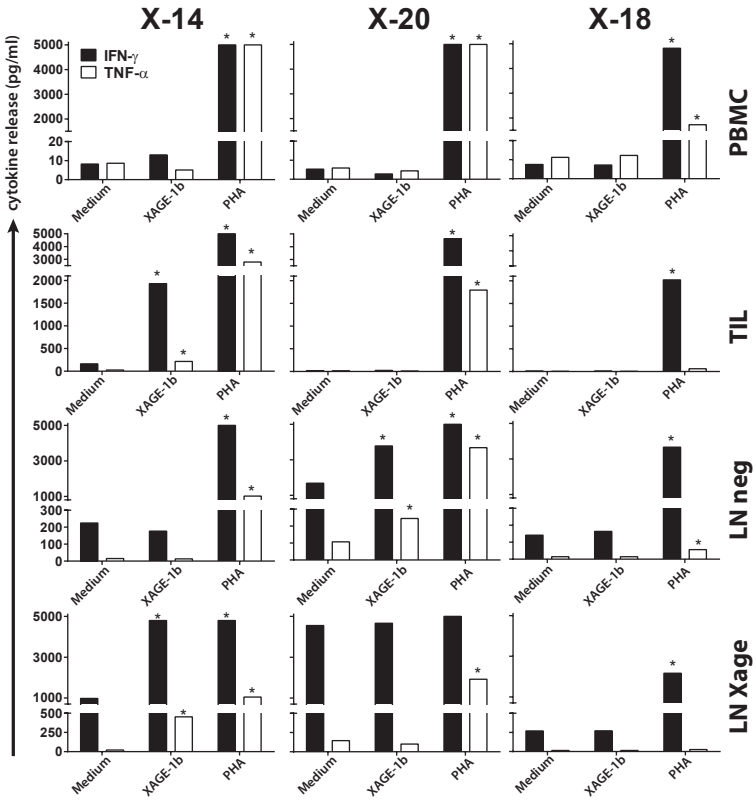

Figure 1. Local XAGE-1b mediated immunity, Th1 response

Day 2 supernatants from PBMC, TIL and LN cells, co-cultured with XAGE-1b pulsed monocytes, were analyzed for Th1 (IFNγ and TNF-α) and Th 2 (IL-5 and IL-10) cytokine release. A positive response (indicated with asterisks) was defined by a cytokine concentration above the cut-off value (20 pg/ml, except for IFNγ, 100 pg/ml) and more than twice the concentration of medium control. Depicted here are the Th1 cytokines. XAGE-1b specific Th1 cytokine release was observed in TIL (X-14) and LN cells (X-14, X-20). Included are the results from one negative (X-18) patient. PHA was used as positive control.

demonstrated potentially due to the high background cytokine production in these activated T cells after culture **(Figure 1)**. Again, despite high cytokine release in the LN neg cells, the XAGE-1b-specific proliferative response was low (SI 1.2, data not shown).

Overall, we found local XAGE-1b mediated Th1/Th2-cell immunity in two of 20 patients tested indicating that XAGE-1b acts as a genuine tumor antigen.

Identification of XAGE-1b B cell epitopes

The ELISA for measuring XAGE-1b-specific IgG antibodies was developed with a high IgG titer serum sample (KLU 187) [13] as positive control. A relatively strong XAGE-1b-specific IgG response was detected in patient X-14 (**Figure 2)**. In total, 3 out of 40 evaluated patients (7.5%) displayed a XAGE-1b-specific IgG response when tested against a mix of the 5 XAGE-1b overlapping peptides. These three serum samples were also tested against the individual XAGE-1b peptides (**Figure 2C**). Although peptide p5 showed the strongest response, the sera showed a response to multiple epitopes in XAGE-1b.

Of these three patients, one patient (X-14) expressed XAGE-1b in the tumor and mounted a local XAGE-1b-specific T cell response. Surprisingly, the second patient (X-27) did not show XAGE-1b expression in the tumor, despite the analysis of 6 separate tumor

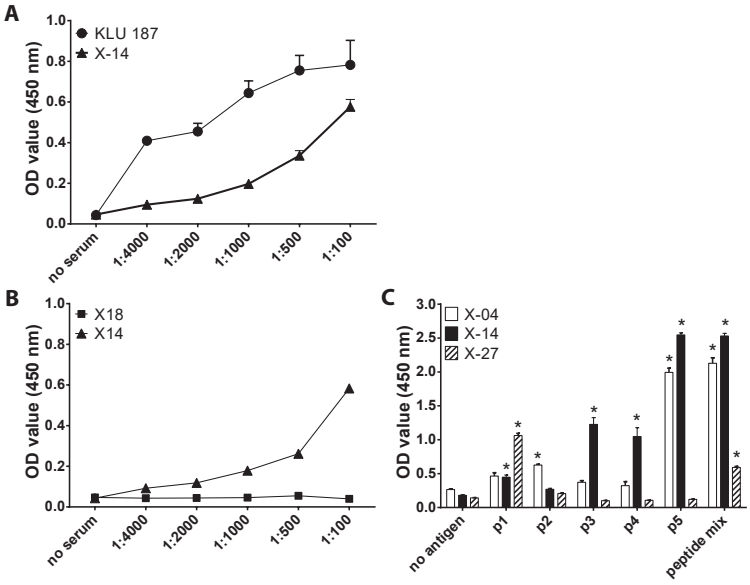

Figure 2. XAGE-1b-specific IgG antibodies in patient sera

A) Example of a XAGE-1b-specific IgG response (X-14) to XAGE-1b peptide mix. KLU 187 represents a serum sample previously shown to have high IgG antibody titers [13] and was used as positive control to set-up the ELISA. Serial dilutions are shown. **B)** Example of a patient (X-18) with no XAGE-1b IgG response to XAGE-1b peptide mix. X-14 was used as positive control. **C)** IgG response (serum 1:100 diluted) to individual XAGE-1b peptides in 3 of the 40 tested patients shows broad recognition. A positive response was defined as at least a 2-fold increase compared to background (no antigen) and is indicated with asterisks.

sections. For the last patient (X-4) the XAGE-1b tumor status could not be assessed due to unavailability of the tumor sample.

XAGE-1b-specific T cells are present in peripheral blood both direct ex-vivo and after in vitro expansion

Since the presence of IgG antibodies indicate an underlying T cell response [13], the three patients (X-4, X-14, X-27) with XAGE-1b-specific IgG were analyzed for circulating XAGE-1b-specific T cells. First, PBMC were stimulated with the pool of XAGE-1b peptides or medium only and examined in a direct *ex-vivo* IFNg-ELISPOT. In one patient (X-4) a strong XAGE-1b-specific IFNg response was demonstrated (**Supplementary Figure 5**) indicative for the presence of circulating XAGE-1b-specific T cells. PBMC of the other patients (X-14, X-27) did not show a direct *ex-vivo* XAGE-1b-specific T-cell response (data not shown).

Subsequently, PBMC samples of these three patients were stimulated with the mix of XAGE-1b overlapping peptides *in vitro* for 10 days, and then tested for XAGE-1b-specific proliferation and Th1/Th2 cytokine production (**Figure 3**). Patient X-4 showed a Th1 response to XAGE-1b reflected by the production of high amounts of IFNγ and low levels of IL-5 and IL-10 to peptide p2 and to the mix of 5 peptides. Similar to our previous observation, the proliferative response of the T cells was modest at best to the peptide mix (SI 2.5) and undetectable to individual peptides. The PBMC culture of patient X-14 produced large amounts of IFNγ when stimulated with peptide p1, p2 or the peptide mix as well as low amounts of TNFα and IL-5 upon stimulation with p1 and the peptide mix. The PBMCs of this patient proliferated when stimulated with p1 (SI 8.5) and peptide mix (SI 8.4) but not when stimulated with p2.

The third patient (X-27) neither showed cytokine production nor a proliferative response specific for XAGE-1b (**Figure 3**). However, we observed an unusually high concentration of CD14+ myeloid cells in the peripheral blood of this patient. Myeloid derived suppressor cells have been shown to be elevated and to have a suppressive effect on T cells in NSCLC [22,23]. Therefore, the $CD14^+$ cells were removed from the PBMC by magnetic-activated cell sorting (MACS) and the remaining cells were stimulated with the XAGE-1b peptide mix. As a result, the 10-day cultured PBMCs produced IFNγ, TNFα and IL-5 and proliferated upon stimulation with p2 (SI 3.9) and the XAGE-1b peptide mix (SI 4.1) (**Supplementary Figure 6**). Thus XAGE-1b-specific T cells are present in the peripheral blood of XAGE-1b IgG positive patients but their reactivity can be obscured by a CD14+ myeloid population.

Type and specificity of the XAGE-1b-specific T-cell response

To type and enumerate XAGE-1b-specific T cells in 8 weeks cultured PBMCs, we assessed the frequency of CD4+ and CD8+ T cells with increased expression of the T-cell

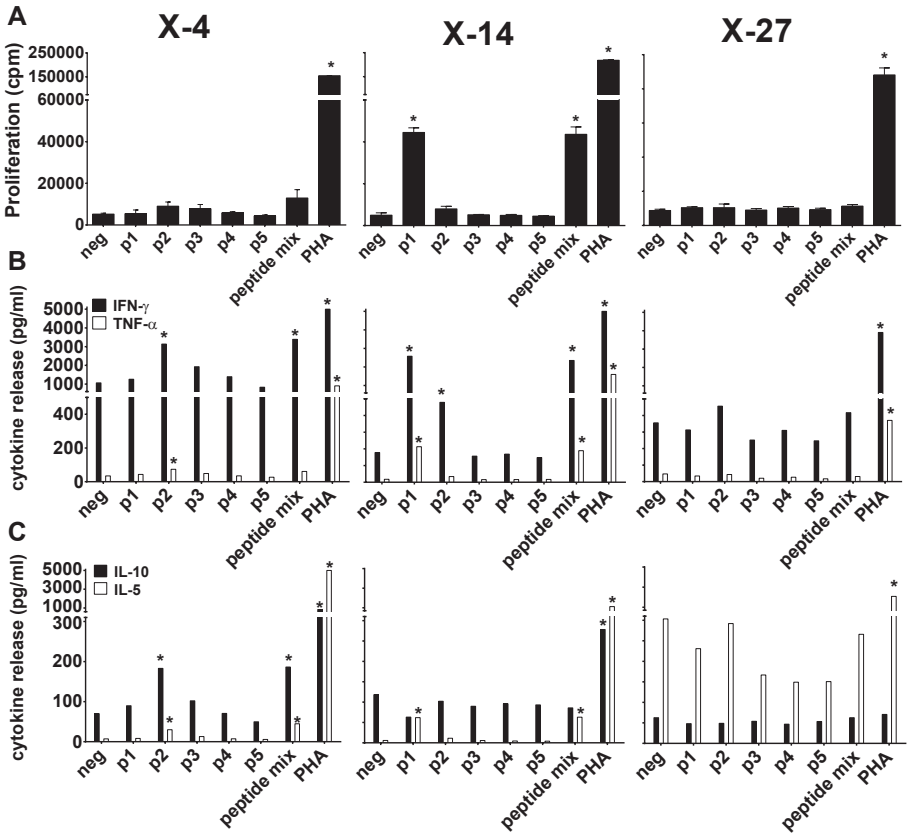

Figure 3. Circulating XAGE-1b specific T cells: proliferation and release of type I/II cytokines
A) PBMC samples from three patients (X-4, X-14, X-27) with XAGE-1b-specific IgG antibodies were stimulated with XAGE-1b overlapping peptides in a 10 day culture and subsequently tested for XAGE-1b-specific proliferation. A positive response (indicated with asterisk) was defined as a SI index of ≥ 3. Patient X-14 showed a response specific for peptide p1 and XAGE-1b peptide mix, X-4 showed a modest proliferative response to the XAGE-1b peptide mix, whereas X-27 showed no proliferative response at all. **B)** Release of Th1 cytokines (IFNγ, TNFα) was observed in two patients (X-4, X14) specific for peptide p1, p2 and peptide mix. **C)** Release of Th2 cytokines (IL-10, IL-5) was observed in two patients (X-4, X14) and was specific for peptide p1, p2 and peptide mix. The third patient (X-27) showed no detectable response (**B,C**).

activation markers CD137 and CD154 after stimulation with antigen-pulsed autologous monocytes (**Figure 4A**). CD4+ T cells of patient X-4 showed a strong response towards stimulation with p1, p2 and the peptide mix. A weak response was observed to p3, p4 and XAGE-1b protein. The type I cytokines IFNγ and/or IL-2 were produced by the double positive (CD137+CD154+) T cells (**Figure 4B**) upon stimulation with p1, p2, p3, p4 and the peptide mix. Unfortunately, the number of expanded CD8+ T cells in this culture was too low to thoroughly assess XAGE-1b-specific CD8+ T-cell reactivity.

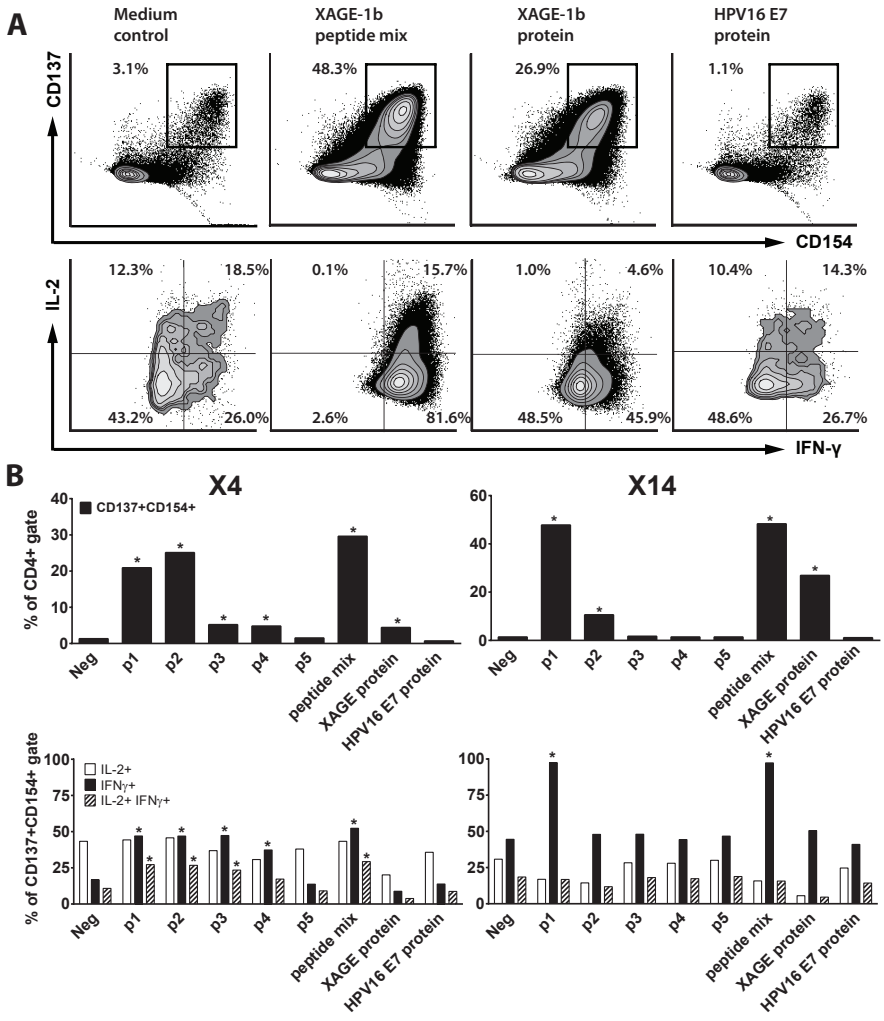

Figure 4. Type and specificity of XAGE-1b-specific CD4 T cell response

A) The CD4+ T cells in the 8 weeks cultured PBMCs of patient X-14 were gated (see **Supplementary Figure 1**). Subsequently, CD4+ double positive (CD137+CD154+) T cells were gated. Finally, the intracellular IFNγ/IL-2 production within this population was plotted. Upon stimulation with XAGE-1b peptide mix and XAGE-1b a specific upregulation of the T-cell activation markers CD137/CD154 and the intracellular expression of IFNγ and IL-2 is demonstrated as compared to medium control and the negative control protein (HPV16 E7). **B)** The specificity of CD4+CD137+CD154+ T cells for individual peptides and XAGE-1b protein and type I intracellular cytokine profile are shown for 2 patients (X-4 and X-14). Asterisks indicate positive responses (at least twice the percentage detected in the medium control). For patient X-4, a CD4+ T-cell response was observed specific for peptide p1, p2 and the peptide mix, as well as a weak response to p3, p4 and p5. Patient X-14 displayed a strong CD4+ T-cell response when stimulated with p1, peptide mix and XAGE-1b protein as well as a moderate response to p2.

The cultured PBMC of patient X-14 showed a strong CD4+ T-cell mediated response when stimulated with p1, peptide mix and XAGE-1b protein as well as a moderate response to p2, corroborating the earlier data (**Figure 4B**). The cytokine profile of double positive T cells mainly showed high IFNγ production specific for p1 and the peptide mix. Notably, the CD8+ T cells within this culture reacted to p1, the peptide mix and to XAGE-1b protein (**Supplementary Figure 7**). Surprisingly, the peptide and protein specific IFNγ production in these CD8 T cells is not accompanied by an increased expression of the activation marker CD137 (**Supplementary Figure 8**).

Subsequently, T cell clones were isolated from the bulk cultured PBMCs from patients X-4 and X-14. During the cloning procedure the expanded PBMCs were kept in culture and, subsequently the established clones and bulk cultured PBMCs were characterized with respect to TCR-Vβ usage and antigen-specific proliferation. Analysis of TCR-Vβ usage showed the presence of at least 11 different TCR-Vβ families of CD4+ T cells in the bulk cultured PBMCs of X-4 (**Supplementary Figure 9A**). Upon stimulation with peptide p1, p2, the peptide mix and XAGE-1b protein, the bulk cultured PBMCs of this patient showed a proliferative response (**Supplementary Figure 9B**). Only one XAGE-1b-specific CD4+ T-cell clone could be established (**Supplementary Figure 9C**). This clone (X-4.6) responded to p1, p2 and the peptide mix. The TCR-Vβ analysis did not lead to the identification of a specific TCR-Vβ family usage (data not shown), indicating that the TCR-Vβ used was outside the range of families covered by the eight sets of antibodies.

The CD4+ T cells in the bulk cultured PBMC of patient X-14 displayed the use of at least 16 different TCR-Vβ-families (**Figure 5A**), of which one was considered dominant (Vβ5.1) and 5 were considered subdominant (Vβ2/3/8/14/21.3). The bulk cultured PBMCs showed a broad response to all 5 overlapping XAGE-1b peptides, the peptide mix and to XAGE-1b protein (**Figure 5B**). A total of 10 XAGE-1b-specific CD4+ T cell clones were obtained of which 8 were analyzed for TCR-Vβ usage. Staining for the dominant TCR-Vβ 5.1 was demonstrated for 4 clones, whereas the subdominant TCR-Vβ 21.3 was expressed by one clone (**Figure 5C**). The established clones showed XAGE-1b specific proliferation when stimulated with p1, p2, the peptide mix and XAGE-1b protein, indicating that CD4+ T-cell clones isolated from this culture recognized their naturally processed cognate antigen. Flow cytometric analysis of intracellular Foxp3 expression by the isolated clones did not reveal the presence of this transcription factor (data not shown).

Taken together, we conclude that T-cell reactivity to XAGE-1b involves polyclonal CD4+ and CD8+ T-cell populations with the capacity to recognize the naturally processed antigen and reactive to peptide epitopes different from those recognized by the IgG antibodies.

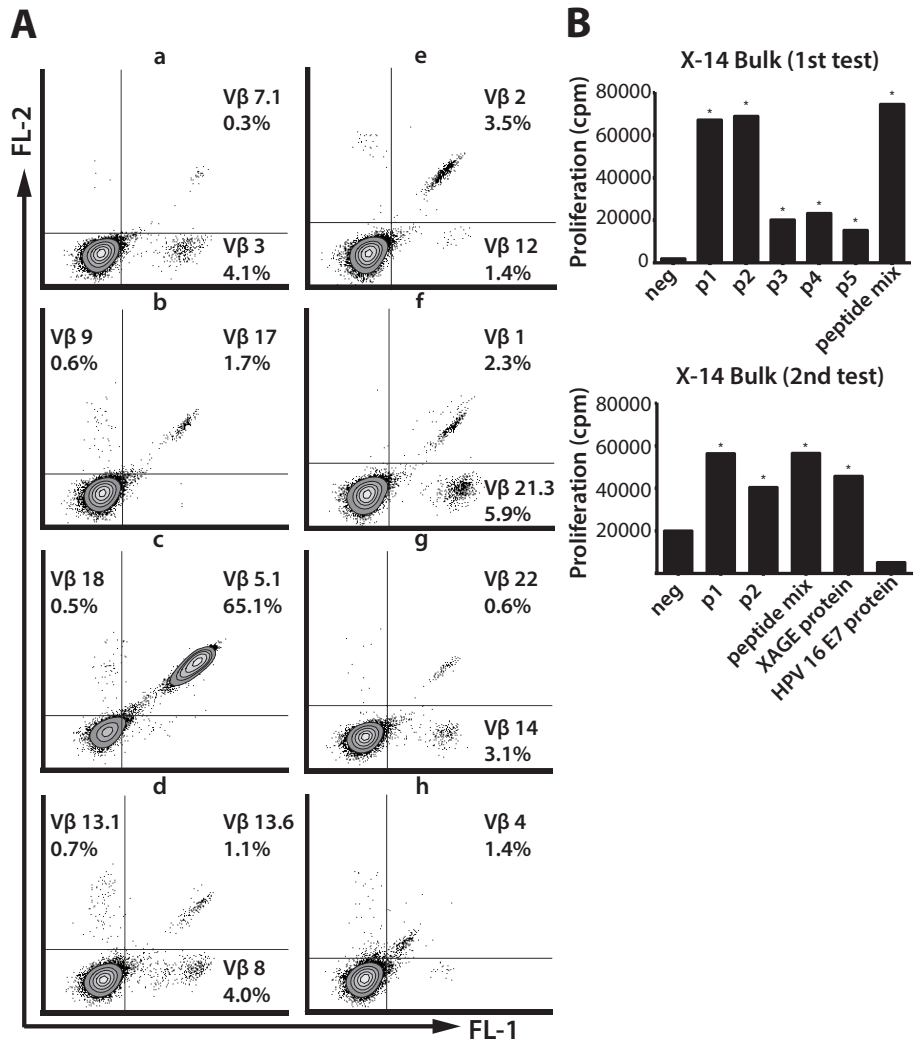

Figure 5. TCR-Vβ expression and XAGE-1b-specific proliferation of bulk cultured PBMCs of patient X-14
A) Sixteen different TCR-Vβ families were discovered in the bulk cultured PBMCs of patient X-14. **B)** XAGE-1b specific proliferation (conducted in two separate assays) demonstrated a broad response to all 5 overlapping XAGE-1b peptides, the peptide mix and to XAGE-1b protein. **C)** In total, 10 clones were isolated from the expanded PBMC; XAGE-1b specific proliferation and TCR-Vβ usage are shown for 3 clones with different patterns of antigen recognition (p1, p2, the peptide mix and XAGE-1b protein) and TCR-Vβ expression (Vβ 21.3 and 5.1).

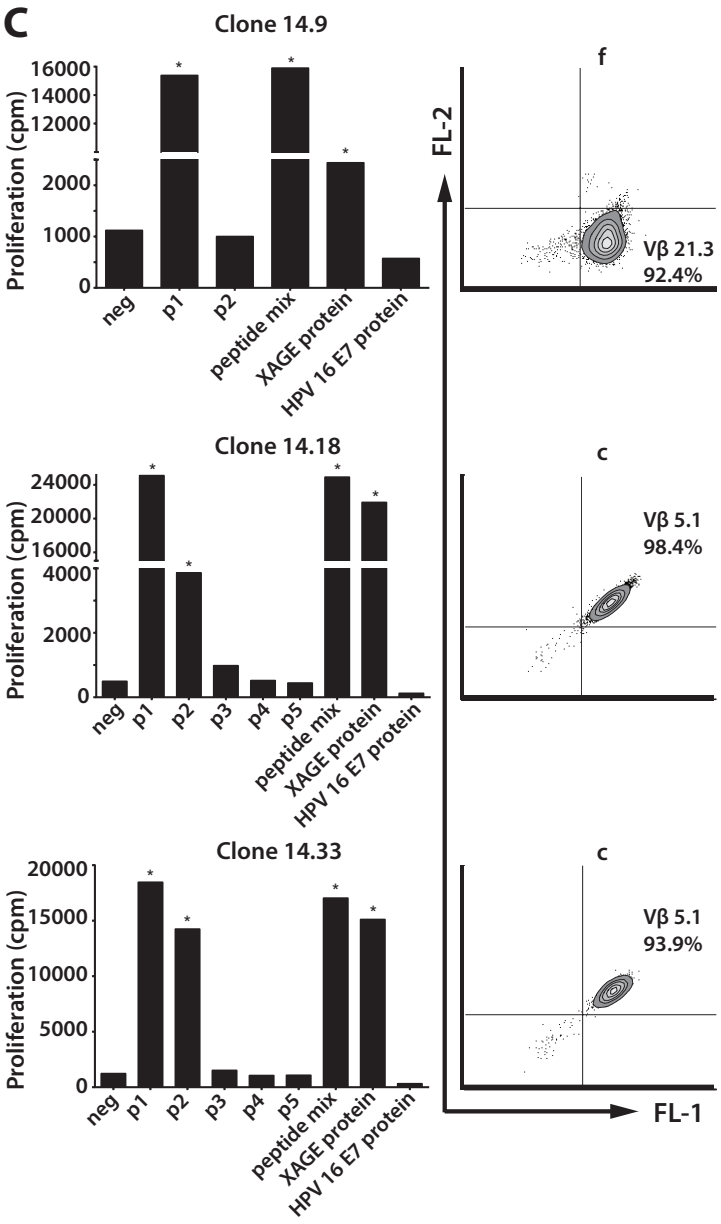

Figure 5. (continued) TCR-Vβ expression and XAGE-1b-specific proliferation of bulk cultured PBMCs of patient X-14

A) Sixteen different TCR-Vβ families were discovered in the bulk cultured PBMCs of patient X-14. **B)** XAGE-1b specific proliferation (conducted in two separate assays) demonstrated a broad response to all 5 overlapping XAGE-1b peptides, the peptide mix and to XAGE-1b protein. **C)** In total, 10 clones were isolated from the expanded PBMC; XAGE-1b specific proliferation and TCR-Vβ usage are shown for 3 clones with different patterns of antigen recognition (p1, p2, the peptide mix and XAGE-1b protein) and TCR-Vβ expression (Vβ 21.3 and 5.1).

DISCUSSION

This is the first study on the immunogenicity of XAGE-1b in a Caucasian study cohort of 40 pulmonary adenocarcinoma patients. We found XAGE-1b protein expression to be present in 43.6% of cases, which is within the frequency range (33-53%) reported for Asian patients [10,11,24]. XAGE-1b was expressed both in primary and metastatic tumor specimens. However, XAGE-1b positive tumors do not show protein overexpression in all tumor samples nor in all fields covering an area that approximates the size of a bronchial biopsy. Hence, the frequency of XAGE-1b overexpressing tumors can be underestimated when only assessing a single section of the tumor and most likely when a biopsy is analyzed. Based on our findings, showing that sometimes only 2 out of 10 randomly analyzed tumor fields stain positive for XAGE-1b, its status is probably best assessed by the analysis of at least two sections of the primary tumor or 5 biopsies. Moreover, our study reveals for the first time the presence of XAGE-1b specific T cells in the primary lung tumor and the tumor-draining lymph nodes from 2 out of 20 evaluated patients. The XAGE-1b reactive T cells displayed both a Th1 (IFNγ/TNFα) and Th2 (IL-5/IL-10) cytokine polarization. These results indicate that XAGE-1b-specific T cells can contribute to the anti-tumor response but the low response rate among patients also implies that the spontaneous activation of T cells to XAGE-1b is limited.

In our cohort, we observed XAGE-1b-specific IgG antibodies in 3 of 40 patients (7.5%). Two previous studies with Asian adenocarcinoma patients also assessed anti-XAGE-1b antibody frequency. The first study [11] found a XAGE-1b IgG frequency of 8.9% in a similar (mainly stage I/II) patient group. The second study [13] found a higher frequency (19%) of XAGE-1b IgG responses but this was in stage IIIb/IV patients. Possibly, XAGE-1b-specific antibodies are more prevalent in patients with a more advanced tumor stage, as also observed for antibodies against p53, NY-ESO-1 and survivin [25].

The presence of XAGE-1b specific IgG antibodies was accompanied by an antigen-specific T cell response in the peripheral blood of all 3 antibody positive patients. In one patient (X-4), an *ex-vivo* IFNγ response to XAGE-1b peptide mix was found by ELISPOT assay, whereas in two patients (X-4 and X-14) Th1 and Th2 cytokine responses were found after one round of *in vitro* stimulation of PBMC with XAGE-1b overlapping peptides. In depth analysis revealed that the XAGE-1b-specific T-cell population comprised both CD4 and CD8 T cells producing both Th1 and Th2 cytokines. The T cells reacted mainly to the overlapping peptides p1 and p2. Recently this N-terminal part of XAGE-1b was shown to comprise a CD4+ T-cell epitope (aa 18-31) and an overlapping CD8+ T-cell epitope (aa 21-29) [13]. Our study confirms this part of XAGE-1b as a T-cell epitope containing domain. Interestingly, while the T-cell response was predominantly directed against sequences present in p1 and p2, the dominant B-cell response was targeted to p5 of XAGE-1b within the small group of patients positive in this study.

In one patient (X-27, **Supplementary Figure 6**), an underlying XAGE-1b specific T-cell response was only detected after removal of a myeloid $CD14^+$ population from the PBMC sample. Immature myeloid cell populations have been shown to inhibit T cell activation [26,27], and their suppressive activity has been specifically reported for NSCLC [22,23,28]. Therefore, removal of the potentially suppressive myeloid cell populations from the peripheral blood of adenocarcinoma patients before analysis of T cell responses might reveal a higher percentage of XAGE-1b specific T-cell responders among Caucasian patients. In addition, based on the current success with co-inhibitory receptor blocking in patients with NSCLC [7] one could consider to test the response in assays where known co-inhibitory receptors are blocked.

In conclusion, our study demonstrates that XAGE-1b acts as a genuine tumor antigen eliciting integrated systemic and/or tumor-infiltrating antigen-specific humoral and cellular immune responses in Caucasian patients with pulmonary adenocarcinoma. As such, this tumor antigen forms an attractive target for active immunotherapy in lung cancer using XAGE-1b based therapeutic vaccines. Based on the presence of these integrated XAGE-1b-specific responses in non-vaccinated patients, it is not to be expected that the induction of such a response by XAGE-1b vaccination will result in safety problems. Based on these results, we have recently started a phase 1 clinical trial with XAGE-1b synthetic long peptides.

ACKNOWLEDGEMENTS

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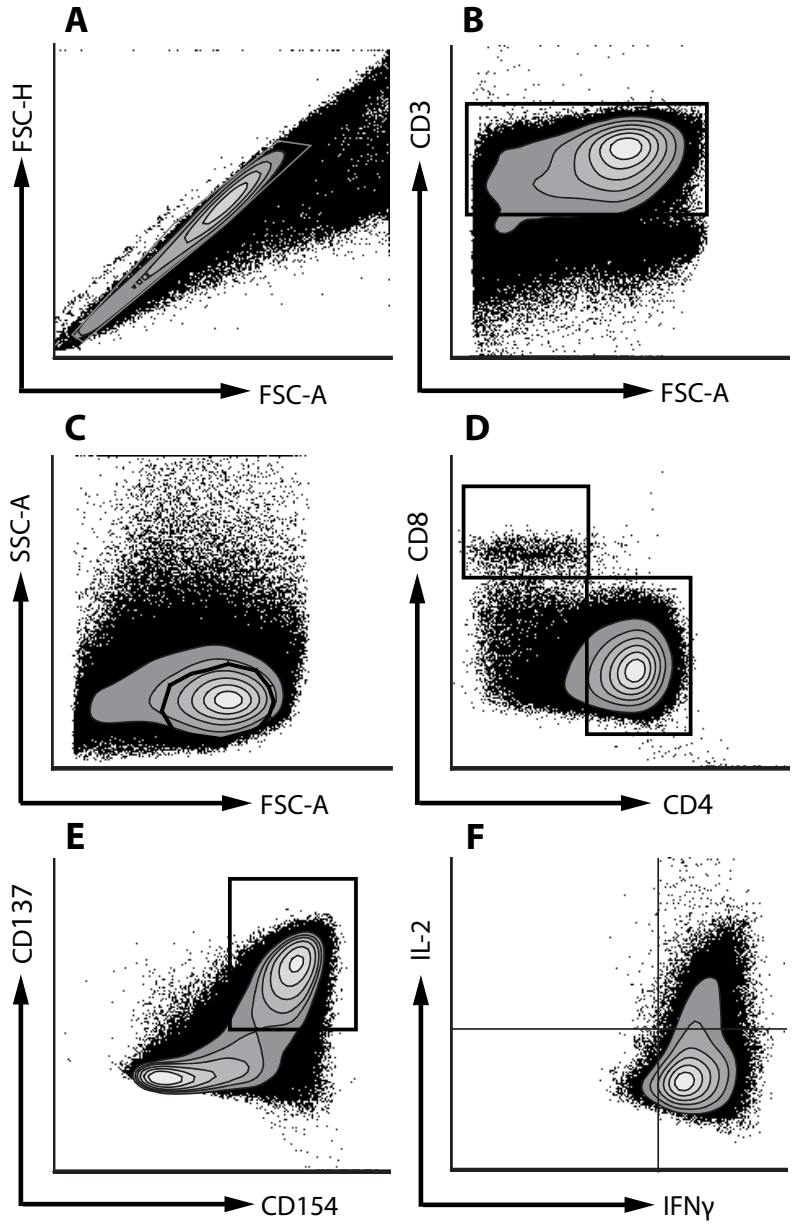
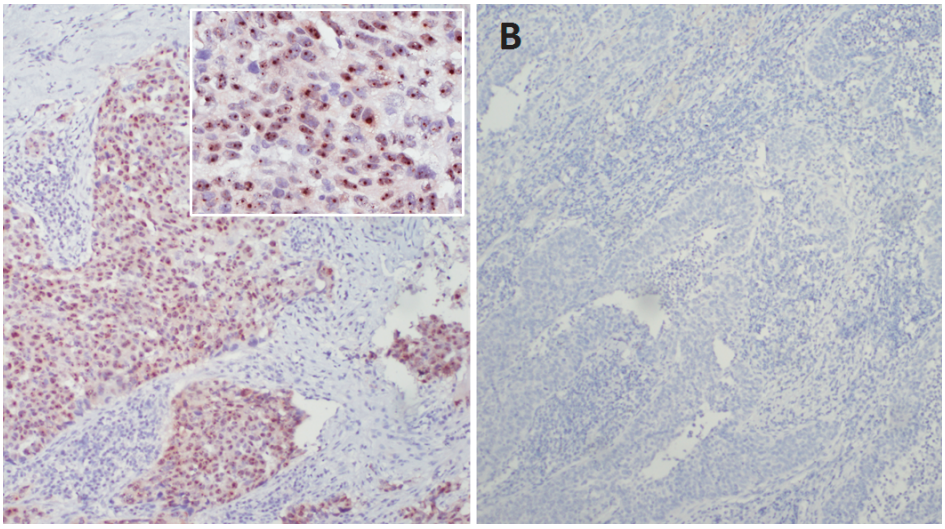
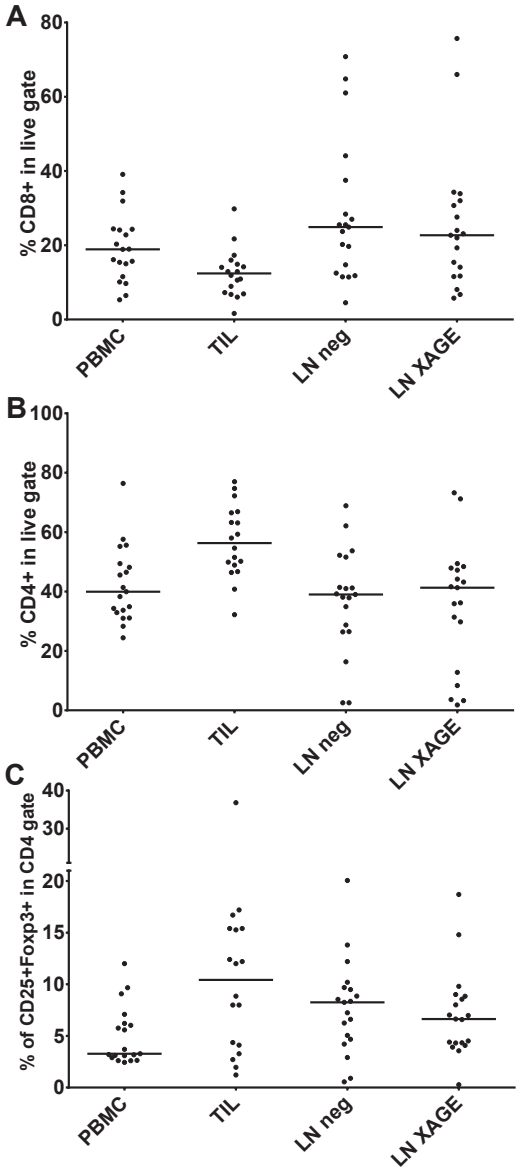


Supplementary Figure 1. Example of gating strategy
First, gating is performed to include only single cells (singlets) (**A**) and CD3+ cells (**B**). After gating on the lymphocyte population (**C**), the CD4 and CD8 gates are set (**D**). The CD4 population is plotted for T cell activation markers CD137 and CD154 (**E**). A gate is set around the double positive population after which IFNγ and IL-2 upregulation within this population is plotted (**F**).

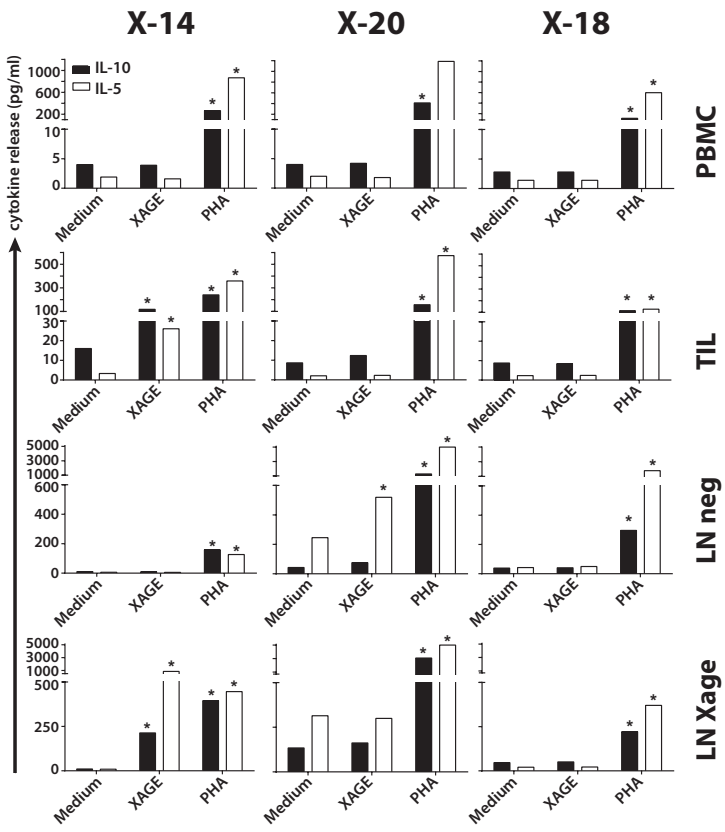


Supplementary Figure 2. XAGE-1b immunohistochemistry
Examples of **A)** a XAGE-1b positive tumor (patient X-14) with a diffuse (>50% tumor cells positive) staining pattern and **B)** a XAGE-1b negative tumor (patient X-18) at a magnification of 200x. The insert in **A)** is a digital magnification showing the typical nuclear staining of XAGE-1b in cancer cells.



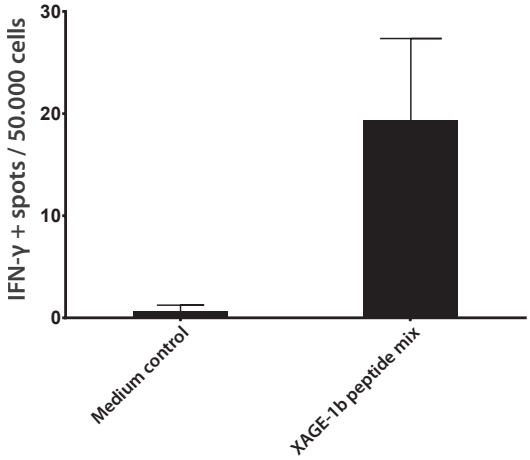
Supplementary Figure 3. Frequency of T-cell phenotypes in peripheral blood, tumor and its draining lymph nodes

T cells were analyzed by flow-cytometry after being stained for CD4, CD8, CD25 and Foxp3. T cells were derived from PBMC (direct ex-vivo), primary tumor (TIL, *in vitro* expanded) and draining lymph node (LN) expanded *in vitro* in the presence (LN XAGE) or absence (LN neg) of overlapping XAGE-1b peptides. Results from 20 adenocarcinoma patients are shown (dots) and the grand median is displayed (horizontal line).
A) $CD8^+$ and **B)** $CD4^+$ T-cell frequency in live cells. **C)** Frequency of $CD4^+CD25^+Foxp3^{high}$ T cells in PBMC, TIL and LN cells. A gating example is displayed in **Supplementary Figure 1**.

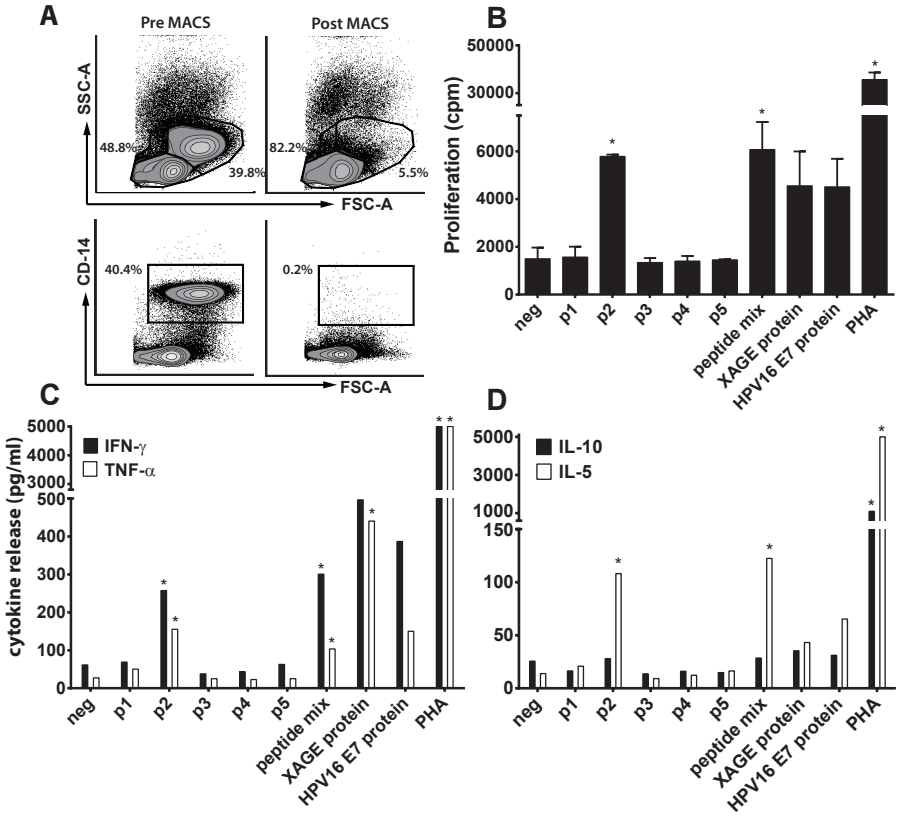


Supplementary Figure 4. Local XAGE-1b mediated immunity, Th2 response

Day 2 supernatants from PBMC, TIL and LN cells tested in a proliferation assay were analyzed for Th2 cytokine release (IL-10, IL-5). A positive response (indicated with asterisks) was defined by a cytokine concentration above the cut-off value (above 20 pg/mL) and more than twice the concentration of medium control. PHA was used as positive control. Results from two positive (X-14, X-20) and one negative (X-18) patients are shown.

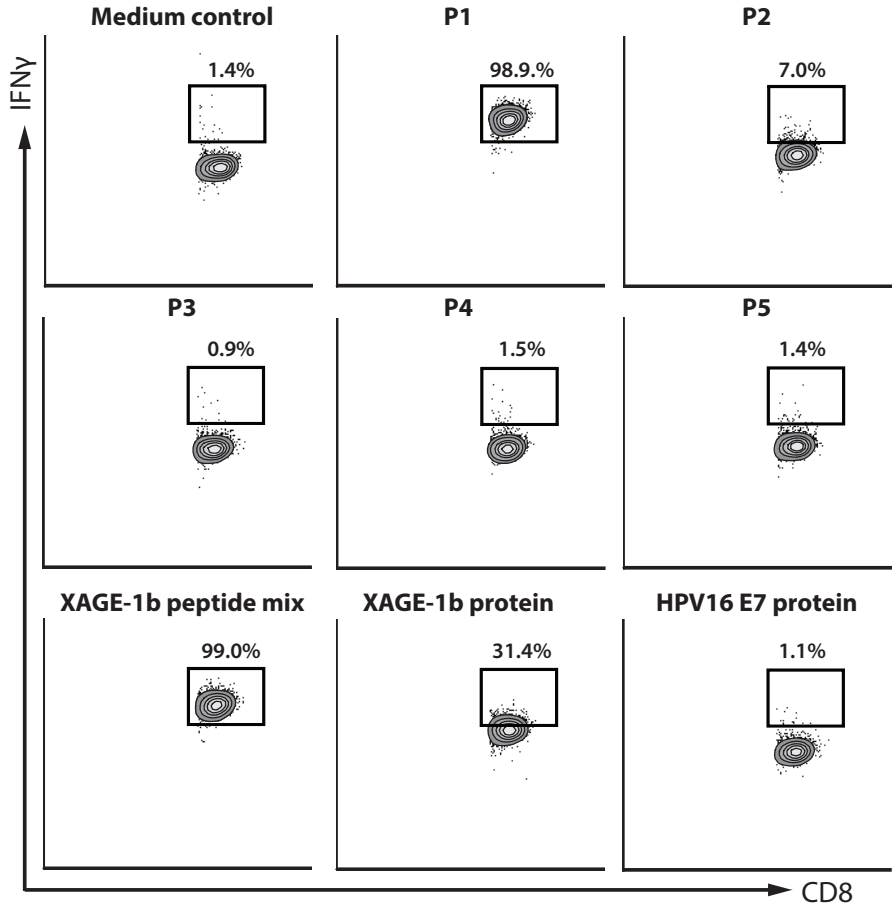


Supplementary Figure 5. Ex-vivo XAGE-1b specific IFNγ response by ELISPOT assay
PBMC samples of patient X-4 were evaluated for XAGE-1b reactivity in a direct ex-vivo ELISPOT assay as described in the Methods section. XAGE-1b specific T-cell frequencies were considered to be increased compared to medium control when frequencies are ≥ 1/10,000 cells.



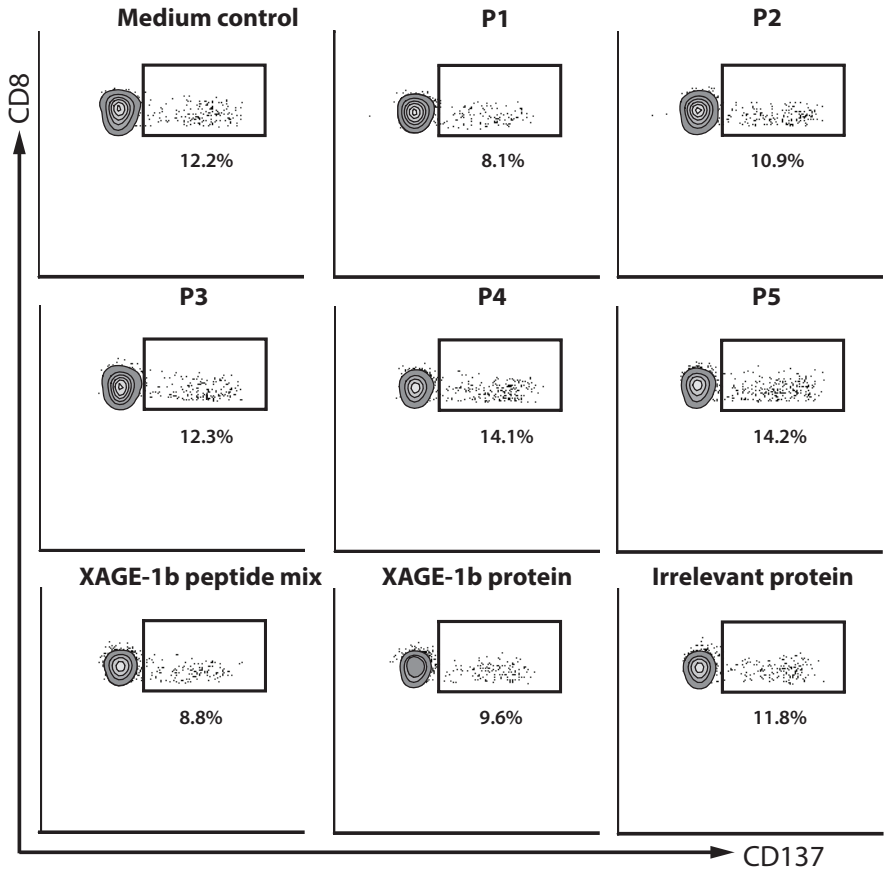
Supplementary Figure 6: Circulating T cells show a XAGE-1b-specific response after removal of CD14+ cells

A) Patient X-27 displayed a high frequency of myeloid cells in PBMCs (39.8%). CD14+ cells were removed from PBMCs by magnetic-activated cell sorting. The contour plots before and after sorting show the removal of the CD14+ cells (5.5% post-sorting) which were the main population of myeloid cells. **B)** The remaining cells were stimulated for 10 days with XAGE-1b overlapping peptides, after which XAGE-1b-specific proliferation was demonstrated for peptide p2 and XAGE-1b peptide mix. The supernatants of these cultures were analyzed for secretion of **C)** IFNγ and TNF-α and **D)** IL-5 and IL-10. Asterisks indicate a positive response.



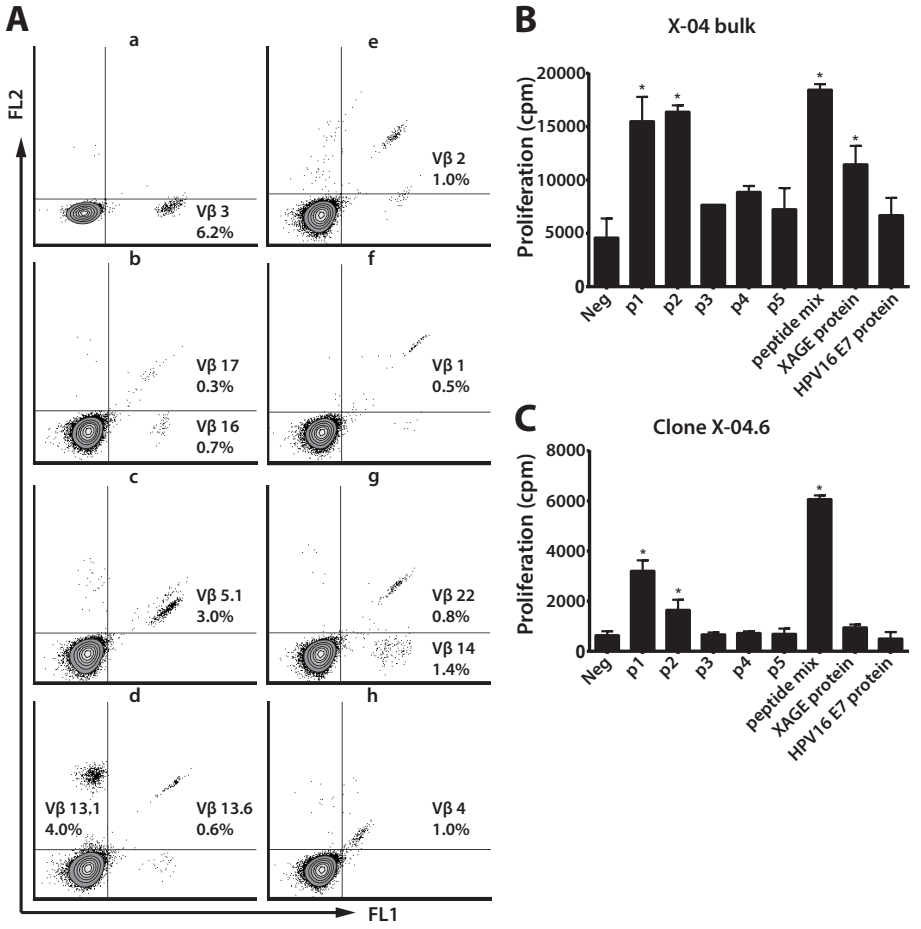
Supplementary Figure 7. XAGE-1b-specific CD8 T cell response

The $CD8^+$ T cells in the 8 weeks cultured PBMCs of patient X-14 were gated (see **Supplementary Figure 1**). These expanded $CD8^+$ T cells showed an IFNγ response when stimulated with APCs pulsed with individual peptide p1, the peptide mix and to XAGE-1b protein, but did not react after stimulation with the other peptides or the negative control protein (HPV16 E7).



Supplementary Figure 8. Expression of T-cell activation marker CD137 in patient X-14

The $CD8^+$ T cells in the 8 weeks cultured PBMC of patient X-14 were gated (see **Supplementary Figure 1**) and tested for XAGE-1b peptide (p1-p5) and protein specific expression of CD8 T-cell activation marker CD137. Although a peptide-specific intracellular IFNγ production was observed particularly for p1, peptide mix and XAGE-1b protein (see **Supplementary Figure 7**), this reaction was not accompanied by an up-regulated expression of CD137.



Supplementary Figure 9. TCR-Vβ expression and XAGE-1b-specific proliferation of bulk cultured PBMC of patient X-4

A) Analysis of TCR-Vβ usage of bulk cultured PBMC of patient X-4. Expression was determined using eight sets of antibodies (a-h), each consisting of three differently labeled antibodies [FITC (FL1), PE (FL2), or FITC-PE combined] specific for 3 different TCR-Vβ families, ultimately covering about 70% of the human TCR-Vβ repertoire. Eleven different TCR-Vβ families (percentage of responding cells indicated in the quadrants) were demonstrated in the expanded PBMCs that were kept in culture during cloning protocol. **B)** Proliferation assay of bulk cultured PBMCs of patient X-4 shows specific response to peptide p1, p2, the peptide mix and XAGE-1b protein. Asterisks indicate a positive response. **C)** Proliferation assay of clone X-4.6 shows a proliferation response to peptide p1, p2 and the peptide mix. Asterisks indicate a positive response.

Chapter 8

Phase I Study: safety and immunogenicity of vaccination with XAGE-1b long peptides combined with poly-ICLC in patients with stage IIIb/IV pulmonary adenocarcinoma

Adapted study protocol with preliminary results
(Protocol ID: NL44189.000.13)

INTRODUCTION

Lung cancer

Lung cancer is the most common cause of cancer mortality in men in the developed world and one of the leading causes in women[1]. The two major forms of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC comprises about 80 % of all lung cancers[2]. NSCLC patients are staged according to the TNM classification system[3-5]. Patients with stage I and stage II non-small cell lung cancer are, when operable, treated with complete surgical resection[6]. Patients with stage III disease are treated with combined chemo-radiotherapy[7]. In the LUMC, stage III adenocarcinoma patients are generally treated for 25 working days by a combination of daily shots of cisplatin followed by radiotherapy. Patients with stage IV disease are treated with palliative chemotherapy[8]. In the LUMC, stage IV NSCLC patients are generally treated with three schemes of chemotherapy (one scheme lasting 21 days) depending on the histology of the primary tumor; squamous cell carcinoma is treated by a combination of carboplatin and gemcitabine, whereas adenocarcinoma is treated by a combination of carboplatin and pemetrexed.

The five-year survival for lung cancer patients is poor. This can in part be explained by the fact that the majority of lung cancer patients present with advanced disease (stage III/IV). For NSCLC patients, the 5-year survival is 14%[2,9]. The medical need for new treatment strategies for lung cancer is therefore high. New anti-cancer therapies have been advocated in recent years, especially for stage IV lung cancer patients. For example, tyrosine kinase inhibitors (e.g. Iressa, Tarceva) have been shown to have the potential of long term suppression of tumor activity in patients with lung adenocarcinoma who have a somatic mutation in the Epidermal Growth Factor Receptor (EGFR) gene[10]. Immunotherapy with therapeutic vaccines targeting specific tumor antigens is another promising treatment strategy for lung malignancies.

Immunotherapy using synthetic long peptides

The immune system plays a major role in cancer. Professional antigen presenting cells (APC), mostly dendritic cells (DC), sample antigen that is secreted or shed by tumor cells. The tumor antigens are processed and presented as peptides by major histocompatibility complex (MHC) class I and II molecules on the DC surface, and recognized by the T-cell receptor (TCR) of T-cells. Major histocompatibility complex (MHC) class I – peptide complexes are recognized by CD8+ T-cells that become activated and differentiate into cytotoxic T-lymphocytes (CTL). MHC class II – peptide complexes are recognized by the TCR of the CD4+ T-helper (Th) cells. Upon stimulation, tumor-specific CD4+ T-cells deliver essential help for tumor-specific CD8+ T-cells by fully activating DC through the CD40-CD40L signaling pathway as well as by secretion of IL-2[11]. Moreover, the tumor-specific

CD4+ T-cells provide help by guiding the homing of CD8+ T-cells and by polarizing the local tumor environment to allow tumor-specific CD8+ T cells to exert their tumoricidal function[12]. In the ideal situation, the close interaction between DC, CTL and Th cells will lead to control of tumor growth and ultimately tumor regression.

At the LUMC, we are currently developing a therapeutic vaccination strategy against HPV16 and p53 expressing malignancies making use of synthetic long peptides (SLP) based on the associated tumor antigens. In experimental models, SLP-based vaccines have shown to be superior to protein vaccines as they induce stronger CTL responses in a direct comparison[13] as well as over viral vector vaccines[14]. The SLP-based vaccines used so far covered non-viral (p53) as well as virus-encoded tumor antigens (HPV-16 E6/E7) and these have turned out to be safe in patients and induced robust T-cell responses in several early phase clinical trials in patients with malignant disease[15-17]. Moreover, vaccination of patients with HPV-16 induced high-grade (premalignant) vulvar intraepithelial neoplasia (VIN 3) with an HPV16-based SLP resulted in complete and durable regressions of VIN 3 lesions[18]. Importantly, a clear link was established between the kinetics and phenotype of the immune response (strong and broad vaccine-prompted HPV16-specific proliferative response with higher IFNγ and IL-5 levels) and complete regression of the lesion[19]. These results showed for the first time that immunotherapy against existing neoplastic lesions by therapeutic vaccination can be truly effective in human beings.

Rationale of XAGE-1b as target for long peptide vaccination in lung cancer

A prerequisite for immunotherapy of lung cancer with SLP-based vaccination is the identification of suitable tumor antigens that provide an immunological window for immunotherapy. Tumors express antigens that – when presented by DCs – can elicit adaptive immune response aimed at eradicating the primary tumor. These so-called tumor antigens can be classified in three categories: 1) antigens that are overexpressed in tumors; 2) antigens that are of viral origin; and 3) antigens that are specifically expressed in several different tumors[11,14].

An interesting group of tumor antigens, frequently recognized by the immune system of cancer patients due to their restricted expression pattern, is encoded by 'cancer-testis'(CT) genes. These CT antigens are present in a significant subset of tumors, including NSCLC. XAGE-1 is a member of the family of CT antigens. The XAGE-1 protein has 4 transcripts (a, b, c and d), of which XAGE-1b (81 amino-acids) is the isoform that is mainly expressed [20-22]. Using a monoclonal antibody against XAGE-1b (USO9-13), immunohistochemical nuclear staining has been observed in 25/47 lung adenocarcinomas, 1/12 hepatocellular carcinomas and 1/11 gastric cancers, but not in adjacent normal tissues[22], indicating highly preferential expression of XAGE-1b in lung adenocarcinoma, a subtype that accounts for 40% of NSCLC. Our preliminary data, using the same monoclonal

antibody (USO9-13), confirm the percentage of XAGE-1b expression in lung adenocarcinoma. Staining of paraffin-embedded resected lung adenocarcinoma tissue revealed positive staining in 4 out of 8 patients (50%). An association of XAGE-1b protein expression in lung adenocarcinomas with clinical prognosis has been suggested[20]. Circulating antibodies generated against XAGE-1b have been observed in 10% of patients with lung adenocarcinoma[21]. The presence of XAGE-1b-specific antibodies is indicative for an underlying XAGE-1b-specific CD4+ T-cell response. In addition, it has been shown that full-length XAGE-1b protein-pulsed human DCs are able to induce specific CTL responses in vitro[23]. These data suggest that (1) there is no immune tolerance against XAGE-1b at the CD4+ and CD8+ T-cell level; (2) the XAGE-1b amino acid sequence comprises CD4+ and CD8+ T-cell epitopes; and (3) this protein is sufficiently immunogenic to spontaneously trigger an immune response in patients.

To confirm these results in a Dutch population, a preclinical study was conducted to detect immune responses against XAGE-1b. Overexpression of XAGE-1b was found in 17 of 39 (43.6%) pulmonary adenocarcinomas by immunohistochemistry. In 20 patients, analysis of T-cells isolated and expanded from the primary tumor and its draining lymph node demonstrated XAGE-1b specific responses in 2 patients. XAGE-1b-specific IgG antibodies (detected by ELISA) were found in serum from 3 out of 40 patients (7.5%). All three antibody positive patients also displayed a systemic T-cell response to XAGE-1b, measured by proliferation, cytokine production and upregulation of T-cell activation markers CD137 and CD154 and intracellular expression of IL-2 and IFN-γ in peripheral blood mononuclear cells (PBMCs). Subsequently established T-cell clones from these bulk cultures showed strongest proliferation against XAGE-1b peptide 1 (amino acid 1-32) and peptide 2 (amino acid 18-42), the N-terminal part of the XAGE-1b protein[24].

These findings are in line with a recent study on the spontaneous immune response against XAGE-1b in non-small cell lung cancer (NSCLC) patients. An antibody response against XAGE-1b was observed in 10% (20/200) of NSCLC patients and in 19% (13/69) of stage IIIB/IV lung adenocarcinoma patients. A CD4+ T-cell response was detected in 88% (14/16) and a CD8+ T-cell response in 67% (6/9) in the XAGE-1b antibody-positive patients examined[23,25].

Rationale of Montanide ISA 51 VG and Hiltonol® as adjuvants to a XAGE-1b SLP vaccine

Therapeutic long peptide vaccines can be improved by adding adjuvants. These are an important component of vaccine formulations since they can enhance immune responses[26]. Montanide ISA 51 VG is defined as a mixture of a highly purified mineral oil (Drakeol 6VR) and a surfactant (Mannide monooleate). When mixed with an aqueous phase in a 50/50 ratio, it renders a water in oil emulsion. This water-in-oil emulsion is frequently used as an adjuvant in numerous clinical trials[27-30]. Most common local reactions

are local pain, tenderness, erythema and granuloma at the injection site. In a less extent, indurations and swelling are described[26]. Trials performed by our group combining a SLP vaccine with Montanide ISA 51 VG induced vaccine peptide specific T-cell responses[15,17]. However, results from the first two trials with p53-SLP showed that vaccine-induced Type 1 T-helper cells produced only low amounts of the key cytokines such as IFN-γ and IL-2, indicating that these responses, although present, were not properly polarized by Montanide ISA 51 VG alone[16,31].

Therefore, to benefit from the tumor-specific T-cells at the tumor site, peptide vaccines should be combined with immune modulating adjuvants that specifically induce polarization Th1/CTL response. This can be achieved by using Toll-like receptors (TLR) agonistic compounds[14,26]. TLR are a class of proteins that play a key role in the innate immune system. These receptors recognize conserved molecules derived from microorganisms and can subsequently induce immune responses[32]. TLR3 is the specific intracellular recognition system that responds to the intracellular presence of RNA virus infection. It recognizes and signals in response to the intracellular presence of dsRNA intermediates, resulting in a local cytokine burst[33]. Polyribosinic:polyribocytidic acid (Poly-IC) is a TLR3 agonist which has been to shown to induce DC maturation and to mediate the release of IFNγ, IL-4, IL-6 and IL-12[34,35]. In a PSA-transgenic mouse model, it could augment both cellular and humoral immune responses to PSA and PSA-anti-PSA immune complex[34]. Importantly, Hiltonol® (Oncovir Inc., Washington DC), a modified poly-IC stabilized with polylysine (poly-ICLC), has been tested and shown to be safe in several recent immunotherapy clinical trials, including studies in which it was combined with antigenic or peptide-based vaccination[36,37].

Montanide ISA 51 VG and Hiltonol® have also been combined. Recently, results were reported of a phase I trial in which ovarian cancer patients were treated with another CT antigen based SLP vaccine (NY-ESO-1 overlapping peptides (OLP)) using Montanide ISA 51 VG and Hiltonol® as adjuvants. The vaccine was generally well tolerated and was not associated with a high rate of severe adverse events (SAE), laboratory abnormalities, abnormalities in vital signs and physical examinations, or decrease in Karnofsky performance status. Most notably, the combination of vaccine, Montanide ISA 51 VG and Hiltonol® displayed the best capacity to induce NY-ESO-1 specific immune responses in patients as opposed to OLP vaccine only or OLP vaccine with Montanide ISA 51 VG[36].

In conclusion, XAGE-1b is an attractive target for immunotherapy due to its preferential expression in lung adenocarcinoma and due to its potential to elicit spontaneous humoral and cellular immune responses in patients with adenocarcinoma. Furthermore, adding Montanide ISA 51 VG and Hiltonol® as adjuvants to a peptide vaccine can help boost antigen specific immune responses. Here, we describe a phase I clinical trial designed to evaluate the safety and immunogenicity of XAGE-1b SLP vaccine combined with Montanide ISA 51 VG and Hiltonol® in patients with lung adenocarcinoma.

OBJECTIVES

Primary objective:

– To evaluate the safety of vaccination with XAGE-1b peptides emulsified in Montanide ISA 51 co-mixed with the adjuvant Hiltonol® (Poly-ICLC) in patients with stage IIIb/IV pulmonary adenocarcinoma.

Secondary objective:

– To evaluate the capacity of the vaccination strategy to induce XAGE-1b-specific humoral and cellular immune responses in lung adenocarcinoma patients, including the migratory capacity of XAGE-1b vaccine-induced T-cells into the vaccine injection site.

STUDY DESIGN

This uncontrolled exploratory phase I study will be performed in 30 patients with stage IIIb/IV primary pulmonary adenocarcinoma patients, who have been previously treated at the department of Pulmonology with combined chemo-radiotherapy (stage III) or with chemotherapy alone (stage IV). An overview of this phase I trial is presented in **Figure 1**.

After obtaining informed consent, patients will be screened for eligibility in the study. The patients will receive 4 vaccinations consisting of 5 synthetic overlapping peptides, covering the entire XAGE-1b protein, emulsified in Montanide ISA 51 and co-mixed with the adjuvant Hiltonol®, by subcutaneous injection in one of the arms. Vaccinations will be given at three week intervals, each at another limb. The first 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma) will be vaccinated with a peptide dose of 50 mg together with an adjuvant dose of 1 mg. The groups will start simultaneously. In the next 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma), the dose of peptides will be simultaneously increased to 150 mg together with an adjuvant dose of 1 mg. The last 2 groups of 5 patients (stage IV and stage IIIb adenocarcinoma) will be simultaneously vaccinated with a peptide dose of 300 mg together with an adjuvant dose of 1 mg. A schedule of events per study patient is displayed in **Figure 2**.

Inclusion criteria

For stage IV pulmonary adenocarcinoma patients:

– Histologically proven pulmonary adenocarcinoma stage IV or stage IIIb according to recent guidelines on TNM classification of NSCLC[4]
– Age ≥ 18 years

- Completion of standard (platinum-based) chemotherapy schedules or combined chemo-radiotherapy schedule (concurrent or sequential)
- No intention for further chemotherapy treatment
- Good WHO performance status (0-2)
- Adequate bone marrow function: WBC ≥ 2.0 x 10^9/l, platelets > 100 x 10^9/l, hemoglobin > 5.0 mmol/L
- Patients of child-bearing potential should test negative using a serum pregnancy test
- Survival expectation > 3 months

Exclusion criteria

- Candidates eligible for restaging and curative surgical resection of the primary tumor after good response to treatment with chemo-radiotherapy
- Progressive disease after finishing standard chemotherapy or chemo-radiotherapy schedule
- Inadequate bone marrow function more than 3 weeks after last chemotherapy treatment.
- Poor WHO performance status (3-5)
- Eligibility for treatment with Tyrosine Kinase Inhibitors (e.g. erlotinib)
- History of an autoimmune disease or other systemic intercurrent disease that might affect the immunocompetence of the patient, or patients receiving immunosuppressive therapy including transplant recipients
- Second primary tumor of non-pulmonary origin
- CD4 cell count < 200/m3 at baseline
- Known seropositivity for Hepatitis B Virus and/or HIV
- History of serious liver or kidney dysfunction, heart condition or thyroid disorder
- Receipt of another investigational product within the previous 4 weeks or at any time during the study period.
- Medical or psychological condition which in the opinion of the treating chest physician and investigator would not permit the patient to participate in or to complete the study

METHODS

Safety will be assessed during the whole study by collecting all adverse events according to the Common Terminology Criteria (CTC) version 3 and by monitoring vital signs, blood chemistry and hematological parameters[38]. A total of 250 ml of fresh blood will be collected from all patients at baseline (100ml), after 6 weeks (50 ml) and after 12 weeks (100ml). Immunological responses will be monitored using PBMCs that are tested

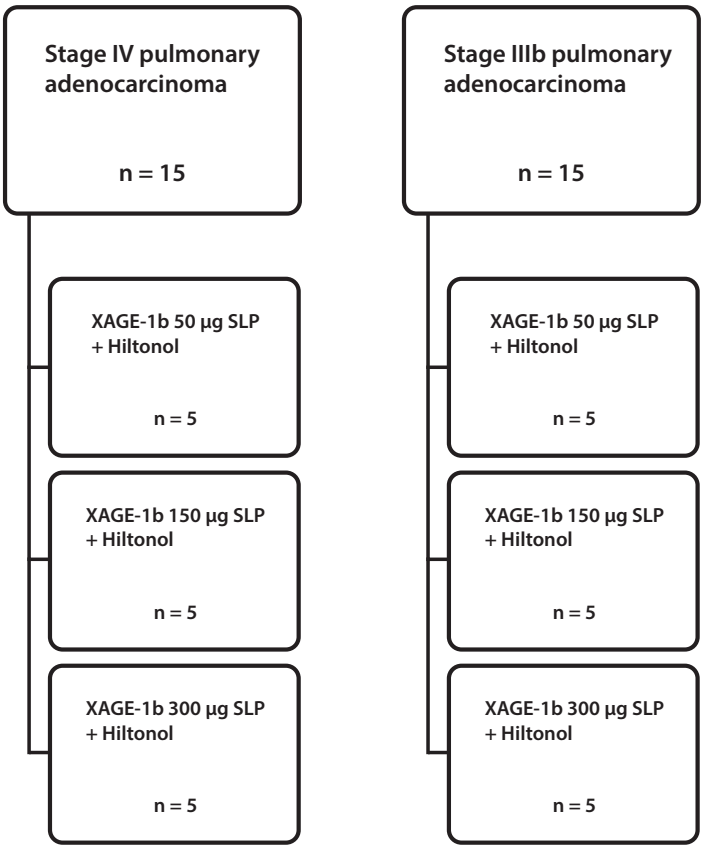


Figure 1. Overview of phase I study. In a dose-escalating fashion (50-150-300 µg), thirty pulmonary adenocarcinoma patients (15 stage IIIB and 15 stage IV) will be given a subcutaneous injection of a vaccine consisting of 5 overlapping peptides covering the entire XAGE-1b protein emulsified in Montanide ISA 51 and co-mixed with Hiltonol® (Poly-ICLC). Safety and immunogenicity of the vaccine will be assessed. Abbreviation: SLP synthetic long peptides

by IFNγ-ELISPOT and intracellular IFNγ/IL-2 staining for directly ex-vivo detection and enumeration of antigen-specific CD4+ and/or CD8+ T-cells, as well as following one round of in vitro stimulation. In addition, proliferation (lymphocyte stimulation test: LST) and associated cytokine production (IFNγ, TNFa, IL-4, IL-5, IL-10, and IL-2) will be assessed. Furthermore, a skin biopsy of the last vaccination site will be used to assess the migratory capacity of vaccine-induced T-cells. Finally, we will collect blocks of paraffin embedded tumor tissue of all patients in order to study the expression of XAGE-1b and other associated tumor antigens.

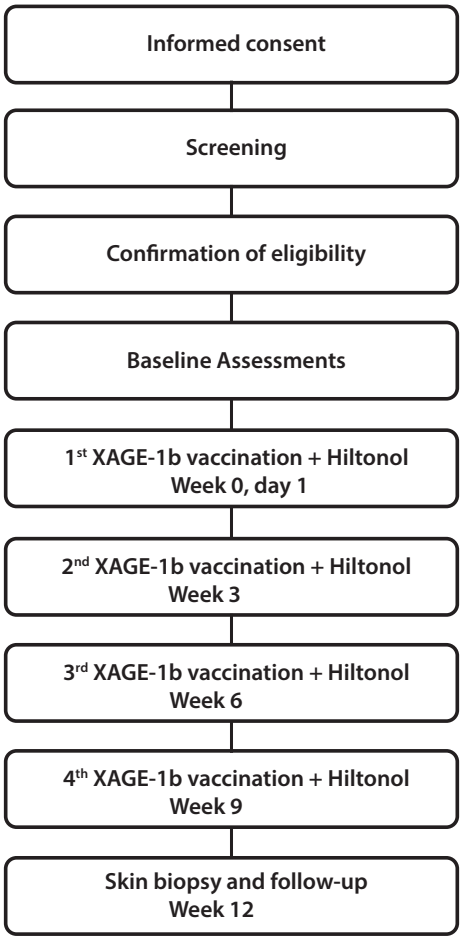


Figure 2. Schedule of events. Adverse events will be monitored throughout the study. For immunomonitoring, blood samples are collected at day 1, 22 and 43, and a skin biopsy of the last vaccination site at week 12.

Immunomonitoring of local changes

A biopsy sample of the last vaccination site obtained 3 weeks after vaccination will be used to monitor the migration of the vaccine-induced T-cells towards antigen-expressing lesions. T-cells will be cultured out of the biopsy and expanded by exposing them to a cytokine cocktail (TCGF, IL-7 and IL-15) as previously described[39] and tested using the same assays as described below for the monitoring of blood samples.

Immunomonitoring of systemic changes

In order to assess the induction of systemic T-cell immunity against XAGE-1b by the vaccinations, the PBMCs in the collected blood samples will be examined for proliferation (LST), cytokine production (IFNγ, TNFα, IL-4, IL-5, IL-10, and IL-2) as well as by

ELISPOT (IFNγ); a set of complementary and qualified assays for ex-vivo detection of antigen-specific responses. Multiparameter flow cytometry will be used to determine the phenotype (CD4+ and/or CD8+) and the percentage of activated XAGE-1b-specific T-cells in combination with IFNγ and IL-2 production, both directly ex-vivo and after one round of in vitro stimulation.

(1) XAGE-1b peptide-specific proliferation assay and cytokine bead array

XAGE-1b-specific proliferation will be assessed using a short-term (6-day) assay in combination with cytokine production by cytokine bead array (IFNγ, TNFα, IL-4, IL-5, IL-10, and IL-2) on supernatants taken at day 6. This combined assay will be performed according to described SOPs and has been published[16,17,19,40]. The average and standard deviation of the 8 medium only control wells are calculated and the cut-off was defined as this average plus 3xSD. The stimulation index (SI) is calculated as the average of 8 test wells divided by the average of the 8 medium control wells. A positive proliferative response is defined as a stimulation index of at least 3 and the counts of at least 6 out of the 8-wells above the cut-off value[40]. A vaccine-induced response is defined as a SI which is at least 3-fold higher than the pre-existing response[17,19]. Positive antigen-specific cytokine production is defined as a cytokine concentration above the detection limit (i.e. 20 pg/ml) and at least 2x the concentration of the medium control. A vaccine-induced response was defined as at least a 3-fold increase in the antigen-specific cytokine production before vaccination.

(2) XAGE-1b peptide-specific IFNγ-ELISPOT for CD4+ T-cells

IFNγ 4-day ELISPOT is our standard assay for measuring (tumor) antigen-specific T-cell responses and will be performed according to described SOPs and has been published[16,17,19]. Spots are counted with a fully automated computer-assisted-video-imaging analysis system (BioSys 5000). Specific spots are calculated by subtracting the mean number of spots in quadruplicate wells + 2xSD of the medium only control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies are considered to be increased compared to non-responders when specific T-cell frequencies are ≥ 1/10,000. T-cell frequencies are considered to be boosted by the vaccine when they were at least 3-fold higher than those prior to vaccination.

(3) Multiparameter analysis of XAGE-1b-specific T-cells by flow cytometry

This assay measures the percentage and polarization of XAGE-1b-specific T-cells. Peripheral blood lymphocytes, which respond to peptide stimulation in vitro, will be simultaneous stained for the T-cell markers (CD3, CD4 and CD8), activation markers (CD137 and CD154) and cytokines (IFNγ, IL-2) followed by acquisition and analysis by a flow cytometer according to described SOPs as has been published before[16,41]. The presence of XAGE-1b-specific T-cells is considered to be positive when the percentage

of XAGE-1b-peptide stimulated CD4+CD154+CD137+ (double activation markers) or CD8+CD137+ (single activation marker) T-cells is at least twice the percentage detected in the medium only control, and the responding cells should be visible as a clearly distinguishable population in the analysis dotplot. The percentage of IFNγ and/or IL-2 producing XAGE-1b-specific T-cells is determined by gating on the activated T-cell population. A vaccine-induced reaction is defined as at least a 3-fold increase in the percentage of antigen-specific T-cells compared to prior to vaccination. Both directly ex-vivo detection and enumeration of antigen-specific CD4+ and/or CD8+ T-cells, as well as following one round of in vitro stimulation, will be assessed.

(4) ELISA to detect vaccine induced XAGE-1b specific humoral responses

Serum samples (obtained at different time-points before, during and after vaccination) are subjected to a XAGE-1b peptide ELISA for detection of XAGE-1b peptide-specific immunoglobulin G (IgG). A 96-well plate (Costar 3590) is coated overnight at 4°C with the individual XAGE-1b peptides (50 µl of 5 µg/ml diluted in 0.1 M carbonate/bicarbonate coating buffer; Merck, Darmstadt, Germany). Then, the plate is washed six times with phosphate buffered saline (PBS; Fresenius Kabi Bad Homburg, Germany) and blocked for 1 h at room temperature (RT) in 100 µl/well PBS+5% FCS (fetal calf serum), which is assigned as blocking buffer. After six washings with PBS, the serum samples diluted in blocking buffer (1:100) are added to triplicate wells (50 µl/well) and incubated at RT for 2 h. As a positive control, a previously obtained serum sample with high XAGE-1b IgG titer (spontaneously induced) will be used. Subsequently, the plate is washed again and 50 µl/well of goat anti-human IgG-Horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL) (diluted 1:3,000 in blocking buffer) is added and incubated for 1h at RT. Finally, after the six washings tetramethyl-benzidine liquid substrate (50 µl/well TMB, Sigma Aldrich) is added for the colorimetric enzymatic reaction. This reaction is stopped by adding 50 µl/well of 2 M H_2SO_4 (Merck) and the plate will be read in an ELISA reader at 450 nm.

The average OD-value of the triplicate uncoated wells (background value) is calculated and values are discarded if these are more than 2SD above this average. A positive XAGE-1b peptide specific IgG response is defined as at least a 2-fold increase compared to the background value. A 2-fold increase of the post-vaccination serum sample over that of the pre-vaccination serum sample is considered a *vaccine-induced* positive XAGE-1b peptide specific IgG response.

Statistical Analysis

To assess the secondary endpoint we will use the Fisher's exact test to analyze the response rate of the patients reacting to XAGE-1b pre- and post-vaccination among all patients (n=30) as well as among stage IIIb (n=15) or stage IV patients (n=15), irrespective of the dose level. The Chi-square test will be used to analyze potential differences in

the response rate of the patients (stage IIIb and IV combined) at each dose level (n=10 per dose level). The response rate will be analyzed for all 4 indicated immune tests separately. To assess differences in the strength of the response, which is defined as the breadth and the magnitude of the measured immune response within one test group, we will use the median specific spot count (ELISPOT), median stimulation index (LST), or median amount of cytokine production (CBA) obtained for all 5 different peptides per patient, of all patients in one dose group, using the nonparametric Mann – Whitney test with use of GraphPad InStat software, version 6.00 or higher. All reported P values will be two-sided and will not be adjusted for multiple comparisons. $P < 0.05$ is considered to indicate statistical significance.

TRIAL STATUS AND PRELIMINARY RESULTS

Patients are being recruited for this phase I study and currently one patient has been included and has finished his vaccination schedule. The results of LST assay of this patient are shown in **Figure 3**. A vaccine-induced proliferative response (SI index >3, indicated by asterisks) to XAGE-1b peptides X2, X3 and X4 was observed after the first vaccination, but not after the second vaccination. This patient demonstrated a strong response throughout vaccination to influenza matrix 1 protein-derived (Flu) peptides and memory response mix (MRM), which are common viral and bacterial recall antigens respectively. These strong recall antigen T-cell responses reflect an adequate general immune status of this patient.

Furthermore, another 2 patients have been included who have not yet finished their vaccination scheme.

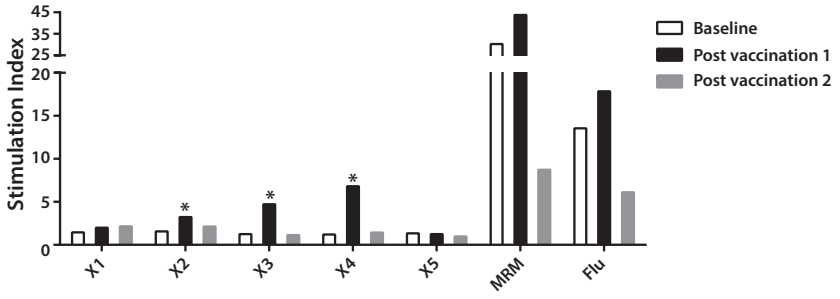


Figure 3. Results from the lymphocyte stimulation test (LST) from the first vaccinated patient (X-VAC-001). A XAGE-1b specific vaccine-induced proliferative response (SI index >3, indicated by asterisks) was observed after the first vaccination, but not after the second vaccination. Flu (influenza matrix 1 protein-derived) peptides and MRM (memory response mix) are common viral and bacterial recall antigens respectively, both of which serve as positive control and as parameters of general adaptive immune status of the patients.

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Chapter 9

Summary and General Discussion

The general introduction of this thesis (**Chapter 1**) gives an overview of epidemiology, classification, the diagnostic process and standard of care treatments of lung cancer. To this day, lung cancer remains a pandemic disease with extraordinarily high morbidity and mortality rates, affecting and killing millions of people around the world[1,2]. Despite this major impact on global health, there is an evident lack of progress in improving the prognosis of this lethal disease, which becomes obvious when examining non-small cell lung cancer (NSCLC) survival rates from the past two decades. In the Netherlands, the five-year overall survival of NSCLC (independent of stage or gender) has improved by a mere 2.6% from 14.8% to 17.4%[3], which reflects a relatively late disease detection of NSCLC patients who generally present with advanced disease[4,5]. Besides the focus on improvements in primary prevention and early diagnosis of NSCLC, research efforts in the field of lung cancer have focused on finding alternative treatment strategies such as immunotherapy[6].

This dissertation deals with two important themes in NSCLC. In **Part I** of this thesis, the main topic is the implementation and optimization of endosonography in the preoperative staging algorithm of NSCLC. In the second part of the thesis (**Part II**), the potential of immunotherapy is assessed, either as an alternative treatment strategy or in combination with standard of care NSCLC therapies.

PART I: ENDOSONOGRAPHY OF LUNG CANCER

Ever since endoscopic ultrasound (EUS), a procedure in which mediastinal lesions are visualized by placing a linear echo-endoscope in the esophagus, was introduced to the field of pulmonology[7,8] several exploratory studies demonstrated promising performance (sensitivity, specificity and accuracy), a strong decline in futile thoracotomies and low complication rates for this new technique[9-12]. However, one common limitation of these studies was that mostly a fixed study design was chosen with preselected patients based on enlarged and/or 18F-fluorodeoxyglucose (FDG) avid lymph nodes on chest computed tomography (CT) and positron emission tomography (PET) scans. There was a lack of knowledge on the impact of EUS when implemented in the routine preoperative staging algorithm of unselected NSCLC patients. To this end, a retrospective study was conducted, the results of which are reported in **Chapter 2**. In a large cohort of consecutive and unselected NSCLC patients (n=152) it was demonstrated that use of EUS prevented futile surgical procedures in 39% of patients and an overall sensitivity of 74%, negative predictive value (NPV) of 73% and accuracy of 85% was found. Based on EUS test characteristics reported from a meta-analysis (pooled sensitivity 83% and NPV 78%) and a recent review (reported sensitivity of 89%) [13,14], one can argue that the EUS test characteristics found in this study are lower than to be expected. This can partly be

contributed to a relatively high proportion of NSCLC patients with small lymph nodes on CT in this study, for whom EUS is known to have a lower sensitivity, and to a relatively low prevalence of mediastinal metastases which is known to diminish NPV[14-16]. Nevertheless, the relatively low sensitivity and NPV, which results in a relatively high false-negative rate, should be considered an adequate reflection of the impact of EUS-FNA on routine NSCLC practice. The fact that in the group of patients, who underwent additional surgical staging procedures (by means of cervical mediastinoscopy), 6 out of 11 mediastinal metastases (55%) were found that went undetected by previous EUS, makes a strong case for confirmatory surgical staging after EUS. Indeed, sensitivity (92%), NPV (85%) and accuracy (95%) were much better in NSCLC patients who underwent both EUS and mediastinoscopy.

The introduction of endobronchial ultrasound (EBUS)[17], a procedure that allows aspiration of hilar, subcarinal and paratracheal lymph nodes under real-time ultrasound guidance from the tracheobronchial tree, was an important step in order to improve the false-negative rate of EUS. By giving access to pre- and paratracheal lymph nodes (station 2 and 4), which are difficult to visualize by EUS due to intervening air in the trachea, EBUS allows tissue staging of the same mediastinal lymph nodes as mediastinoscopy and therefore is complementary to EUS. A recent meta-analysis has indeed confirmed that the combination of EUS and EBUS procedures has higher sensitivity than either procedure alone[18]. An important question that remains is in which cases to perform additional surgical staging. This was addressed in **Chapter 3**, where a study on variables that can predict false negative EUS and EBUS findings is described. A logistic regression analysis of a large retrospective cohort (n = 775) revealed three main predictors for false-negative outcomes for EUS, EBUS and combined EUS/EBUS procedures: central location of the lung tumor, nodal enlargement on CT and FDG-avidity of N2/N3 lymph node stations on PET scans. When combined, several subgroups were identified with low predicted probability for being a false-negative outcome. These determinants can help in guiding clinical decisions on when to perform confirmatory surgical staging procedures in order to decrease the number of futile thoracotomies.

PART II: IMMUNOTHERAPY OF LUNG CANCER

As an introduction to the second main topic of this thesis, namely active immunotherapy of NSCLC, an overview of recent research efforts in the field of therapeutic peptide vaccination is given in **Chapter 4**. Many clinical trials have been conducted in which peptide vaccines were utilized for the treatment of cancer patients, although the number of trials that have been undertaken specifically for NSCLC is limited. Numerous early phase trials managed to induce a vaccine-specific immune response in cancer patients, but few

trials managed to achieve clinical success and a clear link of clinical parameters with the vaccine-boosted immunity was rarely made. Several explanations for these findings are discussed, such as the abundance of early phase trials performed in late-stage cancer patients together with a lack of information on general parameters of adaptive immune status of these patients. Perhaps one underrated aspect is the negative effect of the tumor microenvironment, one of the hallmarks of cancer[19], on immunological parameters and clinical outcomes of therapeutic vaccination of NSCLC. This is reflected by the fact that clinical studies have rarely, if at all, combined vaccine treatment with interventions aimed at depletion of tumor-associated macrophages (TAMs), myeloid derived suppressor cells (MDSC) or regulatory T cells (Tregs). Strategies on how to exploit the tumor microenvironment as a target for cancer therapy are discussed in two recent reviews[20,21].

In **Chapter 5**, the prognostic impact of tumor infiltration by CD8+ T cells in the context of expression of classical and non-classical HLA molecules was assessed. Tumor-infiltration by CD8+ T cells was shown to be correlated to classical HLA class I expression, and a positive effect on overall survival (OS) was demonstrated for tumors with high influx of CD8+ T cells together with high expression of classical HLA class I molecules (HLA-A and HLA-BC). As a singular determinant, a dense CD8+ infiltrate in NSCLC stroma was associated with improved OS, but this effect vanished when tumors had high expression of non-classical HLA-E, which had an expression rate of 70% in this study.

Another noteworthy aspect of the review outlined in **Chapter 4** is that only one study was published in which NSCLC patients were treated with a peptide vaccine combined with a conventional anticancer therapy between 2011 and 2013[22], and only a few of such combinatorial studies since 2014[23-25]. This might be related to scarcity of studies that report on the effect of standard of care NSCLC therapies on the human immune system, which is remarkable since knowledge on this subject is crucial when considering combinatorial approaches of active T cell based immunotherapy with standard of care NSCLC therapies. We have addressed this in **Chapter 6** where we report on a study of advanced NSCLC patients to see how treatment with conventional doublet chemotherapy or chemoradiotherapy influenced systemic immunity. Whereas chemotherapy had no manifest effects on antigen presenting cell (APC) and T cell function as well as on immune cell composition, treatment with irradiation caused a negative functional effect on APCs and T cells which coincided with a persistent drop in lymphoid cells. These findings are in contrast with the predominantly immune-potentiating effects of radiotherapy that are documented in animal models and *in vitro* studies[26]. However, most preclinical studies have examined the effects of radiation on immunity under different circumstances, testing a single high dose radiation as opposed to our clinical study in which patients were treated with multiple fractions of low dose radiation, which is known to induce markedly different gene expression profiles[27,28]. It seems that, to enhance active T cell based immunotherapy, an adjustment of radiation dose and fraction should be con-

sidered. In this light, it is particularly interesting that stereotactic radiotherapy, which utilizes high radiation doses in a few fractions, is now increasingly being used in stage III and IV NSCLC patients, whereas its capacity to cure early stage NSCLC was already well known[29]. Future studies are needed to answer the question whether successful immunotherapeutic approaches such as PD-1/PD-L1 targeting antibodies, now mainly used in advanced NSCLC patients, can be enhanced by radiotherapy, and if so, what the optimal dose and fraction should be.

Another important aspect of successful immunotherapeutic strategies is the identification of novel tumor antigens[30,31]. One such tumor antigen is XAGE-1b, a member of the cancer-testis antigen group which is expressed mainly in pulmonary adenocarcinoma. A study on spontaneously induced XAGE-1b specific local and systemic immune responses in adenocarcinoma patients is presented in **Chapter 7**. In 10% of patients, XAGE-1b specific T cells were detected in the primary tumor or draining lymph nodes and a XAGE-1b specific humoral response was present in 7.5% of patients. All antibody positive patients demonstrated the presence of circulating XAGE-1b specific T cells (both CD4+/ CD8+ T cells secreting both type I and II cytokines). This is the first European cohort of NSCLC patients in which both local and systemic XAGE-1b specific tumor immunity were demonstrated, supporting the notion of XAGE-1b as a novel, tumor specific and immunogenic tumor antigen. In order to translate these preclinical data, a phase I clinical trial is currently recruiting patients with advanced (stage III/IV) NSCLC for treatment with a XAGE-1b based synthetic long peptide vaccine. Safety and immunogenicity of the new XAGE-1b vaccine will be assessed. The study protocol and preliminary results are discussed in **Chapter 8**. How a XAGE-1b vaccine can contribute to cure is discussed below.

FUTURE PERSPECTIVES

What does the future hold with regards to the place of endosonography in lung cancer staging? In spite of claims that mediastinoscopy remains the gold standard of mediastinal nodal tissue staging[32,33], an overwhelming body of evidence points to the opposite. Nowadays, combined EUS and EBUS procedures achieve sensitivity, predictive values and accuracy that can match or even outperform mediastinoscopy[9,34,35] while being more cost-effective[36,37]. It would be much more sensible to consider combined EUS and EBUS as the new gold standard of pathologic staging of the mediastinal lymph nodes, as is reflected by the prominent place of endosonography as the first test of choice in mediastinal tissue staging in recent international guidelines [38-40]. An important prerequisite to maintain this position in the NSCLC staging algorithm is to adequately train pulmonology residents to learn and gain experience in performing EUS and EBUS procedures[41,42]. Furthermore, future studies should attempt to further reduce the number

of false-negatives, either by further improving EUS/EBUS sensitivity or by optimal allocation of NSCLC patients for additional surgical staging procedures. Several false-negative endosonography predictors were reported in this thesis, but nevertheless these results should be externally validated, preferably in a prospective multi-center study.

While endosonography has now been well-positioned in the landscape of pulmonary oncology, this is not yet the case for immunotherapy of lung cancer, which makes research efforts in this field all the more exciting, especially given the recent success of PD-1 blocking antibodies in the treatment of advanced NSCLC[43]. Currently, NSCLC patients are recruited to a multitude of studies testing PD-1 and PD-L1 blocking antibodies in a variety of clinical settings and in combination with conventional therapies and (peptide) vaccines to produce synergistic antitumor responses [44]. These much anticipated trials obviously hold high hopes for the near future. One patient group which is expected to benefit specifically from PD-1/PD-L1 antibody treatment are early stage NSCLC patients. This patient group is treated with curative intent by surgical resection but nevertheless 5-year recurrence rates as high as 24% are reported[45]. Most probably, these patients have minimal but undetected residual disease after resection (e.g. irradical resection or unknown nodal or distant metastasis), eventually leading to recurrence. Adjuvant treatment with PD-1/PD-L1 antibodies could lead to more effective anti-tumor immune responses especially since the tumor burden is low, and hence a suppressive tumor microenvironment is likely absent. It is noteworthy that early stage NSCLC patients have slightly higher rates of HLA class I expression than patients of higher stages (intermediate to high HLA expression, 71-89% stage I vs 61-76% stage II-IV) and, more importantly, stage I NSCLC patients with high HLA class expression have improved overall survival[46-49]. Thus, it makes sense to select resected early stage NSCLC patients based on HLA class I status in order to improve clinical effect of adjuvant PD1-PD-L1 antibody treatment.

However, some critical hurdles leading to non-responsiveness to immunotherapy of NSCLC need to be overcome. Firstly, the high presence of immune suppressive myeloid cells in NSCLC patients is a major impediment to successful immunotherapy of NSCLC. Not only do these cells promote tumor growth and progression, but it is also well known that immune cells with a major role in anti-tumor immunity (such as DCs, NK cells and CD8+ T cells) are inhibited by these immune suppressive myeloid cells. Furthermore, there is evidence that these cells can impair the efficacy of chemotherapy and immunotherapy in treating lung cancer[50]. Depletion of these immune suppressive myeloid cells (or alteration of their function) might therefore be a valuable addition to immunotherapeutic strategies to treat NSCLC. An example of this suppressive effect of myeloid cells was given in **Chapter 7** in a patient whose serum clearly showed XAGE-1b IgG antibodies but in whom we managed to detect XAGE-1b specific T cells only after removal of the abundant CD14+ myeloid population in the PBMC. There is also evidence of this concept of myeloid cell depletion in patients with other types of cancer. In advanced cervical carcinoma patients,

high frequencies of circulating myeloid cells normalized after treatment with carboplatin and paclitaxel which resulted in stronger recall T cell responses[51]. Furthermore, in a recent randomized phase II trial in patients with extensive stage small cell lung cancer (SCLC), treatment with a DC vaccine pulsed with wild-type p53 combined with all-trans-retinoic acid (a metabolite of vitamin A which promotes MDSC differentiation into mature, non-suppressive cells) induced a more than twofold decrease in MDSC, which was not observed in patients treated with the DC vaccine only. Importantly, this decrease in MDSCs resulted in a higher frequency of p53 specific immune responses[52].

Another important focus of future research efforts should be to identify new immunological checkpoints on cancer cells that are able to regulate anti-tumor T cell responses. One potential new checkpoint molecule, non-classical HLA-E, is discussed in this thesis in **Chapter 5**. The HLA-E molecule is expressed in 70% of pulmonary adenocarcinoma specimens. Its ligand is CD94/NKG2A and by engaging this receptor it has an inhibitory effect on T cells and NK cells. Hence, when assessing the clinical effect of peptide vaccines or other forms of active T cell-based immunotherapy targeting NSCLC, HLA-E should be considered as a biomarker and its expression should be taken into account given its potential inhibitory effect on tumor-infiltrating CD8+ T cells. Like its ligand CD94/NKG2A, for which an anti-NKG2A antibody is produced and currently tested in a phase I/II trial with head and neck cancer patients (ClinicalTrials.gov, Identifier: NCT02331875), HLA-E might in the future also become an attractive target for treatment with inhibitory antibodies.

Finally, the quest for novel and highly immunogenic tumor antigens will play an important role on the road to success (or failure) when it comes to immunotherapy of NSCLC. The role of CT antigens in general and XAGE-1b specifically has been discussed, but perhaps a bigger impact will be made by identification of tumor neoantigens which are not encoded in the genome, but arise as a consequence of somatic mutations (either driver or bystander mutations)[53]. Due to advances in next-generation sequencing and epitope prediction, these neoantigens will be increasingly investigated, particularly since treatment of NSCLC patients with PD-1 blocking antibodies improved clinical outcomes when a high nonsynonymous mutation and neoantigen burden was present, and a correlation of clinical response with neo-antigen specific CD8+ T cell response was found[54]. One can envisage that treatment of NSCLC patients with a XAGE-1b peptide vaccine can also lead to a cytotoxic T cell response to neoantigens by means of epitope spreading, since a case report showed that treatment of a metastasized melanoma patient with adoptive T cell transfer of a NY-ESO-1–specific CD4+ T-cell clone led to a T cell response to unrelated tumor antigens MART-1 or MAGE-3[55]. Another possibility would be to design multi-peptide vaccines containing a mix of CT antigen based peptides and personalized peptides based on tumor neoantigens. Either way, in spite of challenges ahead of us, the road ahead is exciting and perhaps can lead us to a better future for the treatment of this devastating disease.

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Chapter 10

Summary in Dutch

De introductie van dit proefschrift (**hoofdstuk 1**) geeft een overzicht van de epidemiologie, de classificatie, het diagnostisch proces en de standaardbehandelingen van longkanker. Heden ten dage blijft longkanker een veelvoorkomende ziekte met hoge morbiditeit en mortaliteit[1,2]. Ondanks de grote impact op de gezondheid van miljoenen mensen wereldwijd, blijft de prognose voor patiënten met deze dodelijke ziekte somber en is er weinig progressie op dit vlak, hetgeen duidelijk wordt uit de overlevingscijfers van het niet-kleincellig longcarcinoom (NSCLC). In de afgelopen twee decennia is in Nederland de vijfjaarsoverleving van NSCLC (onafhankelijk van het stadium of geslacht) slechts met 2,6% toegenomen (van 14,8% tot 17,4%)[3] wat deels voortkomt uit het feit dat op het moment van detectie van NSCLC de ziekte zich doorgaans in een vergevorderd stadium bevindt[4,5]. Naast het verbeteren van de primaire preventie en tijdige diagnose van longkanker, richt het onderzoek in dit veld zich ook op het vinden van nieuwe en alternatieve behandelvormen van longkanker, waarvan immunotherapie een belangrijk en actueel voorbeeld is[6].

Dit proefschrift gaat over twee belangrijke thema's op het gebied van NSCLC. In **deel I** van dit proefschrift wordt ingegaan op de implementatie en optimalisatie van endo-echografie in het preoperatieve stadiëring algoritme van NSCLC. In **deel II** van dit proefschrift wordt het potentieel van immunotherapie besproken, enerzijds als alternatieve behandeling voor NSCLC en anderzijds als adjuvante behandeling gecombineerd met standaardbehandelingen voor NSCLC.

DEEL I: ENDO-ECHOGRAFIE VAN LONGKANKER

Endoscopische echografie (EUS) is een procedure waarbij mediastinale structuren in beeld worden gebracht door plaatsing van een lineaire echo-endoscoop in de slokdarm[7,8]. Sinds de introductie van deze techniek in de longgeneeskunde toonden verschillende onderzoeken aan dat het gebruik van EUS bij patiënten met NSCLC niet alleen een goede sensitiviteit en specificiteit heeft, maar ook leidt tot een sterke daling van retrospectief onnodige thoractomieën en daarnaast gepaard ging met een laag aantal complicaties[9-12]. Wat deze studies echter kenmerkte was dat er veelal gekozen werd voor een vaste onderzoeksopzet waarin voornamelijk gekozen werd voor inclusie van geselecteerde patiënten op basis van vergrote en/of 18F-fluordeoxyglucose (FDG) avide lymfeklieren op respectievelijk computertomografie (CT) scans en positron emissie tomografie (PET) scans. In hoeverre implementatie van EUS de standaard klinische praktijk van preoperatieve stadiëring van (niet-geselecteerde) NSCLC-patiënten zou kunnen beïnvloeden, was nog niet geheel bekend. Om deze vraag te beantwoorden werd een retrospectief onderzoek uitgevoerd, waarvan de resultaten worden besproken in **hoofdstuk 2**. In een groot cohort van opeenvolgende en niet-geselecteerde NSCLC-

patiënten (n = 152) werd aangetoond dat door het gebruik van EUS in 39% van de patiënten chirurgische procedures worden voorkomen. De sensitiviteit werd bepaald op 74%, de negatieve voorspellende waarde (NPV) op 73% en de diagnostische accuratesse op 85%. Dit is lager dan verwacht vergeleken met een recente meta-analyse (gepoolde sensitiviteit 83% en NPV 78%) en review (sensitiviteit van 89%)[13,14]. Dit kan gedeeltelijk worden toegeschreven aan het feit dat dit cohort relatief veel NSCLC-patiënten bevat met kleine lymfeklieren op CT, terwijl bekend is dat EUS een lagere sensitiviteit heeft voor deze patiëntengroep. Tevens heeft dit cohort een relatief lage prevalentie van mediastinale metastasen, hetgeen op zichzelf al een negatief effect heeft op de NPV[14-16]. Toch zou de relatief lage sensitiviteit en NPV, die resulteren in een relatief hoog percentage vals-negatieven, als een juiste reflectie moeten worden beschouwd van het effect van EUS-FNA op de standaard klinische praktijk van preoperatieve stadiëring van NSCLC-patiënten. Het feit dat bij patiënten die aanvullend een cervicale mediastinoscopie ondergingen, 6 van de 11 mediastinale metastasen (55%) werden gevonden die werden gemist door EUS, pleit voor het verrichten van aanvullende chirurgische stadiëring na EUS. Dit werd bevestigd door de verbeterde sensitiviteit (92%), NPV (85%) en accuratesse (95%) bij patiënten bij wie zowel EUS als aanvullende mediastinoscopie werd verricht.

De introductie van endobronchiale echografie (EBUS)[17], een procedure waarbij echogeleide aspiratie van hilaire, subcarinale en paratracheale lymfeklieren plaatsvindt, was een belangrijke stap om het aantal EUS vals-negatieven te beperken. EBUS geeft toegang tot pre- en paratracheale lymfklieren (station 2 en 4), die bij EUS moeilijk te visualiseren zijn door lucht in de trachea, en is derhalve vergelijkbaar met mediastinoscopie qua bereik van mediastinale lymfeknopen, en daarmee in theorie complementair aan EUS. Dit is bevestigd in een recente meta-analyse waarin de combinatie van EUS en EBUS een hogere sensitiviteit oplevert dan beide procedures apart[18]. Een belangrijke kwestie blijft de vraag in welke gevallen aanvullende chirurgische stadiëring is geïndiceerd. In **hoofdstuk 3** werd hierop ingegaan door het rapporteren van een studie over variabelen die EUS en EBUS vals-negatieven kunnen voorspellen. Door middel van een logistische regressieanalyse van een groot retrospectief cohort (n = 775) werden drie belangrijke voorspellers gevonden voor vals-negatieve uitkomsten van EUS, EBUS en gecombineerde EUS/EBUS: centrale ligging van de longtumor, vergrote klieren op CT en FDG-aviditeit van N2/N3 lymfeklierstations op PET-scans. Wanneer deze voorspellende variabelen werden gecombineerd, konden verschillende subgroepen worden geïdentificeerd met een lage voorspelde kans op een vals-negatieve uitslag. Deze predictoren kunnen helpen bij het nemen van klinische beslissingen over wanneer aanvullende chirurgische stadiëring is geïndiceerd om het aantal onnodige thoracotomieën te reduceren.

DEEL II: IMMUNOTHERAPIE VAN LONGKANKER

Ter introductie van het tweede onderwerp van dit proefschrift, namelijk immunotherapie bij NSCLC, wordt in **hoofdstuk 4** een overzicht gegeven van recent onderzoek op het gebied van therapeutische peptide-vaccinatie. Er zijn vele klinische studies uitgevoerd met peptide-vaccins ten behoeve van de behandeling van kankerpatiënten, hoewel het aantal studies specifiek gericht op NSCLC patiënten beperkt is. Verscheidene fase I/II klinische trials zijn erin geslaagd om een vaccin-specifieke immuunrespons bij kankerpatiënten te induceren, maar slechts weinig studies konden daadwerkelijk een klinisch effect bewerkstelligen en een duidelijk verband tussen het klinische behandeleffect en de vaccin-geïnduceerde immuunrespons kon zelden worden gelegd. Verschillende verklaringen voor deze bevindingen worden besproken, zoals de overvloed aan vroege fase klinische studies uitgevoerd in uitbehandelde kankerpatiënten, tezamen met onvoldoende kennis van de algemene immunocompetentie van deze patiënten. Een wellicht onderschat aspect is het negatieve effect van de tumor micro-omgeving, één van de "hallmarks of cancer"[19], op immunologische parameters en klinische resultaten van therapeutische vaccinatie van NSCLC. Dit blijkt uit het feit dat klinische studies zelden, of helemaal niet, therapeutische vaccins gecombineerd hebben met interventies gericht op depletie van tumor-geassocieerde macrofagen (TAM), myeloïde suppressor cellen (MDSC) of regulerende T-cellen (Tregs). Twee recente reviews bespreken mogelijkheden om de tumor micro-omgeving als een doelwit voor kankertherapie te benutten[20,21].

In **hoofdstuk 5** wordt de prognostische impact van tumor infiltratie door CD8+ T-cellen onderzocht in het geval van expressie van klassieke en niet-klassieke HLA-moleculen. Tumor-infiltratie door CD8+ T-cellen bleek te correleren met klassieke HLA-klasse I expressie, en een positief effect op de overleving (overall survival, OS) werd aangetoond voor tumoren met een hoge aanwezigheid van CD8+ T-cellen tezamen met hoge expressie van klassieke HLA klasse I moleculen (HLA-A en HLA-BC). Een hoge aanwezigheid van CD8+ T-cellen in de tumor bleek op zichzelf te zijn geassocieerd met een verbeterde OS, maar dit effect verdween wanneer tumoren een hoge expressie blekken te hebben van het niet-klassieke molecuul HLA-E welke in 70% van de tumoren in deze studie tot expressie kwam.

Een ander opmerkelijk aspect van de reviewstudie beschreven in **hoofdstuk 4** is dat tussen 2011 en 2013 slechts één studie werd gepubliceerd waarin NSCLC-patiënten werden behandeld met een peptide-vaccin gecombineerd met een conventionele behandeling tegen kanker[22], en sinds 2014 kwamen daar slechts enkele studies bij[23-25]. Dit kan te maken hebben met de schaarste aan studies die rapporteren over het effect van standaard NSCLC-therapieën op het menselijk immuunsysteem, hetgeen opmerkelijk is aangezien kennis over dit onderwerp van cruciaal belang is wanneer wordt overwogen om actieve T-cel gemedieerde immunotherapie te combineren met standaard NSCLC

therapieën. In **hoofdstuk 6** wordt op dit onderwerp ingegaan door middel van een studie met laat stadium NSCLC-patiënten waarin het effect van conventionele doublet chemotherapie en chemoradiatie op het immuunsysteem in kaart wordt gebracht. Daar waar chemotherapie geen duidelijke functioneel effect had op antigeen-presenterende cellen (APC) en T-cellen, alsmede op de samenstelling van immuuncellen in het bloed, was dit wel het geval voor radiotherapie waarbij er een negatief effect werd waargenomen ten aanzien van APC en T-cel functie hetgeen samenging met een persisterende daling in lymfoïde cellen. Deze bevindingen zijn strijdig met bevindingen in diermodellen en in vitro studies waar voornamelijk positieve effecten van radiotherapie op het immuunsysteem zijn beschreven[26]. Echter, de meeste preklinische studies hebben de effecten van radiotherapie ten aanzien van het immuunsysteem onderzocht onder andere omstandigheden waarbij voornamelijk het effect van een enkele hoge dosis straling werd gemeten. Dit in tegenstelling tot onze klinische studie waar patiënten werden bestraald met multipele fracties van lage stralingsdoses, waarvan bekend is dat dit een beduidend ander effect heeft op genexpressie profielen[27,28]. Het lijkt erop dat een aanpassing van de dosis en aantal stralingsfracties moet worden overwogen om een synergistisch effect te bereiken met immunotherapie. In dit licht is het interessant dat stereotactische radiotherapie, dat wordt gebruikt in vroeg stadium NSCLC-patiënten en waarbij een hoge stralingsdosis wordt toegediend in enkele fracties, inmiddels ook (in studie-verband) wordt toegepast bij stadium III en IV NSCLC patiënten[29]. Toekomstige studies zijn nodig om de vraag te beantwoorden of het klinisch effect van succesvolle immunotherapieën zoals PD-1/PD-L1 blokkerende antilichamen (nu voornamelijk gebruikt in laat stadium NSCLC-patiënten) kan worden versterkt in combinatie met radiotherapie (en zo ja, wat dan de optimale dosis zou moeten zijn).

Een ander belangrijk aspect op het gebied van immunotherapie van NSCLC is de identificatie van nieuwe tumor antigenen[30,31]. Een voorbeeld van een dergelijk tumorantigeen is XAGE-1b, dat behoort tot de zogenaamde "cancer-testis" antigenen en dat bij NSCLC voornamelijk tot expressie komt in het subtype adenocarcinoom. Een studie van XAGE-1b specifieke lokale en systemische immuunresponsen bij patiënten met adenocarcinoom wordt beschreven in **Hoofdstuk 7**. XAGE-1b-specifieke T-cellen werden gevonden in de primaire tumor of drainerende lymfeknopen van 10% van de patiënten, en een XAGE-1b specifieke humorale respons was aanwezig in 7,5% van de patiënten. Alle patiënten waarin XAGE-1b specifieke antilichamen werden aangetoond, bleken ook circulerende XAGE 1b-specifieke T-cellen (CD4+/ CD8+ T-cellen die zowel type I en II cytokines) in het bloed te hebben. Dit studiecohort is het eerste Europese cohort van NSCLC-patiënten waarbij lokale en systemische XAGE-1b specifieke immuunresponsen zijn aangetoond. Deze studie bevestigt de status van XAGE-1b als een nieuw, tumorspecifiek en immunogeen antigeen. Deze preklinische data zijn gebruikt ten behoeve van een klinische fase I studie waarvoor momenteel patiënten met laat stadium (sta-

dium III/IV) NSCLC worden gerekruteerd voor behandeling met een synthetische lange peptide vaccin gebaseerd op het XAGE-1b eiwit. Veiligheid en immunogeniciteit van dit XAGE-1b vaccin zal worden onderzocht. Het studieprotocol en de eerste preliminaire resultaten worden besproken in **hoofdstuk 8**.

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Appendices

List of publications
Acknowledgments
Curriculum Vitae

LIST OF PUBLICATIONS

1. Standard radiotherapy but not chemotherapy impairs systemic immunity in non-small cell lung cancer
   Talebian Yazdi M, Schinkelshoek MS, Loof NM, Taube C, Hiemstra PS, Welters MJP, van der Burg SH
   Oncoimmunology. 2016 Nov 8;5(12):e1255393

2. The positive prognostic effect of stromal CD8+ tumor-infiltrating T cells is restrained by the expression of HLA-E in non-small cell lung carcinoma
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3. Endosonography for lung cancer staging: predictors for false-negative outcomes
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7. Rapid KRAS, EGFR, BRAF and PIK3CA mutation analysis of fine needle aspirates from non-small-cell lung cancer using allele-specific qPCR

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Lung Cancer. 2010 Jul;69(1):60-5.

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CURRICULUM VITAE

Mehrdad Talebian Yazdi was born on April 19th 1984 in Mashhad, Iran. In 1988, he moved to the Netherlands with his family. In 2002 he completed gymnasium secondary education at the Stanislas College in Delft, the Netherlands. He studied Medicine at Leiden University, from which he graduated in 2009. He combined his medical study with a Bachelor's programme in Philosophy, graduating in 2010. After graduation, he started his PhD project in 2010 at the Departments of Pulmonology and Clinical Oncology (supervisors prof. dr. S.H. van der Burg and prof. dr. P.S. Hiemstra). His research efforts were supported by an AGIKO grant from the Netherlands Organisation for Health Research and Development (ZonMw). In 2014, he started his clinical training at the Department of Internal Medicine of the Reinier de Graaf Hospital in Delft, the Netherlands. In January 2017, he will start his residency in Cardiology at Haga Teaching Hospital at The Hague, the Netherlands.