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Exploring and modulating the tumor immune microenvironment: Towards improving patient outcomes of immunotherapy in lung cancer

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Citation
Theelen, W. S. M. E. (2020, October 21). *Exploring and modulating the tumor immune microenvironment: Towards improving patient outcomes of immunotherapy in lung cancer*. Retrieved from https://hdl.handle.net/1887/137007

Version:	Publisher's Version
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Note: To cite this publication please use the final published version (if applicable).

Cover Page

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Author: Theelen, W.S.M.E.
Title: Exploring and modulating the tumor immune microenvironment: Towards improving patient outcomes of immunotherapy in lung cancer
Issue date: 2020-10-21

Exploring and Modulating the Tumor Immune Microenvironment

Towards improving patient outcomes of immunotherapy in lung cancer

Willemijn Theelen

The work described in this thesis was performed at the Netherlands Cancer Institute-Antoni van Leeuwenhoek, Amsterdam, The Netherlands

Photo on cover: microscopic image of a multi-stained needle biopsy of non-small cell lung cancer
Layout: Willemijn Theelen
Printed by: ProefschriftMaken
IBSN: 978-94-6423-010-9

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Exploring and Modulating the Tumor Immune Microenvironment

Towards improving patient outcomes of immunotherapy in lung cancer

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van de Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 21 oktober 2020
klokke 13.45 uur

door

Wilhelmina Sabina Marilyn Emilie Theelen

geboren te Leiden

in 1981

Promotores:	Prof. Dr. P. Baas Prof. Dr. M.M. van den Heuvel (Radboud UMC, Nijmegen)
Leden promotiecommissie:	Prof. dr. C.A.M. Marijnen Prof. dr. J.G. Keyser-Borst Prof. dr. P.S. Hiemstra Prof. dr. ir. J.J.M. van der Hoeven Prof. dr. D.K.M. de Ruysscher (Maastricht UMC, Maastricht) Dr. A. Becker-Commissaris (Amsterdam UMC, Amsterdam)

Wie één mens redt, redt de gehele mensheid

(Sanhedrin 4:5)

Reason can be fought with reason; how are you going to fight the unreasonable?

(Ellsworth Toohey in The Fountainhead – Ayn Rand)

CONTENTS

CHAPTER 1

General introduction and outline of the thesis

Epidemiology of lung cancer

In 2018, lung cancer was reported as the most common cancer with 2.09 million new cases globally by the World Health Organization. Also, lung cancer was the leading cause of cancer death with 1.76 million deaths that year [1]. In the Netherlands, the incidence of lung cancer is still on the rise. In 2018, approximately 8000 men and 6400 women received a diagnosis of lung cancer. For Dutch men this number has been relatively stable during the last three decades, but for Dutch women the incidence of lung cancer has risen from around 1300 new cases 30 years ago to break through the 6000-barrier for the first time [2]. Tobacco smoking has been strongly associated with the development of lung cancer [3].

In general, lung cancer can be divided into non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). The latter accounts for approximately 15% of lung cancer patients. This thesis focusses on NSCLC only, which again can be roughly divided into adenocarcinoma (AD), squamous cell carcinoma (SCC) and large cell carcinoma not otherwise specified (LCC NOS). Approximately half of the NSCLC patients finds themselves diagnosed with an incurable metastatic stage on first presentation, i.e. stage IV, but also around 30% of patients who initially present themselves with curable disease will eventually develop metastases [4].

Treatment of metastatic NSCLC

Only a very small fraction of patients with metastatic NSCLC can possibly be cured by a more aggressive regimen of a combination of systemic and local treatment, like surgery or radiation therapy (RT). At initial presentation, these patients have few metastatic sites. This situation is referred to as oligometastatic setting and is considered as having a maximum of five metastatic lesions and three organs involved [5].

In general, treatment options in patients with metastatic NSCLC should be considered as palliative. For decades the only systemic treatment option that had shown scientific benefit in NSCLC was chemotherapy [6, 7]. The optimal chemotherapy regimen consists of a platinum doublet, where carboplatin or cisplatin is combined with preferably a third-generation cytostatic compound: a taxane, gemcitabine, pemetrexed or vinorelbine. The only comparison in regard to histology has been made for pemetrexed vs gemcitabine. SCC showed shorter overall survival (OS) with a pemetrexed-platinum combination compared to the gemcitabine-platinum combination, while the opposite effect was observed in non-squamous histology [8]. After progression on first line treatment, only second line mono-chemotherapy can be considered a beneficial treatment option. Local therapy like surgery and especially RT can be applied for palliative reasons on a specific symptomatic tumor site in metastatic disease.

The identification of oncogenic drivers like somatic point mutations or deletions (e.g. EGFR) and gene fusions (e.g. ALK) in NSCLC and the subsequent blockade with specific tyrosine kinase inhibitors has brought impressive tumor responses and prolongation of progression free survival (PFS) compared to chemotherapy [9, 10]. Unfortunately, these improvements in treatment options still only apply to a minority of the lung cancer patients, especially in the Western population, and mostly concern non-smokers.

The era of immunotherapy

More recently, research focusing on unraveling the tumor immune microenvironment has led to significant new insights [11]. Tumors express antigens that arise from mutations within the tumor DNA, the so called neoantigens, which can be recognized by host T cells as non-self. In lung cancer, these mutations are generally caused by smoking, making NSCLC one of the tumors with the highest tumor mutational burden (TMB) [12]. Unfortunately, the triggered immunologic response can generally not overcome progression of tumor growth nor the development of metastatic lesions. The mechanisms of the escape of host immunity by the immunosuppressive environment induced by cancer cells includes down-regulation of cell surface major histocompatibility complex (MHC) class I molecules, secretion of immunosuppressive factors, lack of T-cell co-stimulation, and expression of immune inhibitory pathways [13-16].

The most studied immune inhibitory ligand is the programmed death-ligand 1 (PD-L1). PD-L1 expression has been identified in a wide variety of solid tumors including breast, colon, ovarian, melanoma, bladder, liver, gliomas, thyroid, thymic epithelial, head and neck and lung [17]. Besides aberrant expression by tumors, PD-L1 is also mainly expressed by antigen presenting cells (APCs) and endothelial cells [18]. Its receptor, programmed death 1 (PD-1), is expressed on a variety of cells: T cells, B cells, natural killer T cells, activated monocytes, dendritic cells and even on tumor cells [17]. Binding of PD-L1 to the PD-1 receptor on T-cells activates an inhibitory signal leading to apoptosis or inactivation of the immune cells and thereby allowing the tumor to evade the host immune response.

The high TMB and subsequent presence of neoantigens would make NSCLC highly susceptible to T cell recognition and killing, but based on the high incidence of NSCLC an immune escape mechanism apparently appears to prevent tumor immune attack. Indeed, the development of immune checkpoint inhibitors (ICIs), PD-1/PD-L1 monoclonal antibodies, has led to long-lasting anti-tumor immune responses in patients with metastatic NSCLC [19]. In 2015, the PD-1 inhibitor nivolumab became the new standard of care (SoC) for metastatic NSCLC that had progressed on platinum-doublet chemotherapy [20, 21]. In the same year, registration followed for the PD-1 inhibitor pembrolizumab and the PD-L1 inhibitor atezolizumab for the same indication [22, 23]. All three compounds showed superior OS compared to docetaxel.

Biomarkers for immunotherapy

Overall response rates (ORR) and other patient outcomes were associated with the protein expression level of PD-L1 on tumors as assessed by immunohistochemistry (IHC). The ORR in PD-L1 negative tumors was approximately 8%, but increased to 30% in patients whose tumors expressed high PD-L1 expression defined by PD-L1 expression on 50% of the tumor cells or more. Nivolumab was beneficial irrespective of PD-L1 expression, especially when the more benign toxicity profile of immunotherapy vs. docetaxel is concerned. In the pembrolizumab trial, no patients with PD-L1 negative tumors were allowed to participate. The companion diagnostic tool for PD-L1 assessment in the atezolizumab study also measured PD-L1 expression on tumor-infiltrating immune cells. PD-L1 expression on tumor cells and on tumor-infiltrating immune cells are associated with one another, but both also have been reported to be independently associated with higher response rates on atezolizumab [24]. Still, at the time of

designing the protocol for the PEMBRO-RT trial described in this thesis, assessing PD-L1 expression was not yet readily accessible for clinical use. During that period, profound skepticism had risen about the value of PD-L1 as a useful predictive biomarker for response on ICIs, because PD-L1 negative patient still had an 8% chance of response and the subgroup of patients with the highest PD-L1 expression still a 70% chance of failure of immunotherapy. There are several issues concerning scoring of PD-L1 expression by IHC. Each PD-1/PD-L1 checkpoint blocker has its own PD-L1 assay as companion diagnostic. Cut-off levels are different for each of the assays and where most of them score PD-L1 expression on tumor cells only, the atezolizumab diagnostic tool gives a combined tumor and immune PD-L1 score. The latter assay does not align regarding tumor cell staining with the other assays that do not score PD-L1 on immune cells [25]. Besides different assays, tumor heterogeneity of PD-L1 expression may play a role. In metastatic NSCLC, diagnosis is mainly retrieved based on a small biopsy of the primary tumor or a metastatic site, but PD-L1 expression may not be evenly distributed across all lesions. Investigation of PD-L1 expression as a prognostic biomarker in early stage NSCLC has led to conflicting results and two meta-analyses concluded that no statistically significant association between PD-L1 expression and OS could be established [26, 27].

$CD8^+$ cytotoxic T cells are key players in immunoediting, the process by which tumor cells are eliminated due to antigen mediated killing [28]. Compared to PD-L1 expression, there is reasonable evidence that increased density of tumor infiltrating lymphocytes (TILs) is associated with improved prognosis in NSCLC [29, 30]. In melanoma, infiltration of $CD8^+$ T cells was associated with higher response rate on ICIs [31], but no compelling evidence of a similar association has been published in NSCLC. As to date, opposed to PD-L1 expression, the assessment of CD8 infiltration is not an established clinical biomarker for immunotherapy in NSCLC.

Also, TMB has proven to be a predictive biomarker for response to immunotherapy in NSCLC and height of TMB appeared to be irrespective of the level of PD-L1 expression [32]. Although useful, assessing TMB is still an elaborate effort and therefore not readily accessible for use in a clinical setting.

Exploring the tumor immune microenvironment by gene expression analysis

Previously, gene expression analysis has been used to find prognostic biomarkers especially for early stage NSCLC [33-35]. Also, they have proven to aid in pathological diagnosis of lung cancer [36]. Aside from the PD-1/PD-L1 axis and $CD8^+$ T cells, numerous other immunosuppressive and immunostimulatory mechanisms play a role in the tumor-immune interaction. Gene expression analysis allows us to perform comprehensive immunoprofiling of the tumor immune microenvironment and can assist in dissecting the different components of the immune infiltrate. As mentioned, presence of TILs has shown prognostic benefit in NSCLC probably through the immunostimulatory mechanism they represent [37, 38]. On the other hand, myeloid-derived suppressor cells (MDSCs) and T regulator (Treg) cells have an immunosuppressive effect on cytotoxic T cells and may therefore be associated with NSCLC progression [39]. By defining metagenes for specific immune cell populations based on transcriptomic data, like performed by the Microenvironment Cell Populations-counter (MCP-counter) method and validated by IHC, it becomes possible to evaluate the composition of the tumor immune infiltrate and maybe even allocate some of the established prognostic gene signatures [40]. Much is still

unknown about the optimal composition as well as the unfavorable aspects of the tumor immune infiltrate, let alone how to influence the composition for the NSCLC patients' benefit. Besides a better understanding of the role and ratio of all these components of the tumor immune infiltrate, the localization of these immune cells with respect to tumor cells determined by IHC –stromal and/or intraepithelial- may also contain valuable information on different mechanism of immune-tumor interaction [41, 42].

Abscopal effect of radiotherapy

Although many aspects of the tumor immune microenvironment still need to be unraveled, efforts beyond PD-1/PD-L1 blockade have already been explored as potential immunomodulators to provoke tumor responses in itself or as an enhancement to ICI. In the last decades, an increasing amount of evidence has been gathered proving that ionizing radiation may have potential immunoediting abilities. As mentioned before, RT is frequently used in the palliative treatment of metastatic NSCLC to reduce local symptoms like pain or hemoptysis. However, a rare phenomenon of an out-of-field antitumor effect of RT has been described. Patients who received palliative doses of RT on a specific tumor location showed tumor shrinkage of non-irradiated tumor lesions. This was mostly seen in melanoma patients, but also NSCLC cases have been described [43, 44].

This phenomenon is referred to as the abscopal effect; 'ab scopus' meaning away from the target. The biological rationale for this observation is sought in an antitumor response of the host immune system. When RT manages to induce immunogenic cell death of tumor cells, release of tumor antigens and production of pro-inflammatory mediators is induced [45]. APCs are thereby activated and a subsequent uptake of tumor antigens occurs [46]. These APCs migrate to tumor draining lymph nodes, where presentation of tumor antigens to T cells takes place. Recognition of antigens leads to an increased activation of tumor-specific T cells, which are able to generate an antitumor response within the previous irradiated lesion. Tumor-specific T cells reach the irradiated tumor through the circulation guided by activation of the "stimulator of interferon genes" (STING) signaling pathway through the pro-inflammatory mediators type I interferons in dendritic cells [47]. In general, non-irradiated lesions carry overlapping tumor antigens with the irradiated lesion and therefor recognition of out-of-field tumor lesions can also occur: the biological rationale for the abscopal effect [48, 49].

Combining radiotherapy with ICI

In theory, this seems like a promising systemic treatment, but in reality, only several case reports are known with 'spontaneous' out-of-field responses after local RT. However, this postulation of an abscopal effect makes RT an interesting modality in combination with other immunomodulating agents, like ICIs. In addition to a radiation induced inflammation of the tumor microenvironment and induction of tumor-specific T cell responses, tumor immune escape mechanisms could be tackled by this combination. Tumors can escape recognition by activated T cells through downregulation of MHC class I molecules, which can be found on all cells in the body besides erythrocytes but including tumor cells. These molecules are arbitrary in self-recognition by displaying peptides from normal cellular protein turnover, therefor T cells will not be triggered to attack. If cells present non-self-antigens, like tumor neoantigens, on their MHC molecules, T cells will proceed to cell killing on recognition. By downregulating their MHC

molecules and subsequent loss of neoantigen-presentation tumor cells are able to escape the immune system. RT has proven to upregulate MHC expression on tumor cells [50]. In addition, RT may promote a more pro-inflammatory tumor microenvironment by the elimination of immune suppressive cells. For example, RT has shown to be able to differentiate macrophages from an immune-inhibitory M2 towards an immune-stimulatory M1 phenotype [51]. Unfortunately, induction of immune-inhibitory cells by RT has been described as well, probably due to differences in tumor models or radiation regimens [52]. Interestingly, the pro-inflammatory induction of RT may in itself have detrimental effects on the tumor immune response through subsequent the upregulation of immune checkpoints, like PD-L1, causing tumor immune escape [53].

The described immunomodulating effects -especially the latter being a direct encouragement for this hypothesis- have led to exploring whether the combination of RT with immunotherapy would indeed lead to synergy in pre-clinical in vivo and in vitro solid tumor models [49, 54, 55]. Positive results have led to the development of clinical trials testing the safety and efficacy of this approach.

Outline of this thesis

This thesis sought to obtain a better understanding of the composition of the immune microenvironment in NSCLC and how to modulate this tumor immune microenvironment by RT to induce amplified antitumor immune responses to ICIs in advanced NSCLC patients.

In the first part of this thesis, a multiangular approach of a combination of protein and mRNA expression with clinicopathological characteristics in a large cohort of early stage, resected NSCLC samples will be discussed. The second part focusses on the immune modulating effects of RT, in particular when combined with immunotherapy treatment in metastatic NSCLC.

PART I. Exploring the tumor immune microenvironment

Expression of PD-L1 assessed by IHC is still the most important clinical biomarker to predict response on ICI in NSCLC, but specificity and sensitivity are relatively low. In **chapter 2**, we explored mechanisms of PD-L1 upregulation or to be more precise the lack thereof by comparing PD-L1 expression in tumor cells vs. immune infiltrating cells in early stage resected NSCLC samples. Not only T cells and the PD-1/PD-L1 pathway play a significant role in tumor-immune interactions. In **chapter 3**, an unsupervised exploration based on an expression of a wide variety of immune genes was performed in the same resection cohort, leading to the discovery a 34-gene signature with strong prognostic power in SCC, but not AD.

PART II. Modulating the tumor immune microenvironment

Although long-lasting clinical responses have been observed in responders, only a minority of NSCLC patients respond to ICIs monotherapy. **Chapter 4** provides a review of the immunoediting ability of RT, relevant pre-clinical and clinical data concerning the abscopal effect of the combination of ICIs with RT with a focus on NSCLC. In **chapter 5**, we present the results of the PEMBRO-RT trial, where advanced NSCLC patients were randomized between pembrolizumab alone vs. pembrolizumab after stereotactic

body radiation (SBRT) to a single tumor site. The PEMBRO-RT trial showed benefit of the combined strategy over pembrolizumab alone, but this did not meet our pre-emphasized criteria of meaningful clinical benefit. Finally, in **chapter 6**, a pooled analysis of the PEMBO-RT trial combined with a similar randomized trial performed at the MD Anderson Cancer Center is presented.

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PART I.

Exploring the tumor immune microenvironment

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CHAPTER 2

Absence of PD-L1 expression on tumor cells in the context of an activated immune infiltrate may indicate impaired IFNγ signaling in non-small cell lung cancer

PLoS One. 2019 May 24;14(5):e0216864

ABSTRACT

Background

In non-small cell lung cancer (NSCLC), PD-L1 expression on either tumor cells (TC) or both TC and tumor-infiltrating immune cells (IC) is currently the most used biomarker in cancer immunotherapy. However, the mechanisms involved in PD-L1 regulation are not fully understood. To provide better insight in these mechanisms, a multiangular analysis approach was used to combine protein and mRNA expression with several clinicopathological characteristics.

Patients and methods

Archival tissues from 640 early stage, resected NSCLC patients were analyzed with immunohistochemistry for expression of PD-L1 and CD8 infiltration. In addition, mutational status and expression of a selection of immune genes involved in the PD-L1/PD-1 axis and T-cell response was determined.

Results

Tumors with high PD-L1 expression on TC or on IC represent two subsets of NSCLC with minimal overlap. We observed that PD-L1 expression on IC irrespective of expression on TC is a good marker for inflammation within tumors. In the tumors with the highest IC expression and absent TC expression an association with reduced IFNγ downstream signaling in tumor cells was observed.

Conclusions

These results show that PD-L1 expression on TC and IC are both independent hallmarks of the inflamed phenotype in NSCLC, and TC-negative/IC-high tumors can also be categorized as inflamed. The lack of correlation between PD-L1 TC and IC expression in this subgroup may be caused by impaired IFNγ signaling in tumor cells. These findings may bring a better understanding of the tumor-immune system interaction and the clinical relevance of PD-L1 expression on IC irrespective of PD-L1 expression on TC.

INTRODUCTION

One of the most studied tumor immune escape mechanisms is mediated through the inhibitory programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway. The development of anti-PD-L1/PD-1 monoclonal antibodies has led to long-lasting anti-tumor immune responses in a subset of patients with non-small cell lung cancer (NSCLC). High PD-L1 expression as assessed by immunohistochemistry (IHC) has consistently been reported to be associated with higher responses to anti-PD-L1/PD-1 treatment, resulting in the development of various diagnostic PD-L1 IHC assays [1–3]. The use of various diagnostic PD-L1 IHC assays has led to ambiguity as to how to use this multi-faceted biomarker. In two randomized trials comparing the anti-PD-L1 antibody atezolizumab to docetaxel in second line setting, PD-L1 expression on TC and on infiltrating immune cells (IC) both appeared to be independently associated with response to atezolizumab [3, 4].

Besides PD-L1 expression, wider aspects of the tumor/immune-infiltrating complex are under investigation as biomarkers for immunotherapy. Tumors can broadly be divided into inflamed (hot) vs non-inflamed (cold) tumors. Typically, inflamed tumors show a pre-existing antitumor immune response with abundance of tumor-infiltrating lymphocytes (TILs), IFNγ-producing $CD8^+$ T-cells and high expression of PD-L1. In contrast, non-inflamed tumors are characterized as immune desert: containing hardly any TILs and rarely expressing PD-L1 [5, 6]. The development of gene expression profiling of tumors allows distinguishing ‘hot’ and ‘cold’ tumors by providing prognostic and predictive immune signatures; one example being the T-effector (T_{eff}) signature showing an association with efficacy in the randomized phase II and III trials comparing atezolizumab to docetaxel [3, 4].

Hence, it is important to improve insights in the overlap and differences between PD-L1 expression on TC and/or IC and to relate this expression to other tumor features and markers of the PD-L1/PD-1 axis and T-cell response. In order to do this, we used a multiangular approach by combining protein and mRNA expression with clinicopathological characteristics, including mutational analysis of well-known drivers of NSCLC in a large cohort of clinically annotated resected NSCLC samples.

MATERIALS AND METHODS

Sample collection and patient cohort

Inclusion criteria for this cohort were patients that had undergone a lung resection between 1990 and 2013 at one of four Dutch medical centers. Exclusion criteria were a synchronous primary tumor, unavailability of tumor tissue or patient follow-up data, histology of non-NSCLC, e.g. SCLC or metastasized non-NSCLC. Clinical data about gender, smoking status, neo-adjuvant and adjuvant treatment, age at resection, type of resection, tumor stage, progression free survival (PFS) and overall survival (OS) were collected. No data on treatment after relapse of disease was available. The cohort included 768 samples with adequate patient and tumor characteristics. For all these patients, formalin-fixed, paraffin-embedded (FFPE) tumor samples were collected. After a second pathology revision, samples without sufficient vital tumor material were excluded, leaving 640 samples eligible for further

analysis. All tumors were histopathologically classified according the 2015 WHO classification system [7]. TNM classification was redefined for resections that were done before 2010 according to the 7th lung cancer TNM classification and staging system. Smoking status was defined by pack years (PY). Light smokers were defined by having less than 10 PY, including never smokers. Prior to analysis the samples were de-identified. The Translational Research Board of the Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital approved the use of patient data and material in this study.

Immunohistochemical staining, mutational and gene expression analysis

PD-L1 expression and CD8 staining was assessed in a central laboratory (HistoGeneX, Belgium) using whole slide sections prepared from FFPE resection specimens. Sections were stained using the rabbit anti-human anti-PD-L1 antibody (clone SP142, Spring Bioscience) and the monoclonal mouse anti-human anti-CD8 antibody (clone C8/144B, DAKO) on a Ventana BenchMark XT autostainer (Ventana Medical Systems). PD-L1 expression in TC was assessed as the proportion of TC showing membrane staining of any intensity; expression in IC was assessed as the proportion of tumor area occupied by PD-L1-positive IC of any intensity (Figure 1A-B and S1) [3, 4]. In all specimens, total immune infiltrate and tumor cells were assessed in the tumor area by a certified pathologist based on hematoxylin background staining of the IHC slide and if needed based on the H&E staining. Positive and negative controls were performed using tonsil tissue. The scoring algorithm was developed for the approved VENTANA PD-L1 (SP142) Assay and further details concerning the PD-L1 staining protocol have been described previously [8, 9]. PD-L1 score for expression on TC and IC was available for 615 (96.1%) samples. CD8 staining was reported as the percent CD8-positive tumor infiltrating immune cells in the tumor center, available for 615 (96.1%) samples.

Mutation analysis was performed using a microfluidics-based PCR platform running an allele-specific multiplex test as previously described [10, 11]. The validated panel included a total of 130 hot spot mutations (Table S1). Immunohistochemistry for ALK was performed on a BenchMark Ultra autostainer (Ventana Medical Systems) using clone 5A4 (Abcam). For *ALK* FISH staining and analysis of the results was performed as described by the manufacturer.

DNA was extracted using the Qiagen DNA mini kit (cat. No. 51306) and a minimum of 80ng DNA was shipped to Genentech Inc. for mutation analysis. Gene expression analysis was performed using the NanoString nCounter Analysis system (NanoString) on 80-200ng RNA extracted from FFPE tissue samples. A customized gene panel, including 795 targets including multiple genes of immunologic function and cancer biology and including 4 housekeeping genes was applied. Following thorough assay quality control, data were normalized and underwent analysis. We report here results for CD8 (*CD8A*), PD-L1 (*CD274*), PD-1 (*PDCD1*), PD-L2 (*PDCD1LG2*) and T_{eff} signature that was defined as the mean expression for *CD8A*, *GZMA*, *GZMB*, *IFNG*, *EOMES*, *CXCL9*, *CXCL10* and *TBX21* as previously described [3, 4]. The downstream IFNγ response signature was derived from the DER_IFN_GAMMA_RESPONSE_UP gene set (MSigDB; http://software.broadinstitute.org/gsea/msigdb; 71 genes), where signature expression was calculated by summing the log2-based expression values for genes that are members of the gene set and that are present in the expression data (22 / 71 genes). To calculate the actual minus the expected (residual)

IFNγ signature expression, a linear model based on all samples was created describing the relationship between downstream IFNγ response signature and T_{eff} signature expression. This model was used to calculate the expected IFNγ signature expression. Biomarker high and low subgroups were defined by expression levels at or above various cut-offs, either above or below the median or above or below the 25% or 75% quantile. Gene expression analysis was available for 530 (82.8%) of the samples.

Statistical analysis

All statistical tests were performed in R. Kaplan–Meier methodology was used to construct survival curves. Stratified Cox regression models were used to estimate HRs and 95% CIs in biomarker subgroup populations. For comparison of gene expression data among subgroups, (pairwise) t-tests were performed. For comparison of protein expression data among subgroups, (pairwise) Wilcoxon rank sum tests were performed. For comparison of categorical data among subgroups, Fisher's exact tests or Pearson's Chi-squared tests were used as indicated. The false discovery rate (FDR) was controlled below 0.05 using Benjamini-Hochberg method.

RESULTS

Description of the cohort and distribution of PD-L1 protein expression and CD8 infiltration

The cohort consisted of 640 NSCLC samples: 344 (53.8%) AC, 267 (41.8%) SCC and 29 (4.5%) NSCLC NOS. Only 48 (7.5%) patients were light or never smokers. 83.9% of the cohort was early stage disease (≤ stage II). Median follow-up time was 96.0 months (95% CI: 86–103). All samples were screened for presence of an ALK translocation or mutations of well-known drivers in NSCLC. Mutational analysis was available for 563 (88.0%) samples: 170 mutations were found in 164 patients (29.1%). Six samples harbored two mutations. ALK IHC was available for 630 (98.4%) samples. Four samples (0.6%) were ALK IHC positive and a translocation was confirmed by FISH. *EGFR*, *KRAS*, *BRAF* and *ALK* aberrations were mutually exclusive. Table 1 summarizes the clinicopathological characteristics and genetic alterations of our patient cohort.

In order to investigate the overlap and differences of PD-L1 protein expression between TC and IC, all samples were scored for PD-L1 expression on TC and on IC at all four expression levels. Examples of PD-L1 staining, PD-L1 IHC scoring criteria, the overall prevalence and distribution by overlapping PD-L1 subgroups are presented in Figure 1A–1D and S1. Non-overlapping PD-L1 subgroups are presented in Figure S2. High PD-L1 expression (TC3 or IC3) was present in 132 (21.5%) samples and 74 (12.0%) samples showed no PD-L1 expression (TC0 and IC0 subgroup) (Figure 1C). Only a minority of samples (10.4%) had CD8 infiltration in the tumor center of 5% or higher (Figure 1E).

Inflammatory features like PD-L1 expression may be affected by traditional stratifying criteria (i.e. gender, age, smoking status, histology, tumor stage or *KRAS*/*EGFR* status). In a univariate analysis using the TC and IC scores separately a positive association between heavy smoking and PD-L1 expression on TC ($p = 0.016$) was found, but not for PD-L1 expression on IC. There was no significant difference in PD-L1 expression between the histologic subtypes (Figure S3A-D). PD-L1 expression on

TC was significantly higher for *KRAS* mutant (*KRASm*) tumors compared to *KRAS* wild type (*KRASwt*) tumors ($p < 0.001$) and this was irrespective of smoking status. No difference was found for PD-L1 expression on IC by *KRAS* status (Figure S3E-F). *EGFRm* status was not significantly associated with PD-L1 protein expression (data not shown).

The correlation between PD-L1 protein expression and PD-L1 mRNA expression (encoded by *CD274*) was investigated. Protein expression of both TC and IC was significantly associated with mRNA expression of *CD274* (Figure S4).

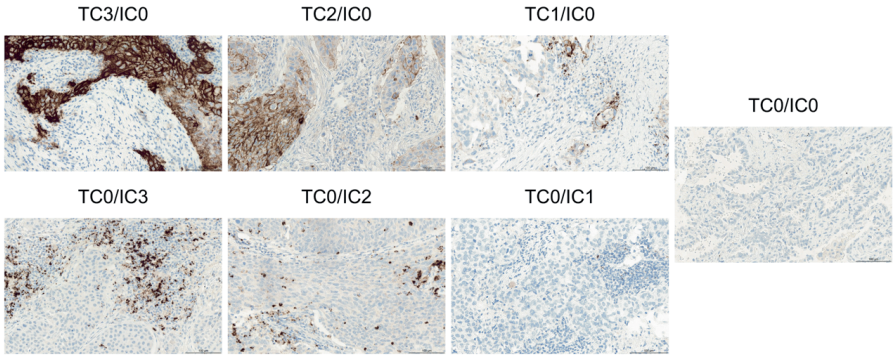
Table 1. Patients' and tumor characteristics of the non-small cell lung cancer cohort.

	AC	SCC	NSCLC NOS
Total (n = 640)	344	267	29
Gender			
Male	163 (47.3%)	188 (70.4%)	17 (58.6%)
Female	181 (52.7%)	79 (29.6%)	12 (41.4%)
Median age at surgery (years, range)	62 (30-84)	67 (38-85)	57 (37-81)
Neo-adjuvant therapy	54 (15.7%)	13 (4.9%)	7 (24.1%)
Chemotherapy	21 (6.1%)	2 (0.7%)	1 (3.4%)
Concurrent chemo radiotherapy	8 (2.3%)	2 (0.7%)	4 (13.8%)
Sequential chemo radiotherapy	3 (0.9%)	0	0
Erlotinib [[56]]	22 (6.4%)	6 (2.2%)	2 (6.9%)
Radiotherapy	0	3 (1.1%)	0
No neo-adjuvant therapy	290 (84.3%)	254 (95.1%)	22 (75.9%)
Adjuvant treatment			
Chemotherapy	49 (14.2%)	45 (16.9%)	8 (27.6%)
Radiotherapy	19 (5.5%)	24 (9.0%)	2 (6.9%)
Chemotherapy + radiotherapy	7 (2.0%)	9 (3.4%)	1 (3.4%)
No adjuvant therapy	244 (70.9%)	160 (59.9%)	14 (48.3%)
Unknown	25 (7.3%)	29 (10.9%)	4 (13.8%)
Smoking			
Light smokers <10PY	42 (12.2%)	4 (1.5%)	2 (6.9%)
Heavy smokers ≥10PY	253 (73.5%)	224 (83.9%)	25 (86.2%)
Unknown	49 (14.2%)	39 (14.6%)	2 (6.9%)
Tumor stage at resection			
Stage I	211 (61.3%)	131 (49.0%)	13 (44.8%)
Stage II	79 (23.0%)	95 (35.6%)	9 (31.0%)
Stage III	44 (12.8%)	34 (12.7%)	7 (24.1%)
Stage IV	10 (2.9%)	7 (2.6%)	0
Genetic alterations*			
EGFR mutated	20 (6.3%)	1 (0.5%)	0
KRAS mutated	110 (34.6%)	7 (3.4%)	3 (10.3%)
ALK translocated	4 (1.3%)	0	0
PIK3CA mutated	10 (3.1%)	14 (6.8%)	0
BRAF mutated	1 (0.3%)	0	0
NRAS mutated	1 (0.3%)	2 (1.0%)	0
HRAS mutated	1 (0.3%)	0	0
No mutation detected	171 (53.8%)	182 (88.3%)	26 (89.7%)
Undetermined^	26	61	0
Mean overall survival (months, range)	71 (0-285)	76 (0-289)	71 (6-273)

* percentages for analyzed samples only. *EGFR* mutations included exon 19 deletions (n=15), exon 20 insertions (n=2) and exon 21 L858R mutations (n=4). No T790M mutations were found. *KRAS* mutations included mutations in codon 12 and 13 (n=116) and codon 61 (n=4). Mutations in *AKT1, ERBB2, FLT3, JAK2, KIT, MYD88* were not present within this cohort. All present *MET* mutations (n=30) were germline single nucleotide polymorphism (SNP).
^ mutation status was undetermined when no sufficient DNA was available or when the microfluidics-based PCR platform lead to an invalid result.
SCC = squamous cell carcinoma, AC = adenocarcinoma, NSCLC NOS = non-small cell lung cancer not otherwise specified, PY = pack years

Figure 1. Examples of PD-L1 staining, scoring criteria, prevalence and overlap between PD-L1 expression on TC and IC and prevalence of CD8 infiltration in the tumor center in NSCLC.

A



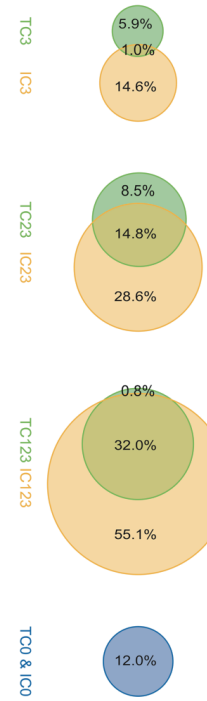
B

PD-L1 tumour cell scoring		PD-L1 tumour-infiltrating immune cell scoring	
Score	Percentage of PD-L1 expressing cells	Score	Percentage of PD-L1 expressing cells
TC3	≥ 50%	IC3	≥ 10%
TC2	≥ 5% and < 50%	IC2	≥ 5% and < 10%
TC1	≥ 1% and < 5%	IC1	≥ 1% and < 5%
TC0	< 1%	IC0	< 1%

C

Overall prevalence	
Subgroup	Proportion in % (n)
TC3 or IC3	21.5% (132)
TC2/3 or IC2/3	51.9% (319)
TC1/2/3 or IC1/2/3	88.0% (541)
TC0 and IC0	12.0% (74)

D



E

CD8 infiltration prevalence in NSCLC	
Percentage of CD8 infiltration	Tumor center (n = 615)
[0, 1)	33.2% (204)
[1, 5)	56.4% (347)
[5, 10)	8.46% (52)
[10, 100]	1.95% (12)

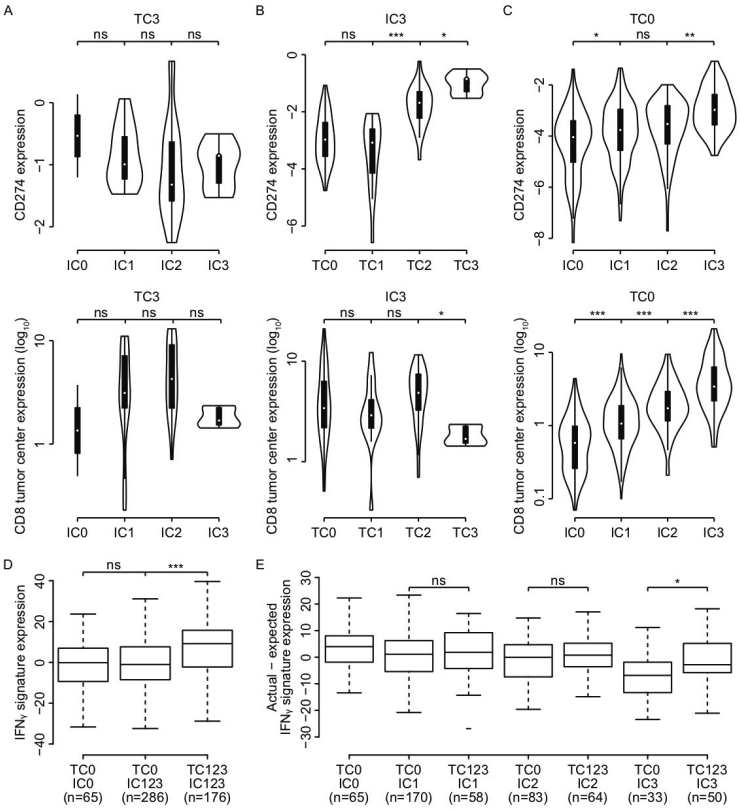
(A) PD-L1 expression by IHC on both TC and IC for each subgroup. (B) PD-L1 IHC scoring criteria on TC and IC [57] (C) Overall prevalence of overlapping PD-L1 subgroups. (D) Percentages in Venn diagrams represent the prevalence of PD-L1 expression by TC and IC in overlapping subgroups. (E) Overall prevalence of CD8 infiltration in the tumor center.

Overlap and differences of PD-L1 protein expression on TC and IC

We then explored the distribution of PD-L1 expression and the overlap and differences between expression on TC and IC. There was minimal overlap between TC3 and IC3 tumors (1.0%, Figure 1D), which might suggest different mechanism of PD-L1 upregulation in tumor cells compared to immune cells. Comparing TC3 tumors to IC3 tumors in regard to clinicopathological features did not reveal significant differences (Table S2). Next, we analyzed potential differences with respect to immunological

features. To correct for potential confounding of the true biology of TC3 and IC3 tumors by the PD-L1 expression in the other compartment, we compared TC3 tumors based on various expression levels of IC (0 to 3) to IC3 tumors based on various expression levels of TC (0 to 3) (Figure 2A–2C and S5). In the TC3 subgroup (n = 39), expression of all inflammatory markers showed a slight increase per increasing IC subgroup except expression of *CD274*, but this was not significant. In the IC3 subgroup (n = 83), we found that only expression of *CD274* increased per increasing TC score, while the other inflammatory markers remained constant, i.e. CD8 infiltration, T_{eff} signature, *CD8A*, *PDCD1* and *PDCD1LG2* expression. Also, when evaluating TC0 samples based on various levels of IC (0 to 3; n = 351) all inflammatory markers, including *CD274*, increased per ascending IC subgroup. The IC score therefore seems to represent a characteristic of true 'hot' tumors.

Figure 2. Associations of mRNA expression of *CD274*, infiltration of CD8 and the IFNγ response signature in non-overlapping PD-L1 expressing subgroups.



(A) Relative mRNA expression of *CD274* and CD8 infiltration in TC3 tumors based on various levels of IC (n = 39). (B) Relative mRNA expression of *CD274* and CD8 infiltration in IC3 tumors based on various levels of TC (n = 83). (C) Relative mRNA expression of *CD274* and CD8 infiltration in TC0 tumors based on various levels of IC (n = 351). (D) Relative mRNA expression of the IFNγ response signature in non-overlapping PD-L1 subgroups: TC0&IC0, TC0/IC123 and TC123/IC123 (n = 530). (E) The actual minus the expected relative mRNA expression of the IFNγ response signature comparing TC negative to TC positive sampels for each non-overlapping IC-subgroup. Expected IFNγ response signature expression was obtained from the level of T_{eff} signature expression based on their linear relationship. ns = non significant, * p = 0.01 – 0.05, ** p < 0.01, *** p < 0.001

As the TC0/IC123 subgroup (n = 286, 55.1%) contains the majority of samples in this cohort (Figure 1D), we then sought to understand why tumors harboring an active immune infiltrate showed no upregulation of PD-L1 on TC. Since IFNγ signaling is an important mechanism for PD-L1 upregulation, we hypothesized that an impairment in downstream IFNγ signaling within tumor cells might explain this phenomenon. It is expected that cytokine production by an active immune infiltrate, represented by the T_{eff} signature, will lead to downstream IFNγ signaling within tumor cells. Therefore, we determined the expression of selected IFNγ target genes, and collectively represented them as an IFNγ response signature. The expression of this IFNγ response signature was significantly lower in TC negative samples compared to TC positive samples ($p < 0.001$, Figure 2D). As this difference was irrespective of the expression of the IFNγ target PD-L1 on IC, this strongly suggests that expression of this IFNγ response signature originated from tumor cells only and not the immune infiltrate. The T_{eff} and the IFNγ response signature showed a linear relationship (Figure S6). We calculated the difference between the expected level of the IFNγ response signature based on this linear model and the actual one (residuals). To overcome confounding by the IC score, again we compared the residuals in TC0 tumors based on various subgroups of IC (0 to 3) (Figure 2E). In the TC0/IC3 subgroup, we observed a significantly lower expression of IFNγ response as would be expected by the linear model compared to the TC123/IC3 subgroup ($p = 0.042$). Expected expression in the TC0/IC3 subgroup was lower compared to all other subgroups. This suggests that the absence of PD-L1 expression on tumor cells in TC0/IC3 samples may be caused by impaired IFNγ signaling in these tumor cells.

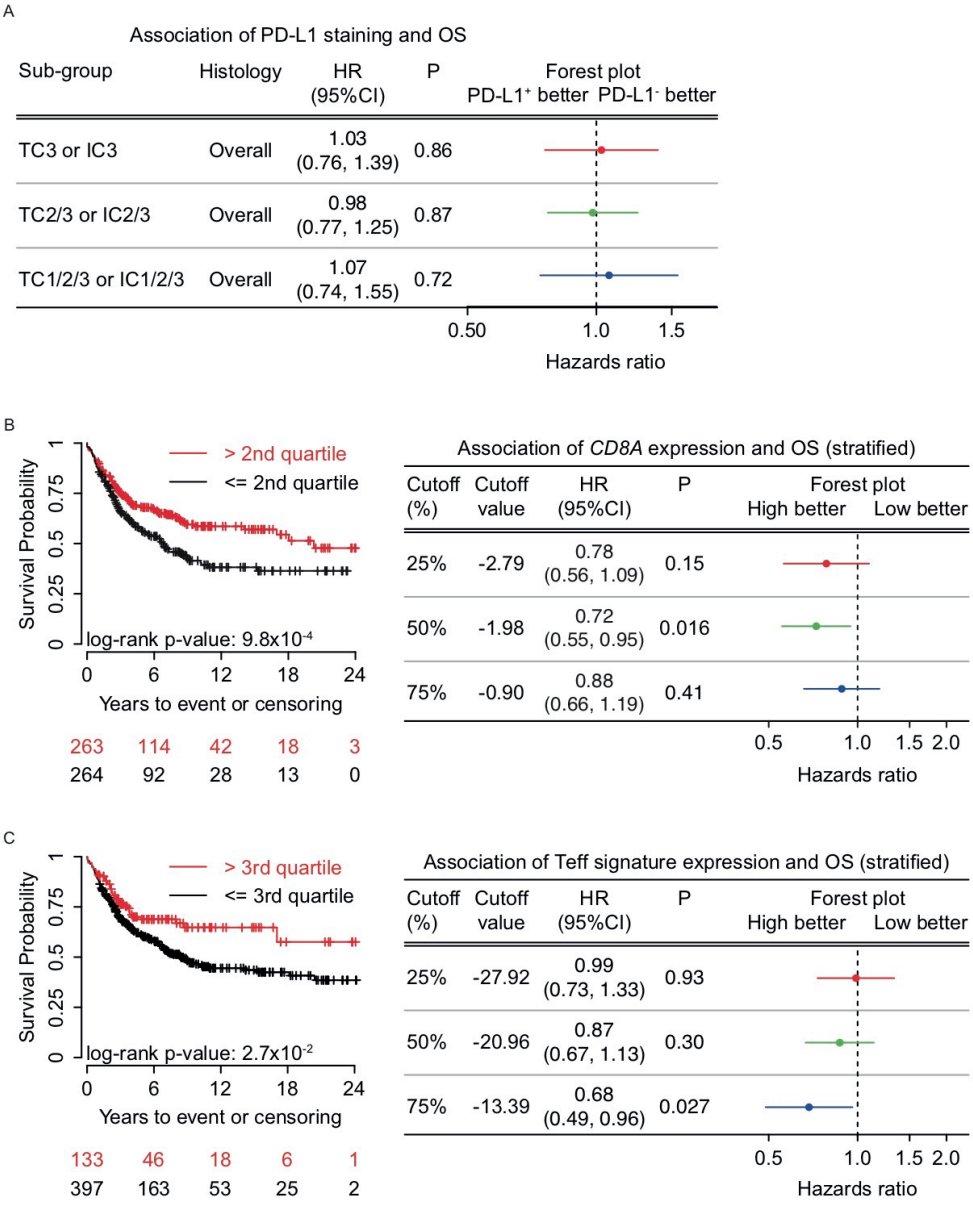
Prognostic value of PD-L1 expression, CD8 infiltration and gene expression

Recent data showed conflicting results concerning the prognostic value of PD-L1 expression in NSCLC. After stratifying for tumor stage, we analyzed the prognostic value of several inflammatory parameters measured in our cohort. PD-L1 protein expression -combined or on TC/IC separately- and mRNA expression of *CD274* had no effect on OS in our cohort (Figure 3A). CD8 infiltration by IHC only showed a trend towards improved OS, but *CD8A* transcript levels were significantly associated with better OS (HR 0.72 (95%CI 0.55–0.95; $p = 0.016$, Figure 3B). High expression of the T_{eff} signature (highest quartile) and *PDCD1* (highest quartile) were both positive prognostic markers (HR 0.68 (95%CI 0.49–0.96; $p = 0.027$), Figure 3C and HR 0.60 (95%CI 0.42–0.85; $p = 0.0035$), data not shown, respectively). Expression of *PDCD1LG2* or the IFNγ response signature had no OS relevance. Based on these results, we conclude that gene expression profiling is a better indicator of prognosis than PD-L1 protein expression.

DISCUSSION

To date, PD-L1 protein expression on tumor cells and on tumor infiltrating immune cells is the most studied biomarker in cancer immunotherapy. This study sought to improve insights in the relation of PD-L1 protein expression with traditional stratifying criteria, like histology and oncogenic driver status, and other markers of the PD-L1/PD-1 axis and T-cell response.

Figure 3. The effect of PD-L1 expression, the expression of *CD8A* and the T_{eff} signature on OS.



(A) Forest plot for overlapping PD-L1 expressing subgroups show no improved OS for higher PD-L1 expression; stratified for tumor stage. (B) Forest plot and Kaplan Meier curve for *CD8A* expression show improved OS for the highest two quartiles; stratified for tumor stage (HR 0.72 (95%CI 0.55-0.95; p = 0.016)). (C) Forest plot and Kaplan Meier curve for quartiles of the T_{eff} signature show improved OS for the highest quartile; stratified for tumor stage (HR 0.68 (95%CI 0.49-0.96; p = 0.027)).

In our cohort, the pattern of inflamed tumors was clearly established: expression of PD-L1 on either TC or IC, infiltration of $CD8^+$ cells and mRNA expression of *CD274*, *CD8A*, *PDCD1*, *PDCD1LG2* and the T_{eff} signature were all associated with one another. Besides this overlap in inflamed features, also differences between PD-L1 expression on TC and IC were found. Co-expression of PD-L1 at the highest level on both TC and IC rarely occurred: prevalence of TC3&IC3 population was only 1%. Fehrenbacher et al. also described this lack of overlap in advanced NSCLC and hypothesized an intrinsic mechanism of PD-L1 upregulation on TC versus an adaptive mechanism on IC [3]. Unfortunately, as opposed to the studies in advanced NSCLC, this early stage cohort contained very few TC123/IC0 samples (< 1%). Therefore, our analyses had several limitations because of the risk of confounding by the PD-L1 expression in the other compartment as we could not compare the true PD-L1 TC positive (TC123/IC0) to the true PD-L1 IC positive (TC0/IC123) tumors. By comparing IC3 tumors based on various levels of TC and TC3 as well as TC0 tumors based on various levels of IC, we observed that inflammatory markers like *CD8A* and the T_{eff} signature correlated most clearly with the IC score. Not unsurprisingly, this was strongest within the TC0 subgroup and shows that the IC score is a good measure for true 'hot' tumors.

By dividing our cohort into three non-overlapping subgroups -TC0&IC0, TC0/IC123 and TC123/IC123- we were able to explore other differences between PD-L1 expression on TC vs IC. We found a significantly lower IFNγ response signature expression in TC negative versus TC positive tumors, suggesting an inability of the tumor cells to upregulate PD-L1 in the presence of an active and IFNγ producing immune infiltrate as is represented by expression of the T_{eff} signature. And as expected after performing further analysis between TC negative and TC positive samples in increasing IC subgroups, strong evidence of a hampered IFNγ-PD-L1 axis in tumor cells within the TC0/IC3 subgroup was found. As to our knowledge, this finding has not been described or looked into before. We observed impaired expression of the majority of the individual IFNγ response signature genes (data not shown), implying that the impaired IFNγ signaling in TC0/IC3 tumors is due to alterations at an early level of the pathway: IFNGR or JAK/STAT. Kowanetz et al. found that the TC0/IC3 subgroup had a response rate to the PD-L1 inhibitor atezolizumab of 22%, which was higher than TC0&IC0 tumors (ORR 8%), but lower compared to the TC3/IC0 subgroup (ORR 40%) [4, 13]. Therefore, it would be interesting to investigate if restoring this impairment might improve the benefit on PD-1 blockade in these patients.

For the PD-L1 staining, the SP142 antibody clone was used according to a validated protocol assessing PD-L1 expression on IC in addition to TC [3, 4]. This enabled a thorough assessment of the differences and overlap between PD-L1 expression on TC versus IC. However, in the Blueprint analysis comparing four PD-L1 IHC assays, the SP142 staining differed significantly by producing a weaker staining on TC and fewer PD-L1 positive TCs compared to the other three assays (22C3, 28–8 and SP263), which were similar in the analytical performance [14]. Based on these differences between the assays it's possible that some of the TC positive tumors may have been unjustly qualified as a TC0 tumor in our cohort in comparison with other PD-L1 assays. This may have resulted in an underestimation of the finding of a hampered IFNγ-PD-L1 axis in our TC0 subgroup and might help explain why we did not find this impairment in the TC0/IC2 or TC0/IC1 subgroup.

In conclusion, these results show the important contribution of PD-L1 expression on IC to identify inflamed tumors. Impaired IFNγ response signaling in tumor cells may explain the absence of PD-L1 expression on TC in the context of an activated immune infiltrate as represented by high PD-L1 IC positivity. These findings may help towards a better understanding of the tumor-immune system interaction and also signify the clinical relevance of PD-L1 expression on IC as a biomarker for immunotherapy in NSCLC patients.

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SUPPLEMENTARY DATA

Table S1. List of hotspot mutations.

Gene	COSMIC ID	cDNA mutation	AA mutation	Gene	COSMIC ID	cDNA mutation	AA mutation
EGFR	6252	2155 G>A	G719S	BRAF	473	c.1798_1799GT>AA	V600K
EGFR	6253	2155 G>T	G719C	BRAF	476	c.1799T>A	V600E
EGFR	6239	2156 G>C	G719A	NRAS	565	c.35G>C	G12A
EGFR	26038	2233_2247del15	K745_E749del	NRAS	562	c.34G>T	G12C
EGFR	13550	2235_2248>AATTC	E746_A750>IP	NRAS	561	c.34G>C	G12R
EGFR	6223	2235_2249del15	E746_A750del	NRAS	563	c.34G>A	G12S
EGFR	13552	2235_2251>AATTC	E746_T751>IP	NRAS	566	c.35G>T	G12V
EGFR	13551	2235_2252>AAT	E746_T751>I	NRAS	564	c.35G>A	G12D
EGFR	12385	2235_2255>AAT	E746_S752>I	NRAS	575	c.38G>C	G13A
EGFR	12413	2236_2248>AGAC	E746_A750>RP	NRAS	570	c.37G>T	G13C
EGFR	6225	2236_2250del15	E746_A750del	NRAS	573	c.38G>A	G13D
EGFR	12728	2236_2253del18	E746_T751del	NRAS	569	c.37G>C	G13R
EGFR	12678	2237_2251del15	E746_T751>A	NRAS	574	c.38G>T	G13V
EGFR	12386	2237_2252>T	E746_T751>V	NRAS	580	c.181C>A	Q61K
EGFR	12416	2237_2253>TTGCT	E746_T751>VA	NRAS	584	c.182A>G	Q61R
EGFR	12367	2237_2254del18	E746_S752>A	NRAS	583	c.182A>T	Q61L
EGFR	12384	2237_2255>T	E746_S752>V	NRAS	582	c.182A>C	Q61P
EGFR	18427	2237_2257>TCT	E746_P753>VS	NRAS	586	c.183A>C	Q61H
EGFR	12422	2238_2248>GC	L747_A750>P	NRAS	585	c.183A>T	Q61H
EGFR	23571	2238_2252del15	L747_T751del	AKT1	33765	c.49G>A	E17K
EGFR	12419	2238_2252>GCA	L747_T751>Q	FLT3	785	c.2503G>C	D835H
EGFR	6220	2238_2255del18	E746_S752>D	FLT3	783	c.2503G>T	D835Y
EGFR	6218	2239_2247del9	L747_E749del	FLT3	784	c.2504A>T	D835V
EGFR	12382	2239_2248TTAAGAGAAG>C	L747_A750>P	FLT3	788	c.2505T>G	D835E
EGFR	12383	2239_2251>C	L747_T751>P	HRAS	480	c.34G>A	G12S
EGFR	6254	2239_2253del15	L747_T751del	HRAS	481	c.34G>T	G12C
EGFR	6255	2239_2256del18	L747_S752del	HRAS	483	c.35G>T	G12V
EGFR	12403	2239_2256>CAA	L747_S752>Q	HRAS	484	c.35G>A	G12D
EGFR	12387	2239_2258>CA	L747_P753>Q	HRAS	487	c.37G>A	G13S
EGFR	6210	2240_2251del12	L747_T751>S	HRAS	486	c.37G>C	G13R
EGFR	12369	2240_2254del15	L747_T751del	HRAS	496	c.181C>A	Q61K
EGFR	12370	2240_2257del18	L747_P753>S	HRAS	499	c.182A>G	Q61R
EGFR	13556	2253_2276del24	S752_I759del	HRAS	498	c.182A>T	Q61L
EGFR	6241	2303 G>T	S768I	HRAS	503	c.183G>C	Q61Hc
EGFR	12376	2307_2308 ins 9 (gccagcgtg)	V769_D770insASV	HRAS	502	c.183G>T	Q61Ht
EGFR	13558	2309_2310complex (ac>ccagcgtggat)	V769_D770insASV	KIT	1216	c.1669T>A	W557R
EGFR	12378	2310_2311 ins GGT	D770_N771insG	KIT	1219	c.1669T>C	W557G
EGFR	13428	2311_2312 ins 9 (gcgtggaca)	D770_N771insSVD	KIT	1290	c.1727T>C	L576P

EGFR	12377	2319_2320 ins CAC	H773_V774insH	KIT	1304	c.1924A>G	K642E
EGFR	6240	2369 C>T	T790M	KIT	12706	c.1961T>C	V654A
EGFR	6224	2573 T>G	L858R	KIT	1311	c.2446G>C	D816H
EGFR	12429	2573-2574TG>GT	L858R	KIT	1310	c.2446G>T	D816Y
EGFR	6213	2582 T>A	L861Q	KIT	1314	c.2447A>T	D816V
PIK3CA	746	c.263G>A	R88Q	MET	710	c.1124A>G	N375S
PIK3CA	754	c.1035T>A	N345K	MET	707	c.3029C>T	T1010I
PIK3CA	757	c.1258T>C	C420R	MET	699	c.3743A>G	Y1248C
PIK3CA	760	c.1624G>A	E542K	MET	700	c.3757T>G	Y1253D
PIK3CA	763	c.1633G>A	E545K	JAK2	12600	c.1849G>T	V617F
PIK3CA	12458	c.1634A>C	E545A	MYD88	85940	c.794T>C	L256P
PIK3CA	764	c.1634A>G	E545G	ERBB2	14060	c.2264T>C	L755S
PIK3CA	765	c.1635G>T	E545D	ERBB2	683	c.2263_2264TT>CC	L755P
PIK3CA	766	c.1636C>A	Q546K	ERBB2	14062	c.2329G>T	L777L
PIK3CA	6147	c.1636C>G	Q546E	KRAS	520	c.35G>T	G12V
PIK3CA	12459	c.1637A>G	Q546R	KRAS	532	c.38G>A	G13D
PIK3CA	25041	c.1637A>T	Q546L	KRAS	512	c.34_35GG>TT	G12F
PIK3CA	773	c.3129G>T	M1043I	KRAS	533	c.38G>C	G13A
PIK3CA	12591	c.3127A>G	M1043V	KRAS	527	c.37G>T	G13C
PIK3CA	776	c.3140A>T	H1047L	KRAS	529	c.37G>C	G13R
PIK3CA	775	c.3140A>G	H1047R	KRAS	528	c.37G>A	G13S
PIK3CA	774	c.3139C>T	H1047Y	KRAS	534	c.38G>T	G13V
PIK3CA	12597	c.3145G>C	G1049R	KRAS	554	c.183A>C	Q61H
KRAS	522	c.35G>C	G12A	KRAS	555	c.183A>T	Q61H
KRAS	516	c.34G>T	G12C	KRAS	549	c.181C>A	Q61K
KRAS	521	c.35G>A	G12D	KRAS	553	c.182A>T	Q61L
KRAS	517	c.34G>A	G12S	KRAS	552	c.182A>G	Q61R
KRAS	518	c.34G>C	G12R	KRAS	520	c.35G>T	G12V

Table S2. Clinicopathological features in TC3/IC<3 vs TC<3/IC3 samples.

	TC3/IC<3	TC<3/IC3	p-value
Total (n = 125)	36	89	
Gender			
Male	15 (41.7%)	45 (50.6%)	
Female	21 (58.3%)	44 (49.5%)	.43
Median age at surgery (years, range)	59 (39-77)	64 (36-82)	.057
Smoking			
Light smokers <10PY	1 (2.8%)	0	
Heavy smokers ≥10PY	32 (88.9%)	70 (78.7%)	
Unknown	3 (8.3%)	19 (21.3%)	.32
Histology			
Adenocarcinoma	15 (41.6%)	52 (58.4%)	
Squamous cell carcinoma	20 (55.6%)	33 (37.1%)	
NSCLC NOS	1 (2.8%)	4 (4.5%)	.14
Tumor stage at resection			
Stage I	21 (58.3%)	40 (45.0%)	
Stage II	9 (25.0%)	30 (33.7%)	
Stage III	5 (13.9%)	17 (19.1%)	
Stage IV	1 (2.8%)	2 (2.3%)	.29
Genetic alterations			
EGFRm	0	2 (2.2%)	.09
KRASm	11 (30.1%)	19 (21.3%)	.71

Figure S1. Examples of PD-L1 co-staining of TC and IC positivity in various subgroups.

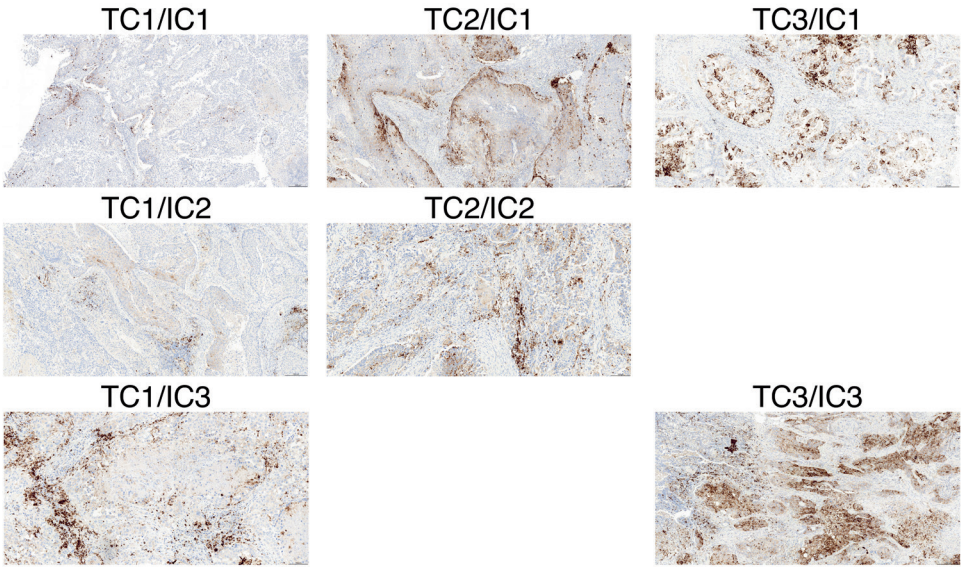


Figure S2. Percentages in Venn diagrams represent the overlap of PD-L1 expression of the TC3 with IC3, the TC2 with IC2 and the TC1 with IC1 subgroups.

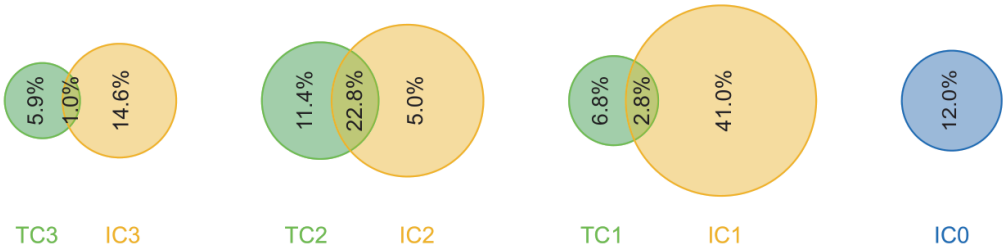
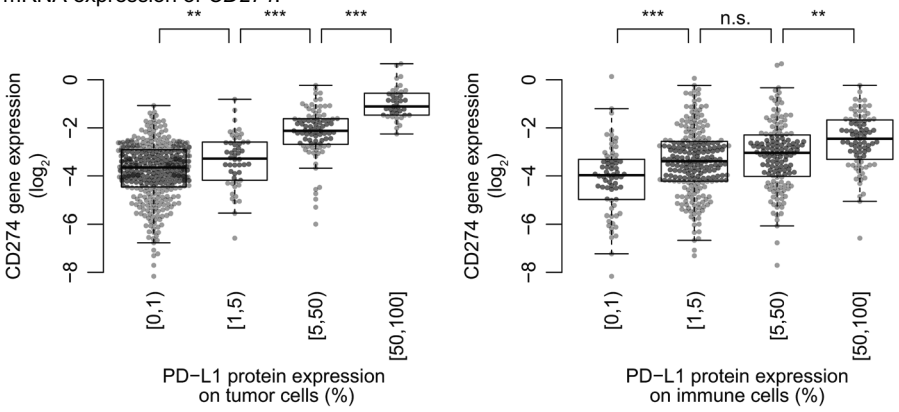
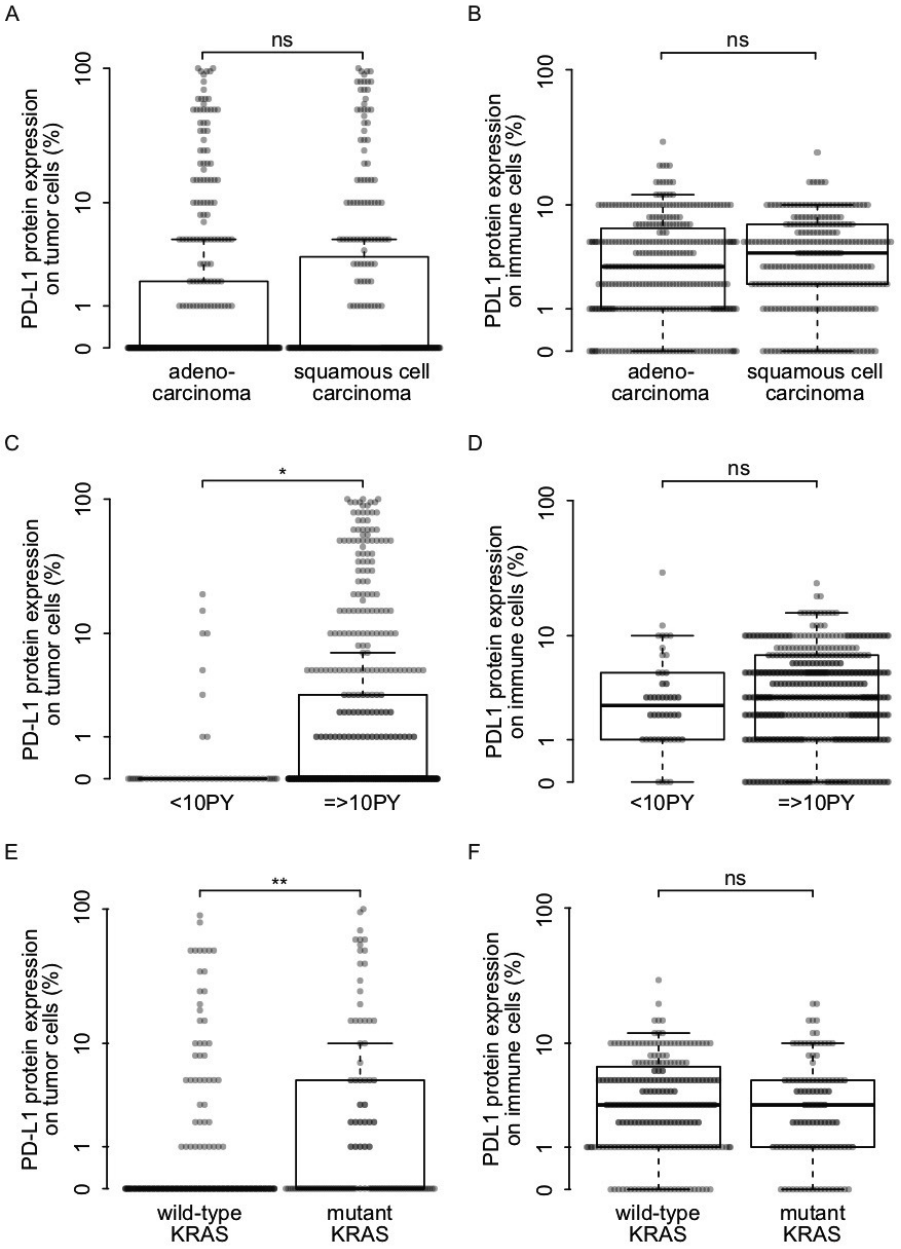


Figure S4. Associations of PD-L1 protein expression on TC and IC in non-overlapping subgroups with mRNA expression of *CD274*.



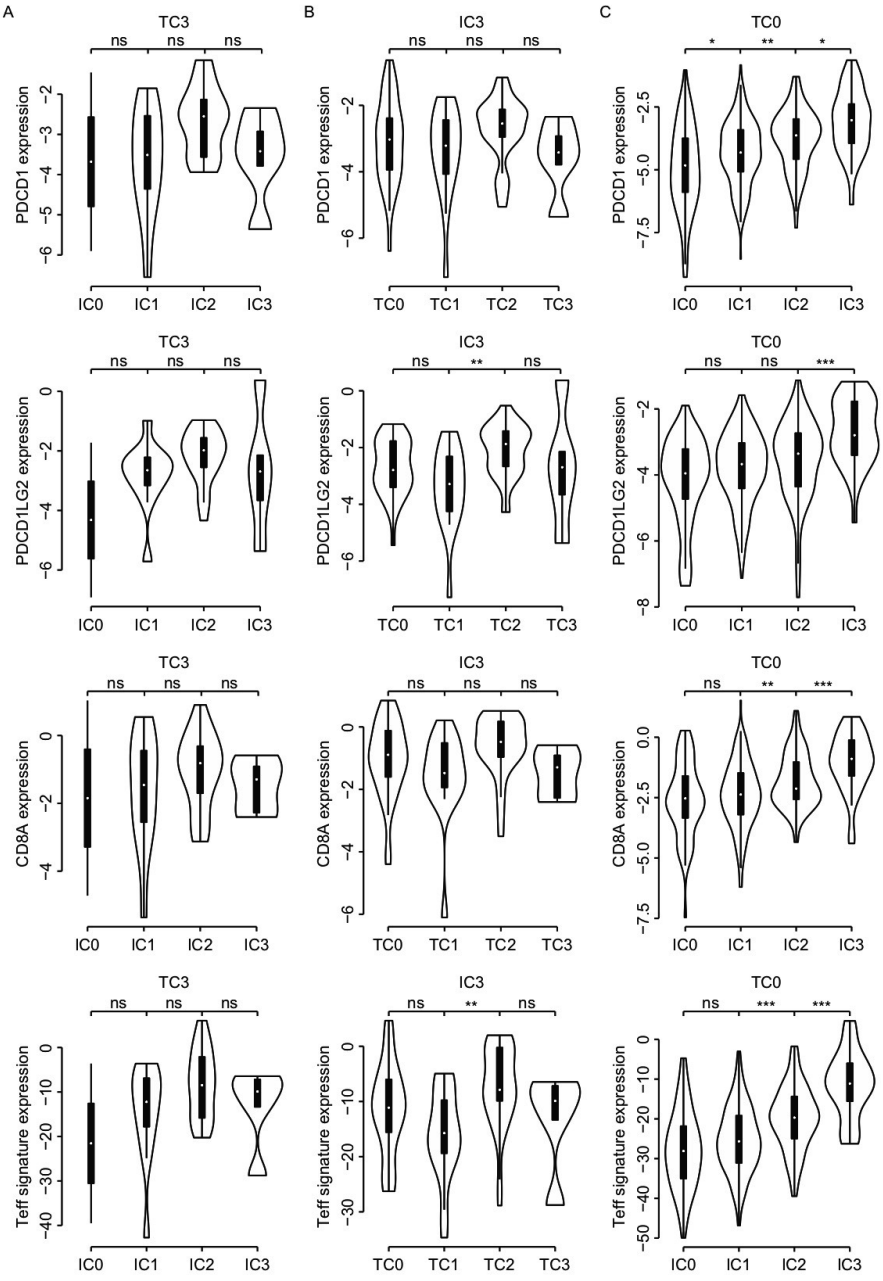
ns = non significant, ** p < 0.01, *** p < 0.001

Figure S3. PD-L1 expression on TC and IC and associations with histology, smoking and *KRAS* status.



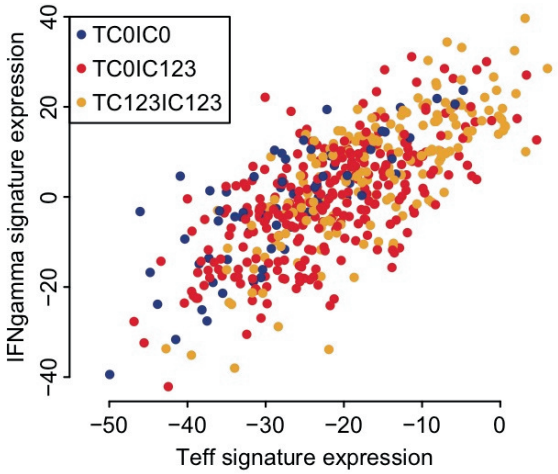
(A, B) No significant difference was seen between SCC compared to AC regarding PD-L1 protein expression on TC or IC (n = 615). (C) PD-L1 protein expression on TC is significantly higher in heavy compared to light smokers (n = 526). (D) No significant difference was seen between heavy compared to light smokers regarding PD-L1 protein expression on IC (n = 526). (E) PD-L1 protein expression on TC is significantly higher in *KRASm* compared to *KRASwt* samples in the AC cohort only (n = 317). (F) No significant difference was seen between *KRASm* compared to *KRASwt* samples regarding PD-L1 expression on IC in the AC cohort only (n = 317). All boxplots were plotted on a hyperlog-transformed y-axis (see Materials and Methods). * p = 0.016, ** p < 0.001, univariate analysis. AC = adenocarcinoma, SCC = squamous cell carcinoma.

Figure S5. Associations of mRNA expression of *PDCD1*, *PDCD1LG2*, *CD8A* and the T_{eff} signature in non-overlapping PD-L1 expressing subgroups.



(A) Relative mRNA expression of *PDCD1*, *PDCD1LG2*, *CD8A* and the Teff signature in TC3 tumors based on various levels of IC (n = 39). (B) Relative mRNA expression of *PDCD1*, *PDCD1LG2*, *CD8A* and the Teff signature in IC3 tumors based on various levels of TC (n = 83). (C) Relative mRNA expression of the *PDCD1*, *PDCD1LG2*, *CD8A* and the Teff signature in TC0 tumors based on various levels of IC (n = 351). ns = non significant, * p = 0.01 - 0.05, * p < 0.01, *** p < 0.001

Figure S6. Expression of the T_{eff} signature vs the expression of the IFNγ response signature.



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*These authors contributed equally to this work

CHAPTER 3

Presence of a 34-gene signature is a favorable prognostic marker in squamous non-small cell lung carcinoma

J Transl Med. 2020 Jul 3;18(1):271

ABSTRACT

Background

The tumor immune microenvironment is a heterogeneous entity. Gene expression analysis allows us to perform comprehensive immunoprofiling and may assist in dissecting the different components of the immune infiltrate. As gene expression analysis also provides information regarding tumor cells, differences in interactions between the immune system and specific tumor characteristics can also be explored. This study aims to gain further insights in the composition of the tumor immune infiltrate and to correlate these components to histology and overall survival in non-small cell lung cancer (NSCLC).

Methods

Archival tissues from 530 early stage, resected NSCLC patients with annotated tumor and patient characteristics were analyzed using the NanoString nCounter Analysis system.

Results

Unsupervised clustering of the samples was mainly driven by the overall level of inflammation, which was not correlated with survival in this patient set. Adenocarcinoma (AD) showed a significantly higher degree of immune infiltration compared to squamous cell carcinoma (SCC). A 34-gene signature, which did not correlate with the overall level of immune infiltration, was identified and showed an OS benefit in SCC. Strikingly, this benefit was not observed in AD. This difference in OS in SCC specifically was confirmed in two independent NSCLC cohorts. The highest correlation between expression of the 34-gene signature and specific immune cell populations was observed for NK cells, but although a plausible mechanism for NK cell intervention in tumor growth could be established in SCC over AD, this could not be translated back to immunohistochemistry, which showed that NK cell infiltration is scarce irrespective of histology.

Conclusions

These findings suggest that the ability of immune cell infiltration and the interaction between tumor and immune cells may be different between AD and SCC histology and that a subgroup of SCC tumors seems more susceptible to Natural Killer cell recognition and killing, whereas this may not occur in AD tumors. A highly sensitive technique like NanoString was able to detect this subgroup based on a 34-gene signature, but further research will be needed to assist in explaining the biological rationale of such low-level expression signatures.

BACKGROUND

In the last decades, it has become increasingly evident that the host immune system has an elaborate interaction with tumor cells. The tumor microenvironment involves a whole range of immune cells together with a wide spectrum of soluble chemokines and cytokines that regulate the infiltrating capacity and the effectiveness of the immune response [1, 2]. The tumor immune microenvironment is a heterogeneous entity, although tumors are often broadly classified as inflamed or 'hot' vs. non-inflamed or 'cold'. Typically, inflamed or 'hot' tumors show an abundance of tumor-infiltrating lymphocytes (TILs), IFNγ-producing $CD8^+$ T cells and high expression of the inhibitory immune checkpoint programmed death-ligand 1 (PD-L1) suggesting a pre-existing antitumor immune response. In contrast, non-inflamed or 'cold' tumors contain hardly any TILs and rarely express PD-L1 [3, 4]. As this is a practical approach, in reality only a small fraction of tumors seems obviously cold or clearly hot, and the level of inflammation seems more like a spectrum.

Aside from TILs, numerous other immunosuppressive and immunostimulatory mechanisms play a role in the interaction of the immune system with tumor cells. Gene expression analysis allows us to perform comprehensive immunoprofiling and may assist in dissecting the different components of the immune infiltrate. Investigating patterns of the separate components could lead to a better understanding of the complex tumor-immune interaction. This is relevant as presence of inflammatory cells has shown prognostic benefit in non-small cell lung cancer (NSCLC) and other solid tumors probably as representation of the immunostimulatory mechanism at work [5, 6]. On the other hand, myeloid-derived suppressor cells and T regulator cells have an immunosuppressive effect on cytotoxic T cells and have been associated with detrimental effects on the anti-tumor immune response [7]. As gene expression analysis also provides information regarding tumor cells, differences in interactions between the immune system and specific tumor characteristics can also be explored. Ultimately, this knowledge may lead towards a better understanding how the immune composition can be influenced for the patients' benefit. This study aims to gain further insights in the composition of the tumor immune infiltrate by nCounter (Nanostring) gene expression analysis and to correlate these components to histology and OS in a large cohort of previously untreated, resected early stage NSCLC samples.

METHODS

Sample collection and patient cohort

The cohort included 641 formalin-fixed, paraffin-embedded (FFPE) NSCLC samples derived from lung resections performed between 1990 and 2013 at one of four Dutch medical centers. Clinical data about gender, smoking status, neo-adjuvant and adjuvant treatment, age at resection, type of resection, tumor stage, progression free survival (PFS) and overall survival (OS) were collected. No data on treatment after relapse of disease was available. All tumors were histopathologically classified according the 2015 WHO classification system. TNM classification was redefined for resections that were done before 2010 according to the 7th lung cancer TNM classification and staging system [8]. Prior to analysis, the

samples were de-identified. The Translational Research Board of the Netherlands Cancer Institute-Antoni van Leeuwenhoek hospital (NKI-AVL) approved the use of patient material in this study.

Mutation analysis and immunohistochemistry staining

Details on mutational analysis and immunohistochemical (IHC) staining for PD-L1 expression and CD8 infiltration was previously reported [9]. Double staining CD3 (yellow) followed by CD56 (purple) of whole slide sections prepared from FFPE resection specimens was performed on a Discovery Ultra autostainer. Slides were deparaffinised in the instrument and heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 32 minutes at 95^0C. The CD3 was detected in the first sequence using clone SP7 (1/100 dilution, 32 minutes at 37^0C, ThermoScientific). CD3 bound antibody was visualized using Anti-Rabbit NP (Ventana Medical systems) for 12 minutes at 37^0C followed by Anti-NP AP (Ventana Medical systems) for 12 minutes at 37^0C, followed by the Discovery Yellow detection kit (Ventana Medical Systems). In the second sequence of the double staining procedure CD56 was detected using clone MRQ-42 (1:2000 dilution, 32 minutes at 37^0C, Cell Marque). CD56 was visualized using Anti-Rabbit HQ (Ventana Medical systems) for 12 minutes at 37^0C followed by Anti-HQ HRP (Ventana Medical systems) for 12 minutes at 37^0C, followed by the Discovery Purple Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems).

Nanostring analysis

Gene expression analysis was performed using the NanoString nCounter Analysis system (NanoString) on 80-200ng RNA extracted from FFPE tissue samples. An input of 5*5µm slides was used. The most tumor-dense area and tumor percentage was assessed by a pathologist on the Hematoxylin and Eosin (H&E) staining and scraped off using a surgical blade. The RNA was isolated using the Roche “High pure RNA paraffin kit” (cat. No. 3270289001) following manufacturers protocol. A customized gene panel (version 0.3), including 531 targets including multiple genes of immunologic function and cancer biology and including 4 housekeeping genes was applied (Additional file 1). For 573 adequate RNA was available for NanoString analysis. To assess the quality of these samples, levels of expression for positive controls and negative controls were retrieved for each sample (Additional file 2). For 18 samples (3.1%) the expression levels were too low and an additional 25 samples (4.4%) failed the NanoString QC, leaving 530 samples for further analysis, consisting of 275 adenocarcinomas (AD), 235 squamous cell carcinomas (SCC) and 20 large cell carcinomas not otherwise specified (NSCLC NOS) (Additional file 3).

Gene expression and statistical analysis

All data analysis was performed in R (version 3.4.3) using CRAN and Bioconductor packages (Huber, Nature methods 2015). Differential gene expression between AD and SCC was assessed with Limma [10]. Heatmaps were generated with a custom version of ‘heatmap.2’ from the gplots package (https://CRAN.R-project.org/package=gplots). Kaplan-Meier plots were generated using the ‘survival’ package (https://CRAN.R-project.org/package=survival).

Validation cohorts

Normalized and clinical data were downloaded for two NSCLC datasets (GSE8894 and GSE14814) from NCBI's GEO database [11, 12]. Z-scores were calculated by centering and scaling the expression data. Expression of the 34-gene signature was computed using the average expression (z-score) of the 34 genes for each sample. To define the '34-gene signature high' and '34-gene signature low' groups for survival analysis the same percentages as in the Nanostring nCounter discovery dataset were used. RNA sequence read count data of lung squamous cell tumor samples (LUSC) from The Cancer Genome Atlas (TCGA) database were downloaded using TCGAbiolinks [13]. Stage I and II samples that were defined as 'Primary solid Tumor' were selected. Statistical analysis of the differential expression of genes was performed using DESeq2 [14].

Correlation of gene signature to immune cell types

To correlate expression of the 34-gene signature with specific immune cell types Microenvironment Cell Population (MCP)-counter was used [15]. To plot the MCP-counter output samples were ordered according to the expression of the 34-gene signature. Correlations between the 34-gene signature and MCP-counter output was calculated using the 'Pearson' correlation.

RESULTS

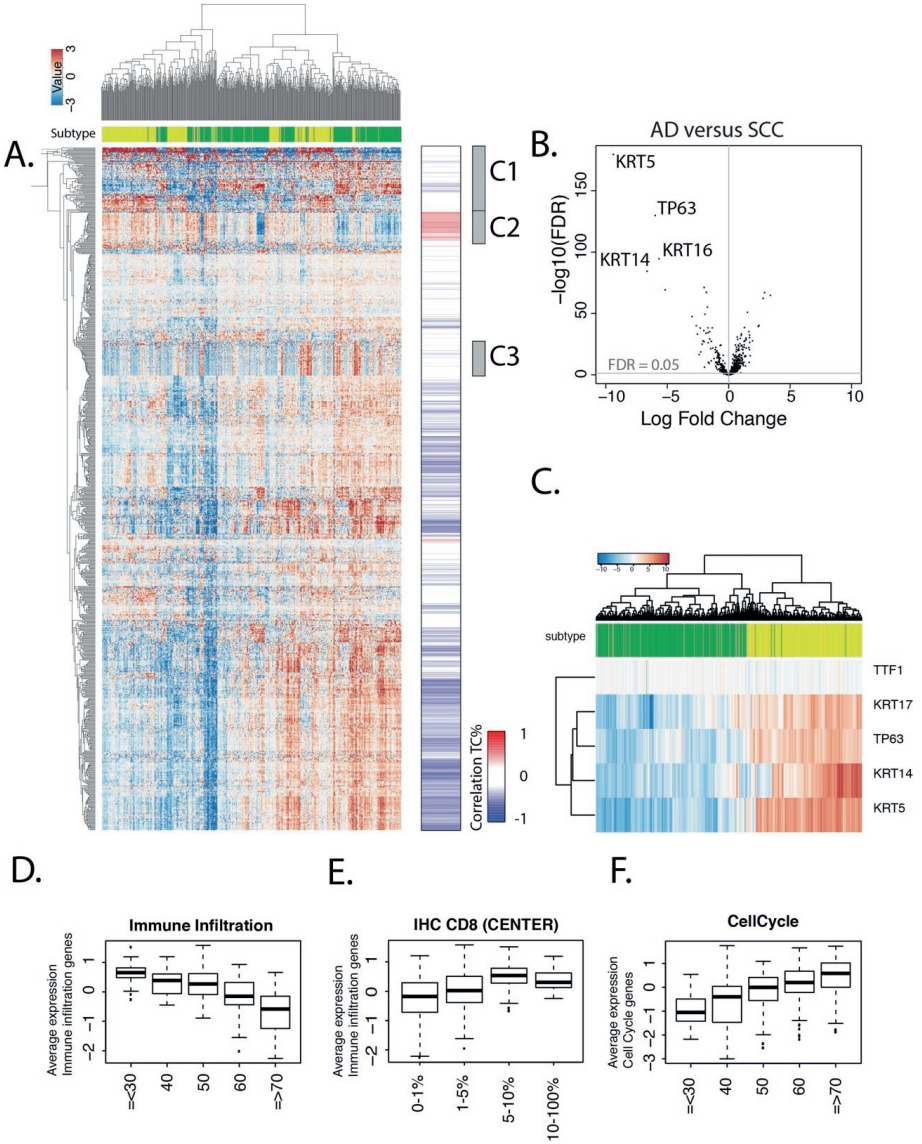
Gene expression analysis

In a cohort of 641 NSCLC archival tissue samples adequate RNA could be isolated from 573 samples and these were sent for nCounter (Nanostring) analysis. Gene expression results were obtained for 530 (92.5%) samples. Despite the large range in age of the FFPE blocks, no association was observed between age of the FFPE blocks or hospital of origin with the QC results. All 530 samples were included in an unsupervised clustering analysis (Figure 1A). Clear differences between the two main histological subtypes AD and SCC were observed (cluster 1). Differential gene expression analysis between AD and SCC showed the largest fold change for KRT5, KRT14, KRT17 and TP63 (Figure 1B). These genes are known to be highly expressed in SCC and KRT5 and p63 IHC are important markers in diagnostics of lung cancer. Interestingly, TTF1 - the most important diagnostic IHC marker for lung AD - was not able to differentiate between histological subtypes on the nCounter platform. Gene expression of TTF1 was higher compared to the negative controls, but at an overall low expression and variance (Figure 1C), suggesting that protein expression of TTF1 as the most important biomarker for adenocarcinoma of the lung may not be represented by high RNA levels. These findings show that the NanoString nCounter platform can be used to robustly perform gene expression analysis, even on old FFPE samples (>20 years).

Immune infiltration is anti-correlated with cell cycle related genes

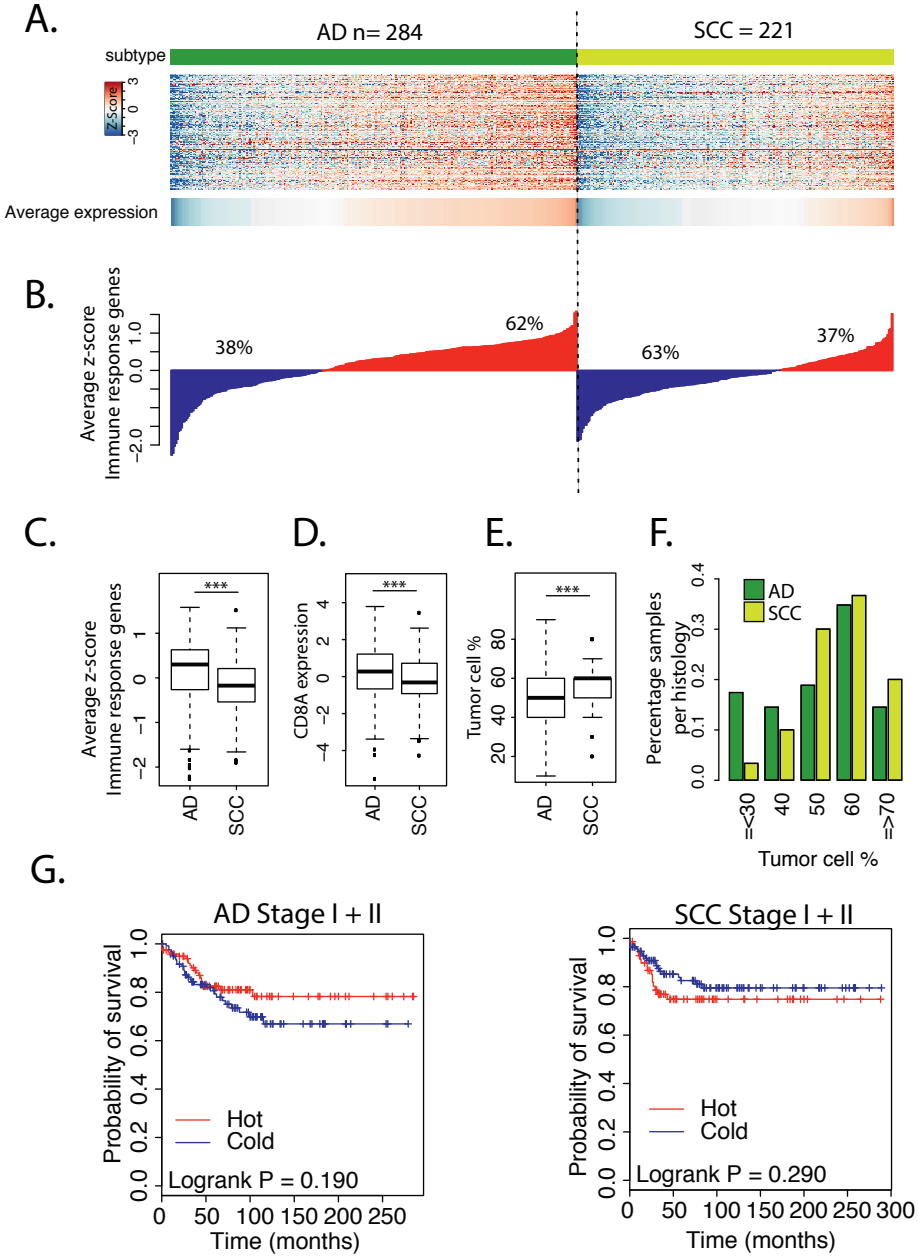
Besides differences between histological subtype, the unsupervised clustering of the samples was mainly driven by the overall level of inflammation; the inflamed or 'hot' samples vs. the non-inflamed or

Figure 1. Gene expression patterns in NSCLC.



A) Heatmap and clustering of all NSCLC samples (n=530) and all genes analyzed using nCounter (NanoString). Top bar indicates the histology as assessed by pathology: green represents AD, yellow SCC. Bar right of the heatmap show the correlation of each gene with the percentage of tumor cells (assessment by a pathologist). Red indicates a positive correlation, blue a negative correlation. Grey boxes indicate the identified clusters that do not correlate with tumor cell percentages B) Volcano plot with the log-fold change on the x-axis and FDR (-log10) on the y-axis. The 4 genes with the highest fold change are indicated. C) Top 4 genes that best differentiate SCC from AD and TTF-1 expression that does not differentiate. Top bar indicates the histology as assessed by pathology: green represents AD, yellow SCC. D) Immune response genes show a negative correlation with the percentage of tumor cells in a sample as assessed by pathology. E) Immune response genes show a positive correlation with the percentage of $CD8^+$ T cells in a sample as assessed by pathology. F) Cell Cycle related genes show a positive correlation with the percentage of tumor cells in a sample as assessed by pathology (cluster 2).

Figure 2. Expression of immune response genes do not provide a survival difference in NSCLC.



A) Heatmap of immune response genes for AD and SCC ordered according to the average expression of the genes. Top bar indicates the histology as assessed by pathology: green represents AD, yellow SCC. B) Waterfall plot of average expression of immune response related genes, both for AD (left panel) and SCC (right panel). Samples above the average are 'hot' tumors (red), the samples below 'cold' (blue). C) Box plot for expression of the immune response related genes per histology. *** $p < 0.001$. D) Box plot for expression of *CD8A* per histology. *** $p < 0.001$. E) Box plot for mean tumor cell percentages per histology. *** $p < 0.001$. F) Bar graph of each tumor cell percentage group for both AD (green) and SCC (yellow) samples. *** $p < 0.001$. G) Kaplan-Meier plots with the probability of survival of 'hot' versus 'cold' tumors in stage I/II tumors, both for AD and SCC.

'cold' samples. The expression of a subset of genes was negatively correlated with genes involved in inflammation (cluster 2). Gene Ontology analysis showed that the genes in cluster 2 were highly enriched for cell cycle related genes (Figure 1A, Additional file 1). As tumor cells tend to proliferate faster compared to stromal and/or most immune cells, this negative correlation between proliferation represented by cell cycle gene expression and the level of inflammation within samples might suggest a relation with the number of cancer cells and the number of immune cells within that same sample. Indeed, the percentage of tumor cells, based on H&E staining by a pathologist, correlated positively with the expression for cell cycle genes (R = 0.47) and correlated negatively with the expression of immune related genes in our cohort (R= -0.57, Figure 1D and F). Apparently, this occurs even though RNA from tumor samples was extracted from tumor-enriched areas designated on the H&E slide by a pathologist in order to increase tumor purity. In addition, these results suggest that not only the number of tumor cells, but also the number of immune cells is represented in the NanoString data and therefore allows for a quantitative measurement of the immune infiltration in these tumor samples. This was confirmed by an increasing expression of immune related genes per increasing number of $CD8^+$ T cells in the tumor-enriched areas (Figure 1E).

Inflammation according to histological subtype

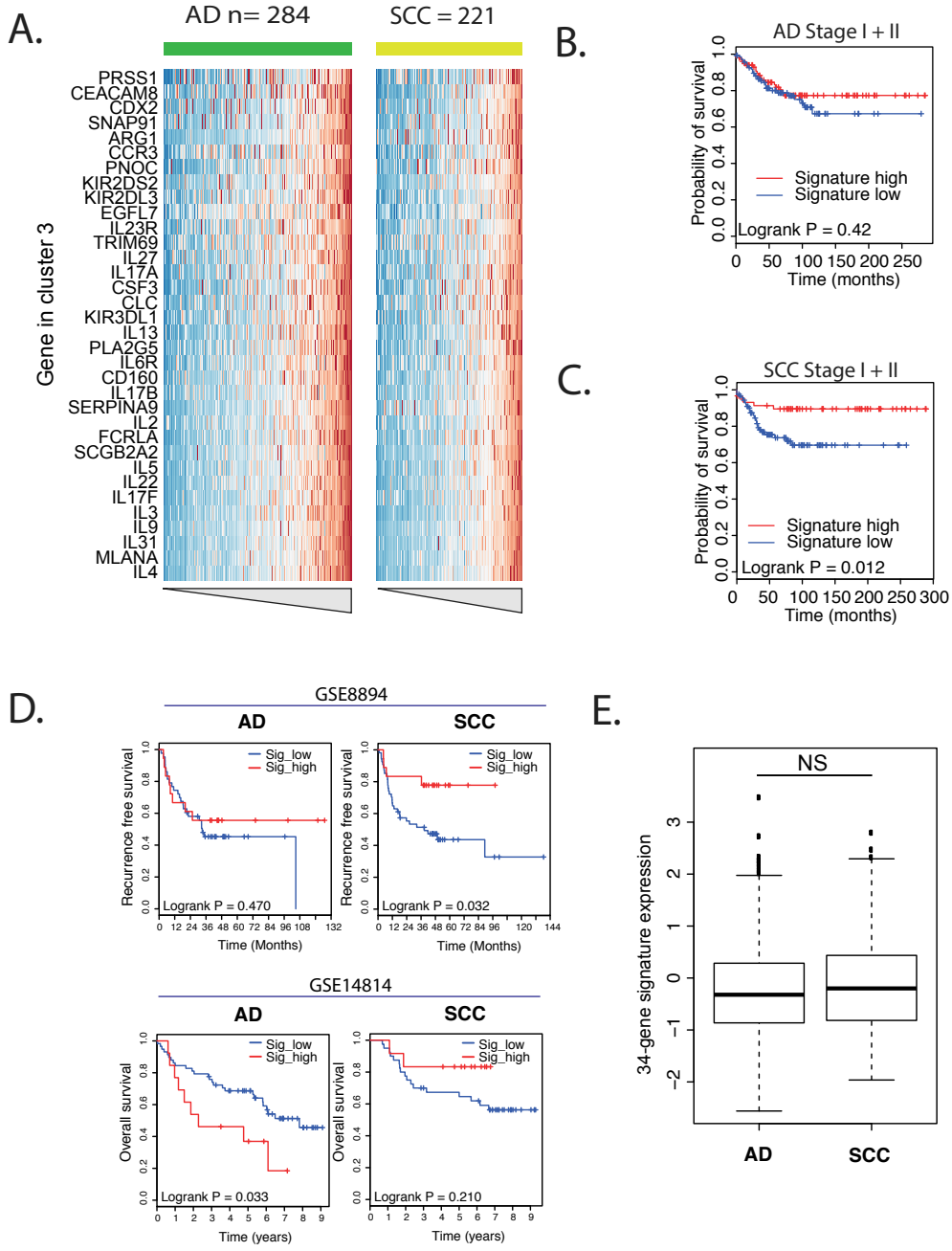
As a proxy to measure the level of 'active' inflammation in each sample as opposed to the quantified immune infiltration in general, we calculated the average expression of genes that are known to be involved in the response to immune signals (the 'immune response genes' as indicated by NanoString), available in the dataset (Additional file 1). Next, we divided the cohort by histological subtype and tested for each sample whether the average expression of immune response genes was above the mean ('hot') or below the mean ('cold') of the dataset. The distribution of samples above the mean was 62% for AD versus only 37% for SCC histology (Figure 2A-B). Based on our previous finding that the level of inflammation is negatively correlated with tumor cell percentage, a comparison between histologies was performed and confirmed our previous result for the 'immune response gene' expression as well: tumor cell percentage is significantly higher in SCC ($p < 0.001$, Figure 2C-D). These findings suggest that the ability of immune cell infiltration and/or the interaction between tumor and immune cells may be different between AD and SCC histology.

Associations between the level of inflammation and OS benefit has been contradictory for NSCLC in the past. No differences in survival were observed between 'hot' and 'cold' tumors in stage I/II samples for neither histologies in our cohort ($p = 0.19$ and 0.29, Figure 2G).

Expression of a 34-gene signature is a prognostic marker in SCC

In addition to the genes that correlated with immune infiltration, histology (cluster 1), and proliferation (cluster 2), the unsupervised clustering of all samples using all genes revealed a third cluster of genes (cluster 3, Figure 1A and Figure 3A). As opposed to the expression of the other immune genes, expression of cluster 3 did not correlate with tumor cell percentage. The expression of the 34-gene signature showed no association with PD-L1 expression and CD8 infiltration (Additional file 4). To check

Figure 3. Gene expression cluster 3 is predictive of response in SCC but not in AD.



A) Zoom-in of cluster 3 of the heatmap from Figure 1A. Samples are ordered on the average expression of the genes per subtype. B) Kaplan-Meier plots of AD samples divided into high (top 1/3) and low (bottom 2/3) expression of the 34-gene signature. C) Kaplan-Meier plot of SCC samples divided into high (top 1/3) and low (bottom 2/3) expression of the 34-gene signature. D) Same analysis as in B and C in two independent validation sets (GSE8894 and GSE14814). E) Boxplot of the expression level of the 34-gene signature in AD and SCC samples (p = 0.534).

whether there is any clinical relevance in the expression of this set of genes, we performed a survival analysis on the stage I/II samples, both for AD and SCC samples separately. In AD samples, no OS benefit was seen between 34-gene signature high (top 1/3) samples and 34-gene signature low (bottom 2/3) samples (p = 0.38, Figure 3B). In contrast, a clear OS benefit was observed in SCC between 34-gene signature high (top 1/3) and low (bottom 2/3) samples (p = 0.012, Figure 3C).

To validate these findings, we downloaded gene expression and associated clinical data from two publicly available NSCLC datasets [11, 12]. Since the expression levels of the genes that comprise the 34-gene signature were generally low, gene expression by RNA sequencing failed to provide accurate read count estimates for the 34-gene signature as tested in the TCGA NSCLC dataset (Additional file 2). Therefore, we were confined to methods with a high sensitivity for gene measurement. Microarray data showed similar sensitivity as our nCounter NanoString panel together with positive correlations between the genes of the 34-gene signature (Additional file 2), providing independent datasets to validate our findings.

In concordance with our large cohort of NSCLC samples, survival analysis on a dataset of 61 AD and 72 SCC samples (GSE8894) showed benefit in recurrence free survival (RFS) between samples with high expression (top 1/3) of the 34-gene signature and low expression (bottom 2/3) in SCC (p = 0.032), but not in AD (p = 0.47, Figure 3D). In the second dataset with 71 AD and 52 SCC samples (GSE14814), survival analysis showed improved OS for the samples with high 34-gene signature expression in SCC albeit not significant (p = 0.21). However, in AD the samples with high expression of the 34-gene signature showed a significant lower OS (p = 0.033, Figure 3D).

Together, these datasets recurrently show a survival benefit in stage I/II SCC patients with high expression of the identified 34-gene expression signature. This, in contrast to AD patients where high expression of the 34-gene signature is either not or negatively correlated with survival.

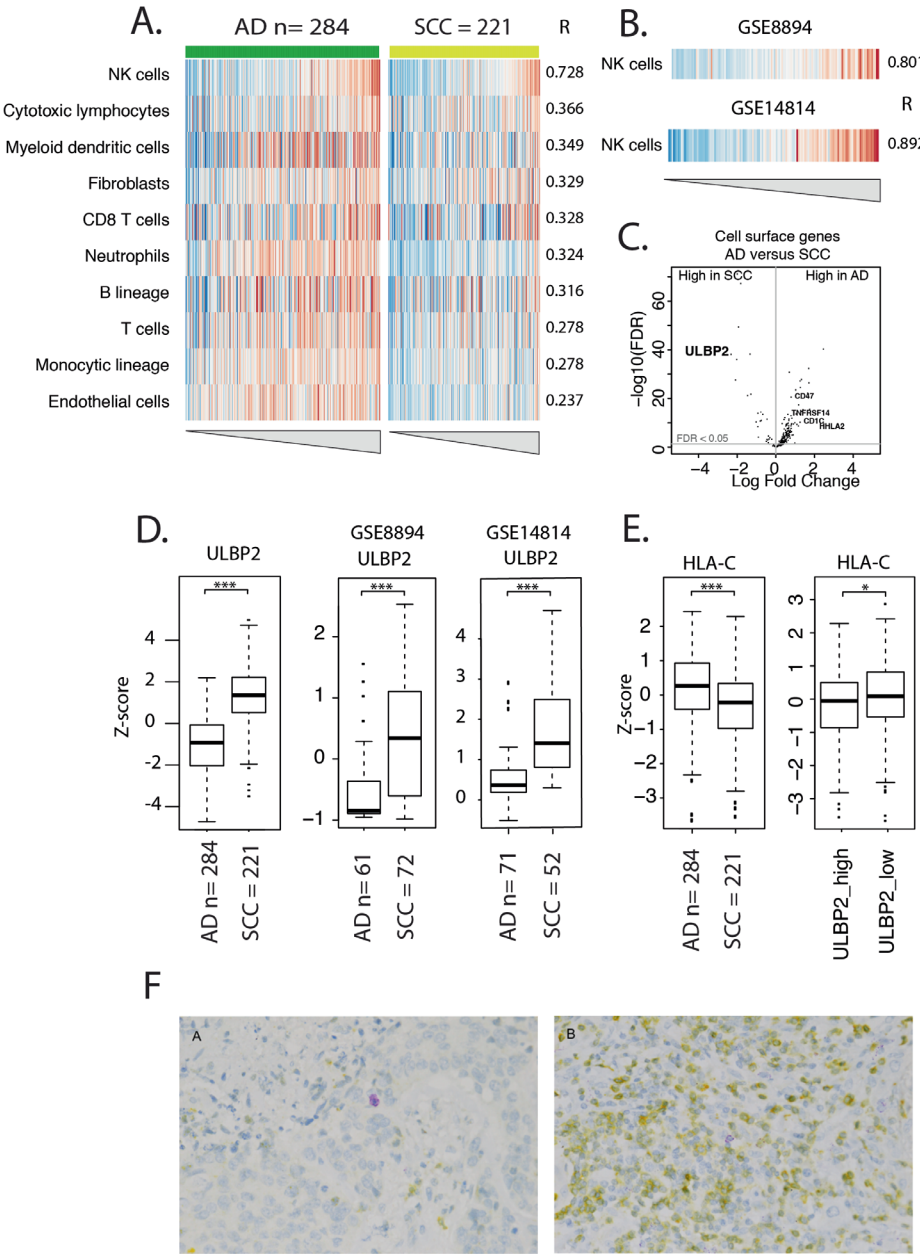
The 34-gene expression signature correlates with NK cell related gene expression

Interestingly, there was no difference in the level of expression of the 34-gene signature between AD and SCC histology (p = 0.53, Figure 3E). However, high expression of the 34-gene signature was only related to improved survival in SCC, suggests a difference in interaction between tumor and immune cells between the two histological subtypes.

To investigate the origin of this beneficial prognostic signal in SCC, we correlated the expression of our 34-gene signature with the presence of specific immune cell populations within the samples. Therefore, we applied MCP-counter on our datasets of 530 samples [15]. The highest correlation between expression of the 34-gene signature and specific immune cell populations was observed for Natural Killer (NK) cells (R = 0.73, Figure 4A). These finding were corroborated in the two independent datasets with again the highest correlation of the NK cell population (GSE8894, R = 0.80 and GSE14814, R = 0.89, Figure 4B and Additional file 5).

Although the expression level was comparable between histologies, but high expression of the 34-gene signature was only related to improved survival in SCC, this may suggest a difference in interaction between tumor and immune cells between the two histological subtypes. To further test whether the

Figure 4. Allocation of the signature.



A) Heatmap of immune cell populations ordered according to expression of the 34-gene signature (cluster 3). B) Correlation of the NK cell population as measured using MCP-counter. Samples are ordered according to the 34-gene expression signature. C) Volcano plot with the log-fold change on the x-axis and FDR (-log10) on the y-axis in AD and SCC for cell surface genes. D) Boxplot for expression of *ULBP2* in AD vs. SCC in our dataset and two independent validation sets. *** $p < 0.001$. E) Boxplot for expression of *HLA-C* in AD vs. SCC ($p < 0.001$) and boxplot with the expression of *HLA-C* in *ULBP2* high vs *ULBP2* low samples. * $p < 0.05$, *** $p < 0.001$. F) Examples of a $CD56^{+}/CD3^{-}$ NK cell in a 34-gene signature high SCC sample (A) and in a 34-gene signature low AD sample (B).

improved survival in SCC, but not in AD, even though expression level of the 34-gene signature was similar in both histologies, could indeed be explained by differences in the interface between tumor and immune cells we analyzed the dataset for cell surface genes and compared their expression between AD and SCC samples (Figure 4C). Interestingly, one of the cell surface genes highly expressed in SCC but not in AD is *ULBP2* (FDR < 0.001, Figure 4D), a marker for NK cell killing. Higher expression of *ULBP2* in SCC was also observed in our validation datasets (GSE8894; FDR < 0.001 and GSE14814; FDR < 0.001, Figure 4D). Also, high expression of *ULBP2* was associated with lower expression of *HLA-C*, one of the genes encoding for major histocompatibility complex (MHC) class I molecules. Furthermore, expression of *HLA-C* was significantly lower in SCC compared to AD (Figure 4E).
To further explore the possible role of NK cell killing in regard to the OS benefit in signature-high SCC opposed to signature-high AD, a double-staining of CD56 and CD3 was performed in a selection of samples. Signature-high and signature-low in both AD and SCC samples were evaluated. Overall, the infiltration of $CD56^{+}/CD3^{-}$ cells was scarce in SCC and only somewhat more frequent in AD, both irrespective of the expression level of the 34-gene signature. This difference between AD and SCC presumably matches the previously mentioned difference in tumor cell percentage and amount of immune infiltrate between histologies, which is overall more pronounced in AD vs. SCC (Figure 4F). These findings might suggest that a subgroup of SCC tumors seems more susceptible to NK cell recognition and killing, whereas this may not occur in AD tumors.

DISCUSSION

In our study, we performed gene expression analysis on a large cohort of early stage resected NSCLC samples. Unsupervised clustering of the samples was mainly driven by the overall level of inflammation, which was not correlated with survival in this patient set. Expression of a 34-gene signature did not correlate with the general inflammation level. This signature provided an OS benefit in SCC, but not in AD. This finding was validated in two independent NSCLC cohorts. The signature showed the strongest association with NK cells based on gene expression profiling, but this could not be validated by IHC, which showed that NK cell infiltration is scarce irrespective of histology.
The expression level of the 34-gene signature was comparable in both histological subtypes, but had a different effect on OS. This histology-dependent OS benefit may suggest a difference in the interaction of the immune system between AD and SCC NSCLC. To understand the biological foundation of the 34-gene signature, the selection of genes in the signature was compared to the gene profiles of eight immune cell populations as established by the MCP-counter method [15]. Our gene signature showed the strongest correlation with the gene profile of NK cells. NK cells have the unique property to revert to cell-killing induced without presentation of tumor specific antigens [16]. Production and release of granules, like perforin and granzyme B, cause lyses of the targeted cell [17]. Inhibition of NK cells occurs through activation of killer cell immunoglobulin-like receptors (KIRs) by recognition of MHC class I molecules on surrounding cells and thereby providing protection against auto-immunity. One mechanism of tumor immune escape is downregulation of MHC class I on tumor cells in order to evade

T cell recognition and killing [18]. However, this may render them vulnerable to NK cell attack. To strengthen the rationale for annotating our signature as possessing NK cell features, we sought for differences between the two histological subtypes in expression of tumor-related genes (as opposed to immune-related genes for which our NanoString panel was enriched). In our cohort, SCC samples showed a significant higher expression of the NK activation marker *ULBP2* and lower expression of the MHC class I gene *HLA-C* compared to AD samples. This may suggest that tumor growth in SCC may be possible because of the tumor immune escape mechanism of evasions of T cell recognition, but that NK cell killing may successfully prevent this escape, eventually leading to improved OS. McGranahan et al. recently found that loss of heterozygosity of HLA (HLA LOH) seemed to be correlated to prior immune activation and to a higher mutational burden in treatment-naïve, resected NSCLC [19]. Even though McGranahan et al. also found a higher overall level of inflammation in AD compared to SCC samples, SCC more often showed HLA LOH and this was associated with a higher expression of two different NK cell signatures from RNA sequencing data.

Unfortunately, there is no clearly validated method for establishing NK cell infiltration by IHC [20]. Because NK cells were defined as $CD56^{+}/CD3^{-}$ in the MCP-counter method, we performed a double-staining with CD56 and CD3 on a selection of samples in this cohort, but very few infiltrating NK cells in either histology were seen [15]. It has been described that even at a low ratio NK cells are able to kill tumor cells due to their specific cytotoxic abilities [21]. As the presence of NK cells in the tumor microenvironment is scarce, it may be difficult to study the role of the innate immune system and NK cells in particular regarding tumor cell attack [22, 23]. Furthermore, by performing only a double-staining with CD56 and CD3 acquiring a differentiating signal from additional subtypes of NK cells could have been missed; nor is it possible to establish the activity-level of these specific NK cells. Infiltration of tumors by NK cells has been previously linked to favorable outcome, although there are limited studies performed in NSCLC [24]. Villegas et al. found improved OS in early-stage SCC NSCLC when more NK cells were present in the tumor as assessed by CD57 staining [25].

However, the NanoString nCounter system used in this study and the microarray-based techniques used in both validation cohorts provide a higher sensitivity compared to standard RNA sequencing. This technique therefor allows discovery of immune gene expression that is present in very low abundance within the tumor microenvironment. Indeed, expression of most genes in the 34-gene signature was low, which precludes accurate measurement of the 34-gene signature in RNA sequencing data sets like TCGA and therefor precludes validation of the prognostic ability of the signature in these available cohorts. Backman et al. found no correlation between IHC of the NK cell marker NKp46 and expression of the corresponding gene *NCR1* measured by RNA sequencing in early-stage NSCLC, which they ascribed to low abundance of NK cells as well [26]. They also noticed that the expression of NK cell genes was not associated with the overall level of inflammation. This NK-enriched subgroup had low expression of T cell markers, low T cell activation and a low tumor mutational burden. Interestingly, the prognosis of this subgroup was similar to the inflamed subgroup, suggesting that not neoantigen-driven T cell recruitment, but a different (immune) mechanism of containing tumor growth may be responsible. Unlike our findings, this OS benefit was irrespective of histology.

Unfortunately, we were unable to provide solid evidence for the annotation of the 34-signature. The signature seemed to have NK cell like features, but although a plausible mechanism for NK cell intervention in tumor growth could be established in SCC over AD, this could not be translated back to IHC or RNA sequencing data. Unfortunately, exploration of additional pathways or gene sets associated with the 34-gene signature was not possible due to the relatively small number of genes in our NanoString panel, which was highly enriched for immune genes specifically, and no additional RNA sequencing data of this cohort was available. Previous NK cell signatures were based on RNA sequencing, sorted cell or single cell RNA sequencing. Due to the low expression level of most genes in the 34-gene signature a formal comparison between signatures that use different techniques seems futile. Maybe single cell sequencing using NanoString or microarray-based techniques may solve the remaining questions regarding the underlying mechanisms of scarce immune cells in the tumor microenvironment.

CONCLUSION

In conclusion, this study identified a subgroup of squamous NSCLC with an OS benefit that seemed not related to infiltration of immune cells in general, suggesting that a different (immune) mechanism of containing tumor growth may be responsible. A highly sensitive technique like NanoString was able to detect this subgroup based on a 34-gene signature, but further research will be needed to assist in explaining the biological rationale of such low-level expression signatures.

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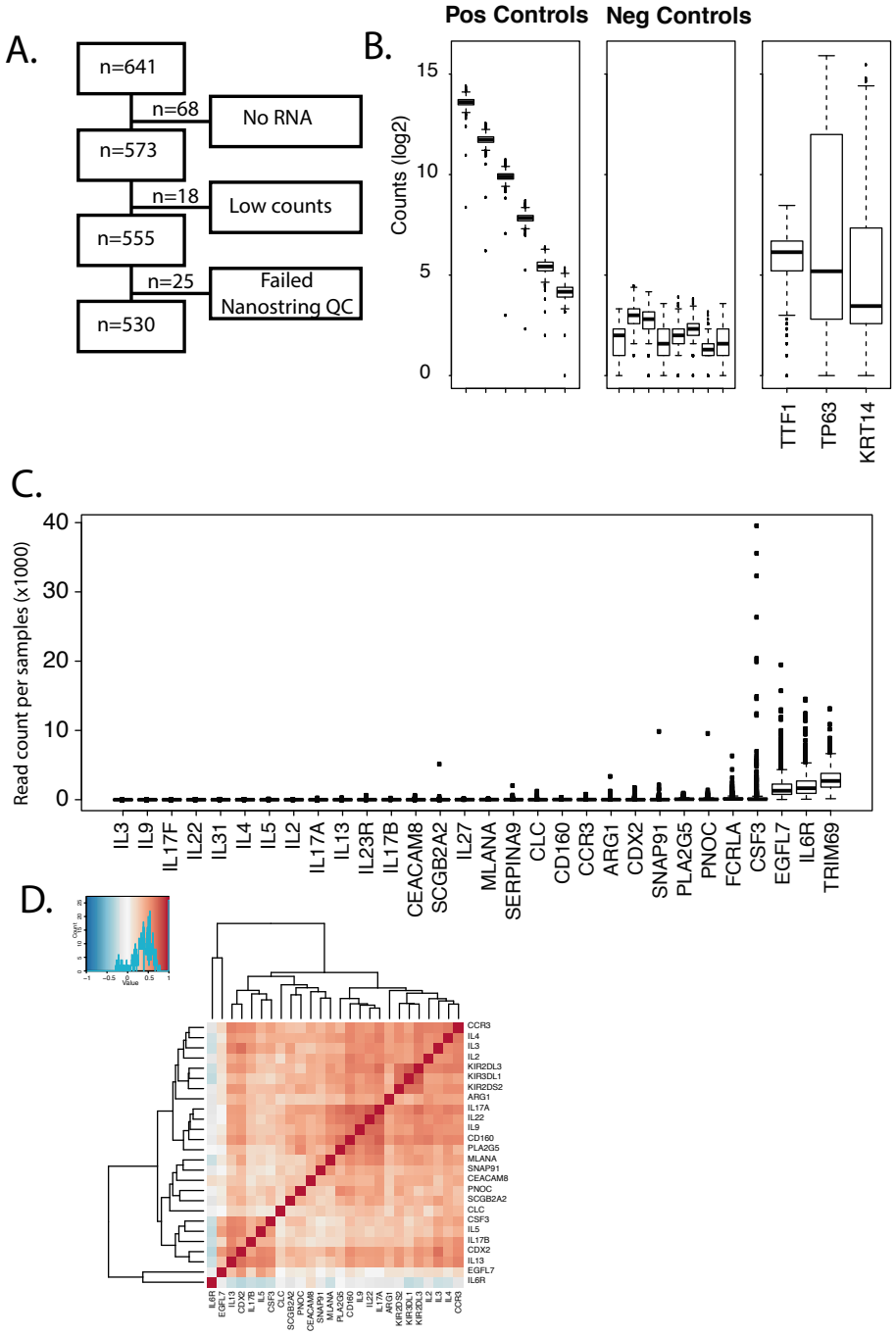
ADDITIONAL FILES

Additional file 1. List of NanoString gene panel.

Gene Cluster 1		Gene Cluster 2	Gene Cluster 3
PI3	FUT2	MKI67	PRSS1
KRT17	MUC1	CCNB1	CEACAM8
TP63	MLPH	UBE2T	CDX2
KRT14	RORC	ECT2	SNAP91
KRT5	C1orf116	ORC6	ARG1
MAGEA3	DPP4	GINS1	CCR3
PRAME	TREM1	RAD51AP1	PNOC
MAGEA4	TNFRSF10C	CDC6	NA
CTAG1B	ENPP3	TYMS	KIR2DS2
KIF1A	HHLA2	RRM2	KIR2DL3
UCHL1	S100B	DTL	EGFL7
DLL3	CD1A	NUF2	IL23R
BEX1	CD207	EXO1	TRIM69
GALNT13	FGFBP2	BIRC5	IL27
CDH2	VTCN1	CENPF	IL17A
NCAM1	S100A9	TOP2A	CSF3
HLA-DQA1	S100A8	MYBL2	CLC
PDZK1IP1	FGFR3	KIF2C	KIR3DL1
LCN2	HAS3	CDC20	IL13
LTF	COL4A6	MELK	PLA2G5
NA	NRG1	NDC80	IL6R
CXCL1	WNT5A	NA	CD160
CCL20	ACKR3	ANLN	IL17B
IL6	JAG1	UBE2C	SERPINA9
SELE	ITGA6	CEP55	IL2
HAS1	FERMT1	CCNE1	NA
PTGS2	TP73	FBXO5	FCRLA
EGLN3	EFS	EZH2	SCGB2A2
ANGPTL4	FGFR2	CENPK	IL5
TNFSF11	ULBP2	PTTG1	IL22
LIF	BBC3	STMN1	IL17F
CXCL3	CDKN2B	BRIP1	IL3
ESM1	CDKN2A	ZNF367	IL9
APLN	SFRP1	GGH	IL31
PROK2		HMMR	MLANA
CMTM2		NA	IL4
AREG		NA	
EREG		LRP4	
FOXA1		HEY1	
KIT		PHGDH	
TMEM45B		E2F7	
CEACAM1		NOS2	

Additional file 2.



A) Flowchart of samples for NanoString analysis. B) QC data NanoString: positive/negative controls and keratin expression. C) The 34-gene signature does not work on TCGA RNA-seq data: unmeasurable or low expression of the majority of the genes. D) Heatmap with correlations (Pearson correlation) of genes from the 34-gene signature in the NSCLC validation set (GSE14814).

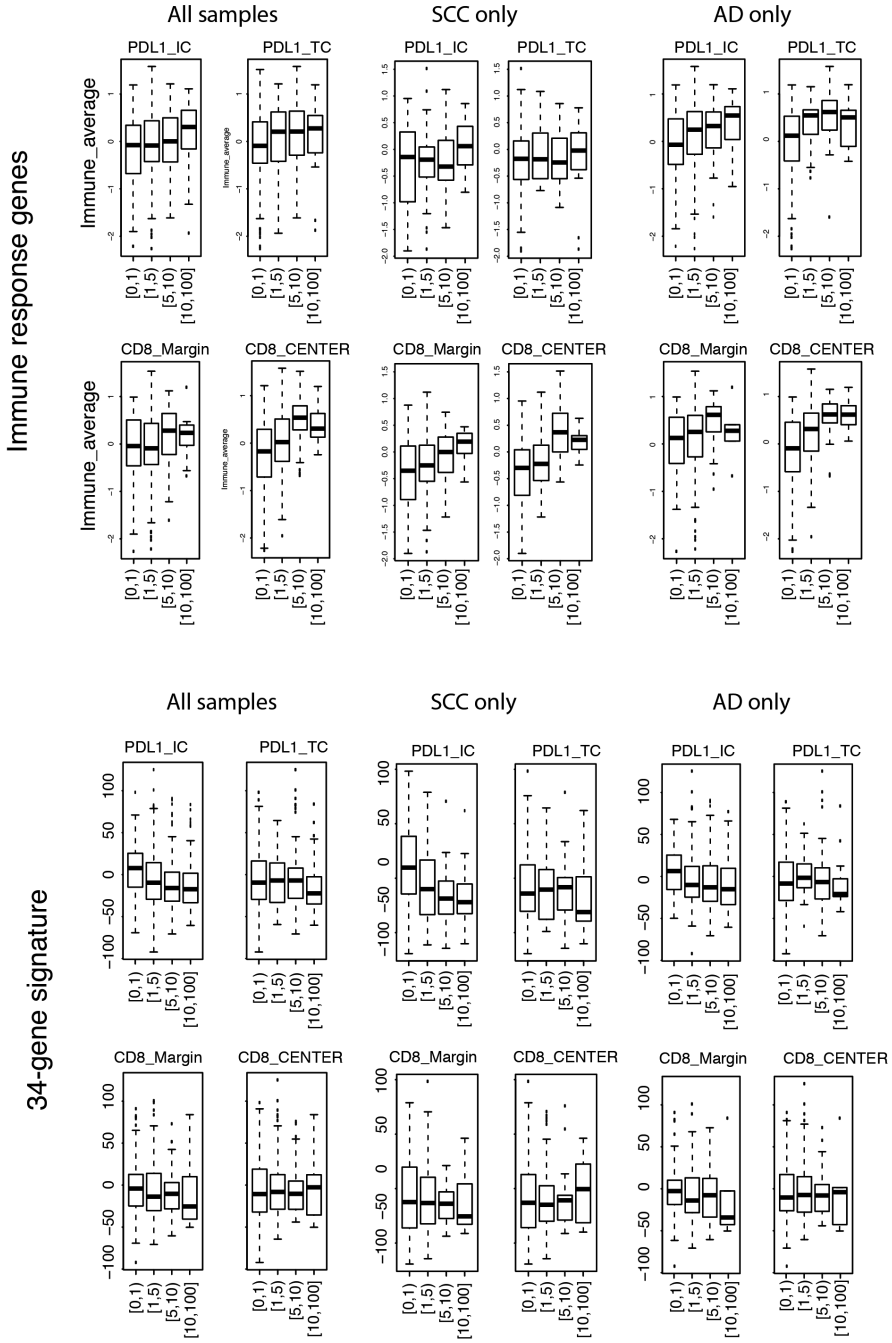
Additional file 3. Patients' and tumor characteristics of the non-small cell lung cancer cohort

	AD	SCC	NSCLC NOS
Total (n = 530)	275	235	20
Gender			
Male	136 (49%)	156 (66%)	11 (55%)
Female	139 (51%)	79 (34%)	9 (45%)
Median age at surgery (years, range)	62 (30-83)	68 (37-85)	58 (37-81)
Neo-adjuvant therapy			
Chemotherapy	16 (6%)	2 (0.8%)	1 (5%)
Concurrent chemo radiotherapy	4 (1.5%)	1 (0.4%)	2 (10%)
Sequential chemo radiotherapy	2 (0.7%)	1 (0.4%)	0
Erlotinib	19 (7%)	6 (3%)	2 (10%)
Radiotherapy	0	1 (0.4%)	0
No neo-adjuvant therapy	234 (85%)	224 (95%)	15 (75%)
Smoking			
Never	21 (8%)	0	1 (5%)
Still	107 (39%)	109 (46%)	10 (50%)
Stopped	141 (51%)	122 (52%)	9 (45%)
Unknown	6 (2%)	4 (2%)	0
Tumor stage at resection			
Stage I	169 (61%)	115 (49%)	8 (40%)
Stage II	64 (23%)	82 (35%)	7 (35%)
Stage III	35 (13%)	32 (14%)	5 (25%)
Stage IV	7 (3%)	6 (3%)	0
Genetic alterations*			
EGFR	16 (6%)	0	0
KRAS	89 (32%)	8 (3%)	2 (10%)
PIK3CA	8 (3%)	10 (4%)	0
BRAF	1 (0.3%)	0	0
NRAS	0	3 (1.3%)	0
HRAS	0	1 (0.4%)	0
Median overall survival (months,range)	49 (0-285)	49 (0-289)	53 (6-259)

* percentages for analyzed samples only. EGFR mutations included exon 19 deletions, exon 20 insertions and exon 21 L858R mutations. No T790M mutations were found. KRAS mutations included mutations in codon 12, 13 and 61. Mutations in AKT1, ERBB2, FLT3, JAK2, KIT, MYD88 were not present within this cohort. All present MET mutations were germline single nucleotide polymorphism (SNP).

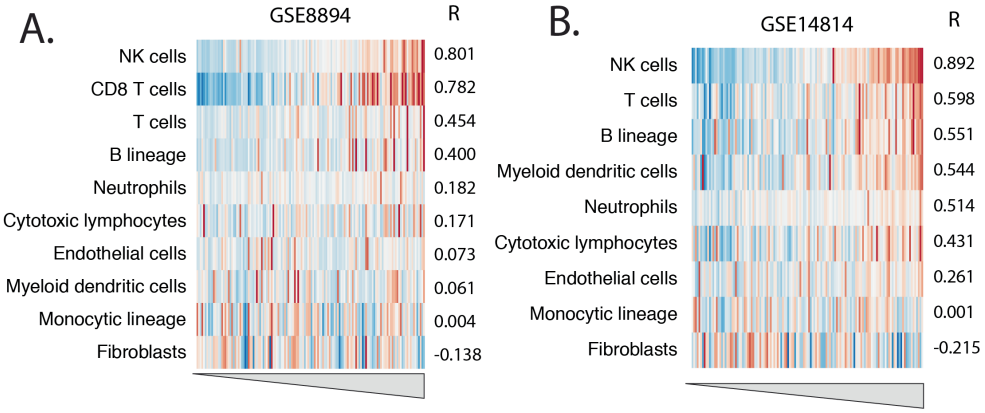
SCC = squamous cell carcinoma, AD = adenocarcinoma, NSCLC NOS = non-small cell lung cancer not otherwise specified

Additional file 4.



Boxplots of the associations between the immune response genes and the 34-gene signature with PD-L1 expression on tumor cells (TC) and immune cells (IC) and CD8 infiltration in the tumor margin and in the tumor center.

Additional file 5.



Heatmaps of the cluster 3 genes with allocated immune cell types per two independent cohort with correlation

PART II.

Modulating the tumor immune microenvironment

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CHAPTER 4

Synergizing systemic responses by combining immunotherapy with radiotherapy in metastatic non-small cell lung cancer: the potential of the abscopal effect

Lung Cancer. 2020 Apr;142:106-113

HIGHLIGHTS

- Immune checkpoint inhibitors are the new cornerstone of metastatic NSCLC treatment
- Several tumor immune escape mechanisms causing failure to ICIs have been postulated
- The immunoediting effect of radiotherapy may overcome some of these mechanisms
- (Pre-)clinical evidence supports augmentation of ICI efficacy when combined with radiotherapy

ABSTRACT

Immunotherapy has obtained a secure place in the treatment of metastatic non-small cell lung cancer (NSCLC) and has made a great impact on prognosis of responders. Unfortunately, not all NSCLC patients derive benefit from this treatment. Several immune escape mechanisms have been postulated, explaining failure of tumor immune attack. A better understanding of these mechanisms helps us to seek treatment strategies to overcome resistance to immunotherapy. Radiotherapy has immunomodulatory qualities capable of enhancing the anti-cancer immune response by tackling a number of these tumor escape mechanisms. In this review, we focus on mechanisms of off-target effects of radiotherapy, the so-called abscopal effect, by describing the current role of immune checkpoint inhibitors (ICIs) in NSCLC, the possible reasons for its failures and evidence on how radiotherapy may be able to counteract these mechanisms. An oversight of pre-clinical and clinical data supporting augmentation of abscopal events by radiotherapy when combined with ICIs is presented. As much remains unclear regarding optimal dose, fractionation, target volume or timing of radiation therapy, future research will need to focus on implementing data from pre-clinical and translational findings in the development of new clinical trials in order to help optimizing the potential of the combination of immunotherapy with radiotherapy.

1. Introduction

In recent years, treatment and prognosis for patients with metastatic non-small cell lung cancer (NSCLC) has changed profoundly due to introduction of immunotherapy. Blocking the programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway has become a new cornerstone in the treatment of advanced NSCLC patients, especially for those without targetable mutations. PD-L1 is mainly expressed by macrophages and endothelial cells, but also in a wide variety of solid tumors [1-3]. Binding of PD-L1 to its receptor PD-1 on T cells or antigen presenting cells (APC) activates an inhibitory signal leading to apoptosis or inactivation of these immune cells, thereby allowing tumors to evade the host immune response. To a lesser extent, interventions in another immune checkpoint mechanism, the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) pathway, have shown efficacy in NSCLC. CTLA-4 is expressed on naïve T cells and by binding to its ligands B7-1 or B7-2 expressed by APCs in lymph nodes, CTLA-4 transmits an inhibitory signal disabling priming of new T cell responses [4].

1.1 Current clinical setting of immune checkpoint inhibitors in NSCLC

Immune checkpoint inhibitors (ICIs) gained their first approval by the US Food and Drug Administration and the European Medicines Agency for the treatment of advanced NSCLC based on two phase III trials in second-line setting comparing the PD-1 antibody nivolumab to standard of care (SoC) chemotherapy, i.e. docetaxel. In both non-squamous and squamous NSCLC, nivolumab showed an improvement in overall survival (OS) over docetaxel [5, 6]. Shortly after, registration followed for the PD-1 antibody pembrolizumab and the PD-L1 antibody atezolizumab due to improved OS over docetaxel in second-line as well [7, 8]. Higher expression of PD-L1 as assessed by immunohistochemistry (IHC) has consistently been reported to be associated with higher response rates to anti-PD-(L)1 treatment. Objective response rates (ORR) varied from 8% in PD-L1 negative tumors up to approximately 30% in tumors with high PD-L1 expression [5, 7, 8]. Nivolumab and atezolizumab were approved as second-line treatment irrespective of PD-L1 expression and pembrolizumab for PD-L1 expression ≥1% only as PD-L1 negative tumors were excluded from the registration trial [7].

Subsequently, anti-PD-(L)1 treatment has found its way into first-line. The KEYNOTE-024 study compared pembrolizumab to platinum-based chemotherapy in patients with previously untreated advanced NSCLC with a PD-L1 expression of ≥50% and found a convincing improvement of OS for pembrolizumab in this group [9]. KEYNOTE-189 and KEYNOTE-407 showed that combining platinum-based chemotherapy with pembrolizumab as first-line regime is superior to chemotherapy monotherapy irrespective of PD-L1 expression [10, 11]. These results have established immunotherapy as the new cornerstone in first-line treatment of NSCLC patients. Two other first-line studies both met their co-primary endpoint of progression free survival (PFS) and OS benefit for the addition of atezolizumab to carboplatin and nab-paclitaxel (IMpower 130) and for the addition of atezolizumab to carboplatin, paclitaxel and bevacizumab (IMpower 150) in non-squamous NSCLC [12, 13].

No phase III trials with CTLA-4 inhibitor monotherapy have been performed in NSCLC. In the multi-arm CheckMate-227 study, the combination of nivolumab with the CTLA-4 antibody ipilimumab improved OS compared to platinum-based chemotherapy in first-line NSCLC [14]. This was significant in both the PD-

L1 ≥1% subgroup, which was the primary endpoint of this study, and in the PD-L1 negative subgroup. Another immunotherapy combination of the PD-L1 antibody durvalumab and anti-CTLA-4 drug tremelimumab showed no PFS or OS benefit over first-line chemotherapy in patients with advanced NSCLC [15]. The role of the addition of CTLA-4 antibodies in advanced NSCLC therefore remains unclear, and no approval of anti-CTLA-4 treatment in NSCLC has been granted to date.

Due to the success of PD-(L)1 inhibition in stage IV disease, immunotherapy was also tested in earlier and curable stages of NSCLC. In stage III NSCLC, the PACIFIC-trial compared one-year adjuvant durvalumab to placebo for patients that had not developed progression after concurrent chemoradiation (CRT). Patients in the adjuvant durvalumab arm experienced improvement of PFS and OS over placebo and this adjuvant treatment is now SoC [16]. Recently published studies in this setting were mainly focusing on safety and translational issues. Other trials with neo-adjuvant and adjuvant treatment for early stage NSCLC are ongoing, so more specific data on efficacy is eagerly awaited.

The introduction of ICIs has made a great impact on clinical outcomes for patients with advanced NSCLC as long-lasting anti-tumor immune responses on monotherapy have been described [17, 18]. The combination with chemotherapy in first-line setting increased response rates to an impressive 48-58% depending on histology, leading to further improvements in survival for advanced NSCLC patients [10, 11]. The addition of adjuvant immunotherapy to CRT transferred benefits to curable stage III patients [16]. Unfortunately, primary –as well as secondary- resistance to immunotherapy is still common and no clear second-line systemic treatment option has momentarily been established.

1.2 What may cause failure to immune checkpoint blockade?

In recent years, many insights were obtained in the interaction between the immune system and solid tumors. Alterations gained in tumor DNA may lead to expression of mutated proteins. Some of these mutated antigens can serve as so-called neoantigens. They can be recognized as non-self by APCs and when phagocytized and presented to circulating T cells a tumor specific immune reaction can be induced. Apparently, mechanisms of tumor immune escape have to be in existence in order to allow tumor growth to occur. Evasion of immunological destruction has now been recognized as an emerging hallmark of cancer [19]. Several of these immune escape mechanisms have been postulated (Table 1): 1) low tumor mutational burden (TMB) may prevent the presence of adequate neoantigens for recognition by APCs or T cells; 2) a low spill of neoantigens due to lack of excessive cell death, for example in slow progressing tumors, could compromise the induction of an immune response; 3) a lack of penetration of APCs into the tumor bed will prevent the ability of antigen presentation; 4) tumor cells may create a hostile environment to prevent infiltration of cytotoxic T cells into the tumor bed; 5) in order to become activated, T cells require inflammatory stimuli like danger signals after immunogenic cell death. In immune ‘cold’ tumors these stimuli are often absent; 6) recognition of neoantigens by cytotoxic T cells may be impaired through hampering of neoantigen presentation by oncogenic downregulation of MHC class I molecules on tumor cells or through a lack of diversity of the T cell receptor (TCR) repertoire; 7) presence of immune suppressive cells, like myeloid-derived suppressor cells (MDSCs), tumor-associated macrophages (TAMs) and T regulator cells (Tregs) or immune suppressive cytokines can disrupt the ongoing anti-cancer immune reaction; 8) upregulation of immune inhibitory pathways

like the PD-L1/PD-1 axis can cause further suppression and secondary tumor immune escape; 9) also, T cell exhaustion or a too large a tumor load for the immune system to handle may eventually lead to renewed tumor progression [20, 21].

Table 1. Mechanisms of tumor immune escape.

• Low tumor mutational burden and lack of adequate neoantigens
• Low spill or exposure of neoantigens
• Lack of antigen presenting cell penetration into the tumor bed
• Lack of cytotoxic T cell infiltration into the tumor bed
• Absence of inflammatory stimuli for T cell activation
• Oncogenic downregulation of MHC I molecules on tumor cells
• Lack of diversity of the T cell receptor repertoire
• Presence of an immune suppressive tumor microenvironment
• Oncogenic upregulation of immune inhibitory pathways
• Exhaustion of cytotoxic T cells

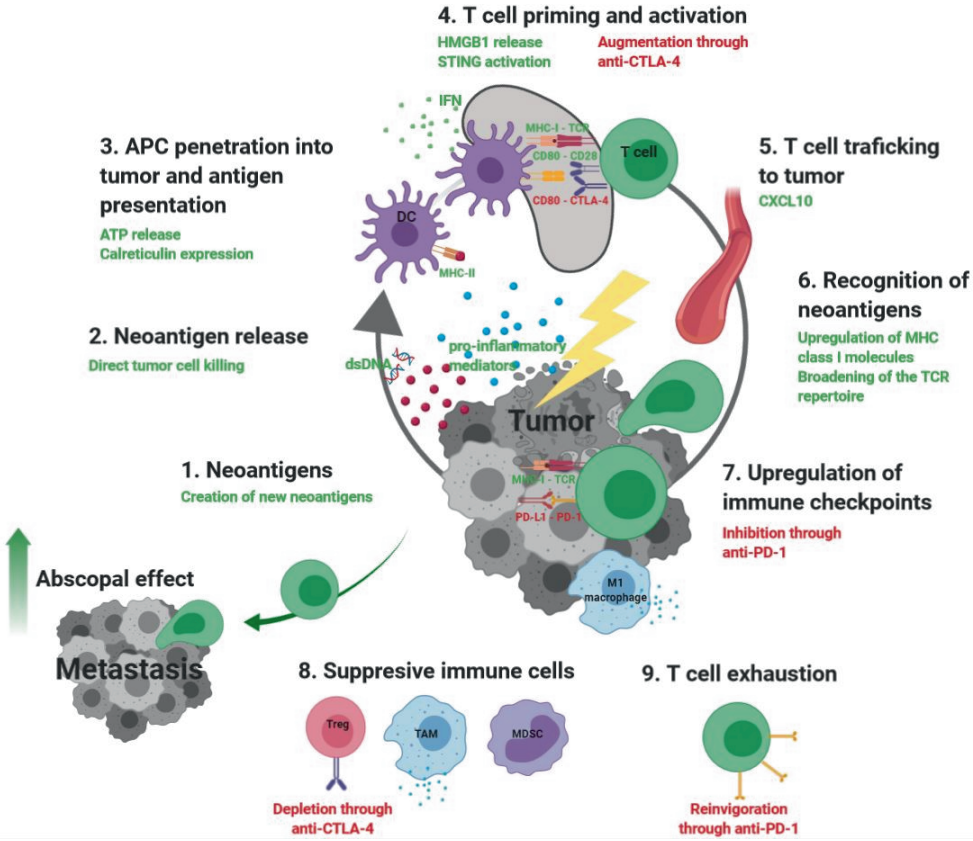
ICIs seem to be able to overcome some of these escape mechanisms, but unfortunately, resistance still forms a big challenge in daily patient care. It is therefore important to explore ways to further enhance the effect of anti-PD-(L)1 therapy to overcome this.

1.3 The immunomodulatory effect of radiotherapy

There has been a growing amount of in vitro and *in vivo* evidence that ionizing radiation has strong immunomodulatory potential, which provides a biological rationale that radiotherapy might be successful in making tumors more vulnerable to immune attack. A direct toxic effect of radiotherapy is decreasing tumor burden through the induction of tumor cell death. Furthermore, radiotherapy is able to induce immune responses that can both be pro-inflammatory and antitumor as well as immunosuppressive and protumor [22, 23]. When radiotherapy causes immunogenic cell death however, the subsequent effects may be able to counteract many of the tumor immune escape mechanisms mentioned above (Figure 1). Immunogenic cell death is characterized by the release of tumor antigens, production of pro-inflammatory mediators like ATP and high mobility group box 1 (HMGB1), and enhancement of surface expression of calreticulin [24]. Secretion of large amounts of ATP stimulates recruitment and activation/maturation of APCs. Exposure of calreticulin acts as an ‘eat-me’ signal, hence promoting the uptake of dead cell-associated antigens by APCs [25]. Radiotherapy also has the potential to create novel proteins that can be presented by APCs and thereby increases the pool of neoantigens [26]. This immunogenic cell death leads to increase of antigen presentation in the tumor draining lymph nodes, where tumor-specific T cells can become activated and then make their way to the newly inflamed tumor bed [27, 28]. The activated T cells are guided by the secretion of CXCL10 by tumor cells, a stimulator of T cell recruitment. The release of HMGB1 promotes the synthesis of pro-inflammatory factors including type I interferons (IFN), which are responsible for this CXCL10 production [25].

Also, radiotherapy causes release of double-stranded DNA and RNA, thereby activating the “stimulator of interferon genes” (STING) signaling pathway in dendritic cells through type I IFNs. When STING pathway activation in dendritic cells was blocked, priming of T cells would not occur, suggesting that

Figure 1. Tumor immune escape mechanisms and immunomodulatory effects of radiotherapy.



Mechanism of tumor immune escape are summarized in black text. The immunomodulatory effects of radiotherapy are presented in green text and grouped per escape mechanism. In red text the additive immune stimulatory effects of immune checkpoint inhibition are given. APC = antigen presenting cell; HMGB1 = High mobility group box 1; STING = stimulator of interferon genes; CTLA-4 = cytotoxic T-lymphocyte-associated protein 4; TCR = T cell receptor; PD-1 = programmed death 1; PD-L1 = programmed death-ligand 1; Tregs = T regulator cells; TAM = tumor-associated macrophages; MDSCs = myeloid-derived suppressor cells. The image was constructed using biorender.

STING signaling plays an essential role in generating an adequate immune response [29]. Furthermore, radiotherapy can improve the ability of tumor cell recognition by increasing upregulation of MHC class I molecules on tumor cells and by broadening the TCR repertoire [26, 30].

However, reports on the effect of radiotherapy on the presence of immune suppressive cells, like MDSCs, TAMs and Tregs, have been conflicting. A shift towards a more pro-inflammatory tumor microenvironment has been described as well as an increase in immune suppressive cell populations after radiotherapy [31, 32]. The reason for these discrepancies might be due to differences in tumor models or radiation regimes.

Low-dose radiotherapy has shown to have some specific immunomodulatory effects. Given the high radiosensitivity of leucocytes, low doses of radiotherapy are able to rid a tumor of immunosuppressive cells [33]. Additionally, low-dose radiotherapy keeps the local vasculature intact, while facilitating

extravasation of leucocytes by upregulation of ICAM-1 on endothelial cells is able to differentiate macrophages towards an immune-stimulatory M1 phenotype [34, 35].
Upregulation of immune checkpoints, like PD-L1 or IDO1, by radiotherapy with subsequent rebound immune suppression has been described in several pre-clinical studies [28, 36-38]. This upregulation strengthens the hypothesis that radiotherapy, when applied in the right way, can be an accessible and capable immunomodulator in combination with ICIs.

2 Rationale of combining radiotherapy with immune checkpoint inhibition

Besides a local synergistic antitumor effect between radiotherapy and the immune system, rare cases of systemic phenomena of interaction have also been observed. Local radiotherapy in itself can induce a systemic response by showing tumor shrinkage of untreated cancer lesions in patients [39, 40]. The notion of a systemic response by local radiation treatment is referred to as the abscopal effect; ab scopus meaning away from the target. These findings led to further research as to how localized radiotherapy could induce tumor-specific T cell activity and whether combination with ICIs could create a synergistic effect and even overcome primary resistance to ICIs.

2.1 Pre-clinical evidence of synergy between immuno- and radiotherapy

Several in vitro and *in vivo* studies in solid tumors compared ICIs combined with radiotherapy to either of the regimes alone. In an *in vivo* model of glioblastoma multiforme, the combination of a PD-1 antibody and radiation showed increased infiltration of cytotoxic T cells, reduced Tregs and improvement of tumor control compared to either regime alone [41]. In similar experiments in other *in vivo* solid tumor models, tumor regression was more pronounced when radiation was combined with anti-PD-1 treatment in the radiated lesion as well as in the non-radiated lesion [28, 37, 42, 43]. The synergistic response disappeared after depletion of T cells, suggesting that T cells are mandatory players in the abscopal effect [28]. The authors concluded that the necessity of additional anti-PD-1 treatment to establish a meaningful abscopal effect is because of upregulating of the PD-1/PD-L1 axis as an immune suppressive ‘side effect’ of radiation [37].
Less is known about the optimal dose, fractionation, target volume or timing of radiation therapy. As mentioned previously, low-dose radiotherapy may lead to pro-inflammatory and antitumor effects, but both low and high doses of radiotherapy are able to evoke local or abscopal immune responses, presumably through various mechanisms. Abscopal responses to low-dose radiation have been reported in mice [36]. However, (moderately) hypofractionated radiotherapy has been hypothesized to give stronger abscopal responses in various mice/tumor models. Morisada et al. compared the effect of a low-dose fractionated (10x2Gy) to a high-dose hypofractionated regime (2x8Gy) in an oral cavity and a colon carcinoma model. They found suppressed antitumor immunity with low-dose radiotherapy, but high-dose hypofractionated radiation led to preservation of peripheral and tumor-infiltrating effector immune cells, reduction of immunosuppressive immune cells and enhancement of tumor-specific immune responses [44]. Both radiotherapy strategies showed a reduction in MDSCs, but not in Tregs. They also found that addition of anti-PD-1 treatment after high-dose hypofractionated radiotherapy

reversed adaptive immune resistance, but this did not occur after low-dose fractionation. In a lung cancer model, Camphausen et al. had already confirmed that 5x10Gy provided increased tumor growth reduction in a non-radiated tumor lesion compared to low-dose 12x2Gy [45]. Several studies showed that a fractionated dose led to more signs of the abscopal effect compared to a single dose [46-48]. The experiments by Vanpouille-Box et al. also suggested that there might be a maximum dose as a dose of 8-10Gy per fraction induced interferon signaling, whereas higher doses lead to TREX1 upregulation and abrogation of interferon signaling [48]. However, Lee et al. noticed vigorous priming and expansion of effector T cells after a high single dose of 20Gy altering the tumor microenvironment from immune-suppressive to immune-activating in a melanoma mouse model [49].

The ideal timing of radiation with ICIs has been the focus of investigation as well. When fractionated radiotherapy was combined sequentially vs concomitantly with anti-PD-L1 treatment in a variety of syngeneic mouse models of cancer, lead-time of more than one week between radiation and start of immunotherapy rendered T cells anergic [36]. The survival benefit shown in the concomitant regime was lost in the sequential regime suggesting that anti-PD-1 treatment should be started within one week after radiation. The optimal timing may also be dependent on the specific effect of the immunotherapy agent. Applying anti-CTLA-4 treatment before radiation showed longer survival compared to adjuvant treatment *in vivo*. However, anti-OX40, a co-stimulatory agonist, did not provide any antitumor effect when given before radiation, but tumor reduction and improved survival was established when anti-OX40 was given within 24 hours after radiotherapy, suggesting that this compound is effective only in the antigen presentation phase [50]. In melanoma mouse models, Twyman-Saint Victor et al. found a 58% complete response rate after treatment with radiotherapy and doublet immunotherapy: CTLA-4 and PD-L1 inhibition. Again, CTLA-4 antibodies were only effective when given before radiation. Based on tumor and blood analyses, they postulated that CTLA-4 antibodies may downregulate Tregs, that anti-PD-L1 antibodies reinvigorate exhausted CD8 T cells and that radiotherapy broadens the TCR repertoire, illustrating why this proposed 'triple-regime' might even further improve patient outcomes [51].

The number, size and location of the target volume(s) could all possibly influence the immunogenicity of radiotherapy. Since most animal experiments are carried out with an irradiated tumor in one flank and an 'abscopal' unirradiated tumor in the other flank, experiments that irradiate multiple metastases or metastases in various organs are difficult to control and set up in mice.

Also, not just irradiation to various tumor locations, but also irradiation to normal tissues could influence response to (radio-)immunotherapy. As priming of antitumor T cells takes place in tumor draining lymph nodes (TDL), research on the consequences of nodal radiation on the synergistic effect of radiation and ICI seems relevant [52]. Marciscano et al. compared radiation of the tumor only to radiation of the tumor together with the TDL, called elective nodal irradiation (ENI) [53]. It was shown that radiation of the TDL attenuated the influx of antigen-specific intratumoral T cells and adversely affected local tumor control of the radiated tumors in mice. ENI restrained the adaptive immune response and addition of PD-1 antibodies could not restore this lack of T cell trafficking. The addition of CTLA-4 antibodies however, did show improvement of local tumor control and survival after ENI in these mice. Noteworthy, in patients, radiotherapy to the thorax or spine can increase the risk of severe lymphopenia, which was associated with poorer survival in patients treated with ICIs [54].

Thus, besides decreasing tumor load, increasing neoantigen release, presentation and repertoire, increasing APC influx, stimulating T cell recruitment, upregulation of MHC class I molecules and broadening the TCR repertoire, radiotherapy also showed to be able to provide necessary immune stimuli to augment response to immunotherapy (Figure 1). Still, much work needs to be performed concerning optimization of this synergistic effect. Whether these in vitro and *in vivo* successes are reproducible in clinical setting and will eventually lead to relevant clinical benefit, is still under elaborate investigation.

2.2 Clinical endeavors in NSCLC

2.2.1 Search strategy

We performed a systemic literature search in PubMed to obtain studies reporting on clinical outcomes of the combination of radiotherapy with ICIs in (NSCLC) patients. The search including several terms: “lung cancer”, “NSCLC”, “radiotherapy”, “radiation”, “irradiation”, “SABR”, “SRS”, “SBRT”, “immunotherapy”, “immune checkpoint inhibitors”, “anti-CTLA-4”, “anti-PD-1”, “anti-PD-L1”, “abscopal effect”. The final search was performed in January 2020. After removing duplicates, 513 publications were identified and assessed based on title and abstract. Non-English articles, conference abstracts, editorials, reviews and case reports were excluded. The studies selected for this review comprised of retrospective and prospective series on sequential and/or concurrent ICI-radiation treatment. Series without NSCLC patients and series where all tumor lesions were radiated and therefore no abscopal effect for non-radiated lesions could be established were further excluded, leaving 18 studies eligible for this review (Table 2).

2.2.2 Safety and efficacy data

As radiotherapy induces a locally and systemically inflammatory response, the combination could harbor the risk of increased toxicity. Retrospective series and prospective single arm studies have focused on safety aspects of combining radiotherapy with ICIs. Luke et al. performed a prospective study evaluating the safety of stereotactic body radiation therapy (SBRT) on 2-4 tumor lesions before start of pembrolizumab in 73 patients with solid tumors. They found grade 3 or higher toxicity in 8% (6/73) of patients. Four of these experienced toxicity within the irradiated field: 3 cases of pneumonitis and 1 case of colitis. These numbers are comparable to toxicity from ICI monotherapy and the authors concluded that the combination of radiotherapy with ICI seemed to be well tolerated with acceptable toxicity. Evidence of the abscopal effect was represented by a significant correlation of expression of interferon-gamma-associated genes from post-SBRT tumor biopsies from responding non-radiated tumor lesions [55]. Several retrospective series have been published investigating the possible toxicity risk of ICIs and radiotherapy, but auto-immune toxicities in the radiation field were few and overall well manageable [56-62]. This safety profile was also established for patients with advanced NSCLC with brain metastases who received cranial radiation specifically [63, 64], but there is also concern about an increase in the development of radiation necrosis in ICI-treated patients [65, 66].

Table 2. Clinical results of radiotherapy and ICI combination in metastatic non-small cell lung carcinoma

Author	Study type	Histology	N	IT agent	RT target	RT dose (Gy/fraction)	Treatment sequence	ORR, abscopal	mPFS (months)	mOS (months)	Toxicity Grade ≥3
Luke et al. 2018 [55]	Phase I	Various solid tumors	79	pembro	Various	30-50Gy/3-6 to 2-4 lesions	Sequential	13%	3.1	9.6	10%
Bang et al. 2017 [56]	Retrospective	NSCLC, MEL, RCC	133	aPD-(L)1 and/or aCTLA-4	Various	8-66Gy/1-15	Concurrent or sequential	NR	NR	NR	8%
Mohamad et al. 2018 [62]	Retrospective	Various solid tumors	59	nivo +/- ipi, pembro or atezo	Various	6-54Gy/1-5	Concurrent or sequential	26%	6.5	not reached	20%
v. Reibnitz et al. 2018 [57]	Retrospective	Various solid tumors	79	aPD-(L)1 and/or aCTLA-4	Thoracic	NR	Concurrent or sequential	NR	NR	NR	NR
Hwang et al. 2018 [58]	Retrospective	NSCLC	73	aPD-(L)1	Thoracic	8-54Gy	Concurrent or sequential	NR	NR	12.1	NR
Verma et al. 2018 [59]	Retrospective	Various solid tumors	60	ipi or pembro	Various	45-54Gy/15 50-60Gy/4	Concurrent	NR	NR	NR	25%
Miyamoto et al. 2018 [61]	Prospective	NSCLC	6	nivo	Lung	25-48/3-4	Sequential	75%	NR	NR	17%
Lesueur et al. 2018 [60]	Retrospective	NSCLC	104	nivo	Various	20-36Gy/1-10	Concurrent or sequential	NR	2.7	11.1	10%
Hubbeling et al. 2018 [63]	Retrospective	NSCLC	50	pembro, nivo or atezo	Intracranial	NR	Concurrent or sequential	NR	NR	NR	9%
Chen et al. 2018 [64]	Retrospective	Various solid tumors	79	ipi, pembro or nivo	Intracranial	15-24Gy/1-3 25Gy/5	Concurrent or sequential	NR	2.3	24.7 conc; 14.5 seq	16%
Martin et al. 2018 [66]	Retrospective	NSCLC, MEL, RCC	115	ipi, pembro or nivo	Intracranial	18-20Gy/1 25-30Gy/5	Concurrent or sequential	NR	NR	NR	20%*
Colaco et al. 2016 [65]	Retrospective	Various solid tumors	42	aCD137, aPD-1, aCTLA-4, IL-2	Intracranial	NR	Concurrent or sequential	NR	NR	NR	33%*
Shaverdian et al. 2017 [67]	Retrospective	NSCLC	42	pembro	Various	NR	Sequential	NR	4.4	10.7	2%
Formenti et al. 2018 [68]	Phase II	NSCLC	39	ipi	Various	6Gy/5 or 9Gy/3	Concurrent	18%	NR	7.4	50%
Tang et al. 2017 [69]	Phase I	Various solid tumors	35	ipi	Lung or liver	50Gy/4 60Gy/ 10	Concurrent or sequential	10%	3.2	10.2	34%
Welsh et al. 2019 [70]	Phase II	Various solid tumors	106	ipi	Lung or liver	50Gy/4 60Gy/10	Concurrent or sequential	9%	2.9	not reached	34%
Srivastava & Huang 2017 [71]	Retrospective	NSCLC, MEL	50	pembro or nivo	Intracranial	NR	Concurrent or sequential	NR	NR	NR	NR
Theelen et al. 2019 [72]	Phase II	NSCLC	76	pembro	Various	8Gy/3	Sequential	36%	6.6	15.9	14%

IT agent = immunotherapy agent, RT = radiotherapy, ORR = overall response rate, PFS = progression free survival, OS = overall survival, NSCLC = non-small cell lung cancer, MEL = melanoma, RCC = renal cell carcinoma, aPD-1 = anti-programmed cell death protein-1; aPD-L1 = anti-programmed death-ligand, aCTLA-4 = anti-cytotoxic T-lymphocyte-associated antigen 4, pembro = pembrolizumab, nivo = nivolumab; atezo = atezolizumab; ipi = ipilimumab, IFN = interferon, IL-2 = interleukin-2, Concurrent = radiotherapy was applied during ongoing ICI treatment, sequential = ICI treatment was started >1 day after radiotherapy was applied, NR = not reported. * symptomatic radiation necrosis

Several trials have tried to improve insights in the efficacy of a possible radiotherapy augmentation on ICI responses. A retrospective analysis of NSCLC patients in the phase I KEYNOTE-001 evaluated the effect of previous radiotherapy given anywhere during the disease period on clinical outcomes. Patients that had received previous radiotherapy showed a significant PFS and OS benefit over patients that were never irradiated. Toxicity data of the radiation group was comparable to treatment with ICI monotherapy [67]. Another trial investigated the combination of ipilimumab with radiation in advanced NSCLC patients. In this heavily pre-treated cohort, ORR measured in non-radiated lesions only was relatively high with 18% (7/39). Functional analysis in one responding patient showed the rapid *in vivo* expansion of CD8 T cells recognizing a neoantigen encoded in a gene upregulated by radiation, supporting the hypothesis that part of the explanation for the abscopal response is radiation-induced exposure of immunogenic mutations to the immune system [30].

In a phase I trial comparing several SBRT regimes -concurrently vs sequentially in two different fractionated schemes and lung vs liver lesions- together with ipilimumab in solid tumors of which 8/36 (23%) NSCLC patients, showed clinical benefit in 23% of patients, which was associated with an increase in peripheral CD8 T cells count [68]. T-cell activation measured by the expression of stimulatory signals -ICOS, GITR, and 4-1BB- by peripheral T cells was more pronounced after radiation of a liver lesion compared to lung lesions, leading the authors to the suggestion that the site of SBRT may be of relevance. However, their phase II trial including 30/106 (28%) NSCLC patients showed considerably higher disease control rate at 6 months with ipilimumab and sequential radiotherapy on a lung lesion (42%) compared to concurrent radiation on a liver lesion (5%) [69]. Therefore, we should be aware of a possible bias in selection of radiation-site due to metastatic pattern in these trials.

Besides location of SBRT, timing should be carefully addressed as well. Unfortunately, not much clinical data regarding this aspect has yet been generated. In a retrospective series of patients that received stereotactic radiosurgery (SRS) for brain metastases of several solitary tumors, mostly NSCLC or melanoma, local control and distant brain control were better when immunotherapy was started within 3 weeks of SRS [70].

Our group recently published the results from the PEMBRO-RT study, a hypothesis-generating phase II trial, where patients in second-line, metastatic NSCLC setting were randomized to either pembrolizumab alone vs pembrolizumab within one week of SBRT (3x8Gy) to a single tumor lesion [71]. The ORR at 12 weeks doubled in the SBRT arm and this led to an increase of PFS and OS without increase in treatment related toxicity. Although these improvements did not meet the predefined clinical endpoints and randomization was not stratified based on PD-L1 expression, subgroup analyses showed a significant benefit in PFS and OS from the addition of radiotherapy in patients with PD-L1 negative tumors (<1%). Translational research on collected blood samples, baseline and on-treatment biopsies is still ongoing to further explore in more detail as to what extend SBRT has improved patient outcomes in this trial.

2.2.3 Adjuvant ICIs after ablative radiotherapy

A subgroup of NSCLC patients can be defined as having oligometastatic disease. Treating these patients with locally ablative therapy to all tumor sites as adjuvant to platinum-doublet chemotherapy

has been associated with improved PFS and OS [72, 73]. Bauml et al. reported superior outcomes of patients that had received LAT after chemotherapy for oligometastatic disease (≤4 metastasis) and treated with one year of adjuvant pembrolizumab compared to historic controls [74]. This strategy was deemed safe.

As already mentioned, the PACIFIC-trial investigated the role of adjuvant ICI after CRT in curable stage III NSCLC [16]. Besides improved patient outcomes, PACIFIC showed a slight increase in toxic effects in the durvalumab group compared to the placebo arm, but the rates of severe immune-related adverse events, and of pneumonitis in particular, were not significantly different. Also, patients showed lower recurrence of disease when durvalumab was initiated within ≤2 weeks of last radiation dose rather than >2 weeks after radiation. This may suggest that a short window between radiotherapy and start of ICI should be pursued, but it cannot be excluded that this is a selection bias where the 'best' patients are the ones that are able to start sooner with adjuvant treatment [75]. The single arm LUN 14-179 study investigating adjuvant pembrolizumab after CRT showed improvement of recurrence rate and PFS compared with historical controls with no significant immune-related toxicity increase [76].

However, in adjuvant setting where all tumor localizations are treated with local radical radiotherapy for oligometastatic or stage III disease, proving an abscopal effect will be difficult. As there is no residual disease for response measurement, time to disease recurrence or OS are the only assessable clinical endpoints. But the synergistic effect between radiation and ICIs, the 'true' abscopal effect, is indistinguishable from a lack of residual disease -cure by CRT alone- or earlier onset of successful systemic treatment for occult metastatic disease irrespective of radiotherapy synergy. We will need dedicated translational research to help us bring better insights in these non-overlapping patient categories as this might warrant a more individualized approach.

2.3 Future perspectives

Many NSCLC trials are currently ongoing to further assess the safety and efficacy of the combination of radiation and immunotherapy in metastatic setting. Unfortunately, most of these trials do not focus on comparison of different radiation regimes, timing, anatomical site or number of radiated lesions, so the question as how to optimize this synergistic opportunity might remain unsolved for some time to come. A multi-arm optimal dose/fractionation-finding study based on clinical outcomes and translational endpoints would be a reasonable next step. Besides anti-PD-(L)1 treatment, the effect of immunotherapy combinations could be explored in such a setting as well. And with new radiotherapy modalities becoming available, it would be interesting to compare FLASH radiotherapy to heavy ion or photon beam radiotherapy in regards to the immunomodulation. Brooks et al. claimed that maybe more than one lesion should be radiated to obtain a maximum abscopal effect as this could help tackle tumor heterogeneity and clonality of metastases, differences in immunogenicity of lesions or local immune suppressive effects and a decrease in tumor load [77]. McGee et al. compared immune response generated by SBRT on metastatic lesion of solid tumors in different organs in a small prospective patient series. They found that irradiation to lung and liver metastases showed an induction of immune response, whereas this was not observed for radiotherapy to bone or brain metastases, endorsing further development of these comparative trial setups [78].

Retrospective evidence of re-invigoration of ICI responses through the addition of radiotherapy after development of secondary resistance has recently been described [79, 80]. It would be interesting to investigate this ability of radiotherapy in a prospective manner.

Currently, the role of ICIs in locally advanced stage III NSCLC is being investigated beyond adjuvant durvalumab. The first safety data shows that concomitant addition of immunotherapy, nivolumab and atezolizumab, to concurrent CRT is safe and tolerable [81, 82]. A randomized phase III study of CRT with concomitant durvalumab, the PACIFIC-2 study [ClinicalTrials.gov identifier: NCT03519971], is currently ongoing. The phase II BTCRC-LUN16-081 trial [NCT03285321] is including patients to investigate the safety and efficacy of a one-year adjuvant ICI-combination nivolumab and ipilimumab after CRT. Based partly on pre-clinical evidence that addition of anti-CTLA-4 treatment may have a better abscopal effect before the application of radiation [50, 51], our group is now recruiting patients for a phase I trial, evaluating safety of neo-adjuvant durvalumab and tremelimumab before CRT, the Induction-1 study [NCT04287894]. Again, as all known disease locations in these settings will be radically treated, it will be difficult to differentiate the abscopal and therefore synergistic effect from the advantage of moving a possible beneficial effect of immunotherapy to an earlier time point in disease treatment. Nevertheless, many new insights will be gained from these trials.

3. Conclusion

Pre-clinical research has provided convincing evidence that radiotherapy has immunomodulatory qualities leading to synergistic effects when combined with ICIs. Still, much remains unclear regarding optimal dose, fractionation, target volume or timing of radiation therapy. Patients/tumors with different mechanisms for treatment failure, might benefit from different immune-stimulating radiotherapy regimens. Pre-clinical and translational experiments that focus on one aspect of the radiation regime could hopefully answer some of these specific questions. Also, establishing assessable biomarkers for immunogenic cell death or radiotherapy-induced anti-tumor immune responses will help to bring research in this field forward. Although biomarker research for response to immunotherapy is a fast-evolving field with new pieces of the puzzle generated almost daily, the many elements of the immune system involved together with a diverse interaction with tumor cells makes this a difficult enterprise. Future clinical research will need to focus on implementing data from pre-clinical and translational findings in the development of new clinical trials in order to proof reproducibility in a patient setting and to help optimizing the abscopal potential.

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CHAPTER 5

Effect of pembrolizumab after stereotactic body radiotherapy vs pembrolizumab alone on tumor response in patients with advanced non-small cell lung cancer: results of the PEMBRO-RT phase 2 randomized clinical trial

JAMA Oncol. 2019;5(9):1276-1282

KEY POINTS

Question

Does stereotactic body radiotherapy enhance the effect of immune checkpoint inhibition by increasing tumor response in nonirradiated lung cancer lesions in metastatic non–small cell lung cancer?

Findings

In this phase 2 clinical trial of 76 patients with recurrent metastatic non–small cell lung cancer randomized to either pembrolizumab alone or pembrolizumab after stereotactic body radiotherapy on a single tumor site, the overall response rate at 12 weeks was 18% in the control arm vs 36% in the experimental arm.

Meaning

Stereotactic body radiotherapy prior to pembrolizumab was well tolerated; although a doubling of the overall response rate was observed, the results did not meet the study criteria for meaningful clinical benefit.

ABSTRACT

Importance

Many patients with advanced non–small cell lung cancer (NSCLC) receiving immunotherapy show primary resistance. High-dose radiotherapy can lead to increased tumor antigen release, improved antigen presentation, and T-cell infiltration. This radiotherapy may enhance the effects of checkpoint inhibition.

Objective

To assess whether stereotactic body radiotherapy on a single tumor site preceding pembrolizumab treatment enhances tumor response in patients with metastatic NSCLC.

Design, setting, and participants

Multicenter, randomized phase 2 study (PEMBRO-RT) of 92 patients with advanced NSCLC enrolled between July 1, 2015, and March 31, 2018, regardless of programmed death–ligand 1 (PD-L1) status. Data analysis was of the intention-to-treat population.

Interventions

Pembrolizumab (200 mg/kg every 3 weeks) either alone (control arm) or after radiotherapy (3 doses of 8 Gy) (experimental arm) to a single tumor site until confirmed radiographic progression, unacceptable toxic effects, investigator decision, patient withdrawal of consent, or a maximum of 24 months.

Main outcomes and measures

Improvement in overall response rate (ORR) at 12 weeks from 20% in the control arm to 50% in the experimental arm with $P < .10$.

Results

Of the 92 patients enrolled, 76 were randomized to the control arm ($n = 40$) or the experimental arm ($n = 36$). Of those, the median age was 62 years (range, 35-78 years), and 44 (58%) were men. The ORR at 12 weeks was 18% in the control arm vs 36% in the experimental arm ($P = .07$). Median progression-free survival was 1.9 months (95% CI, 1.7-6.9 months) vs 6.6 months (95% CI, 4.0-14.6 months) (hazard ratio, 0.71; 95% CI, 0.42-1.18; $P = .19$), and median overall survival was 7.6 months (95% CI, 6.0-13.9 months) vs 15.9 months (95% CI, 7.1 months to not reached) (hazard ratio, 0.66; 95% CI, 0.37-1.18; $P = .16$). Subgroup analyses showed the largest benefit from the addition of radiotherapy in patients with PD-L1–negative tumors. No increase in treatment-related toxic effects was observed in the experimental arm.

Conclusions and relevance

Stereotactic body radiotherapy prior to pembrolizumab was well tolerated. Although a doubling of ORR was observed, the results did not meet the study's prespecified end point criteria for meaningful clinical

benefit. Positive results were largely influenced by the PD-L1–negative subgroup, which had significantly improved progression-free survival and overall survival. These results suggest that a larger trial is necessary to determine whether radiotherapy may activate noninflamed NSCLC toward a more inflamed tumor microenvironment.

Trial registration
ClinicalTrials.gov identifier: NCT02492568

INTRODUCTION

In recent years, treatment for non-small cell lung cancer (NSCLC) has changed significantly owing to the introduction of immunotherapy. The programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway is one of the most studied tumor immune escape mechanisms [1]. Targeting the PD-L1/PD-1 pathway with immune checkpoint inhibitors has produced long-lasting anti-tumor immune responses in a subset of NSCLC patients [2-5]. Unfortunately, most patients with NSCLC do not benefit from this treatment owing to primary resistance, possibly because certain tumor antigens are not recognized.

Stereotactic body radiotherapy (SBRT) is the delivery of a high radiation dose in generally 3 to 5 fractions with high accuracy to a single tumor site. SBRT may synergize with immunotherapy. Several preclinical studies reported an increased tumor antigen release, improved antigen presentation and T-cell infiltration in irradiated tumors. Combining radiotherapy with immune checkpoint inhibition showed more pronounced tumor regression in several solid tumor types, including in the nonirradiated tumors, than provided by either of these treatments alone [6-12].

We present the results of the PEMBRO-RT study, the first randomized study, to our knowledge, of pembrolizumab, a highly selective humanized PD-1 monoclonal antibody, with or without prior SBRT to a single tumor site in patients with metastatic NSCLC. This study evaluates whether SBRT enhances the effect of immune checkpoint blockade by increasing tumor response in nonirradiated lung cancer lesions on PD-1 immune checkpoint blockade.

METHODS

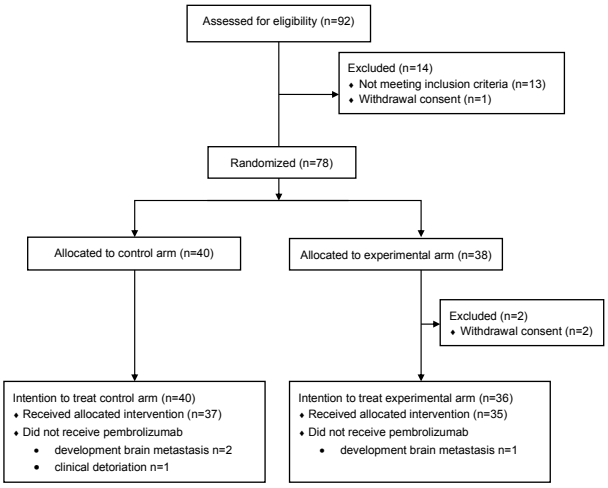
This multicenter, phase 2 randomized clinical trial was conducted at 3 medical sites in the Netherlands. Patients 18 years or older were eligible to participate if they had histological or cytological confirmed metastatic NSCLC that progressed after at least 1 regimen of chemotherapy but who were immunotherapy naive and had an Eastern Cooperative Oncology Group (ECOG) performance status of 1 or lower. At least 2 separate lesions were required, one of which was measurable according to Response Evaluation Criteria in Solid Tumors (RECIST) and suitable for biopsy, and the other of which was amenable to irradiation. Patients were ineligible if they had (1) radiotherapy to any tumor site within 6 months prior to randomization; (2) known, active central nervous system metastases and/or carcinomatous meningitis; (3) untreated driver alterations of epidermal growth factor receptor or anaplastic lymphoma kinase; or (4) active autoimmune or interstitial lung disease. The trial protocol and all amendments were approved by the institutional review board or independent ethics committee of the Netherlands Cancer Institute-Antoni van Leeuwenhoek Hospital, Amsterdam. The trial was conducted in accordance with the provisions of the Declaration of Helsinki and the Good Clinical Practice guidelines of the European Medicines Agency and the US Food and Drug Administration. All patients provided written informed consent before enrollment.

Patients were randomly assigned using a 1:1 ratio to receive treatment with pembrolizumab either after SBRT to a single tumor site (experimental arm) or without SBRT (control arm). Randomization was

stratified to smoking status (<10 pack years vs ≥10 pack years). Pembrolizumab was administered intravenously at 200 mg every 3 weeks. In the experimental arm, the first course was given within 7 days after completion of SBRT, which consisted of 3 doses of 8 Gy delivered on alternate days to a single tumor site that did not overlap with the biopsy site and was deemed most safe and/or convenient for the patient. Response evaluation was done according to RECIST, version 1.1, by an independent reviewer. The irradiated lesion was excluded from RECIST measurements and therefor reviewers could not be blinded for the treatment arm. Tumor response was assessed with CT-scans every 6 weeks for one year and every 8 weeks thereafter. Patients were allowed to continue treatment beyond initial radiologic progression in the absence of clinical deterioration. If the subsequent CT scan did not confirm progression, the initial progression was considered to be pseudo-progression, and the patient was allowed to continue treatment with pembrolizumab. Pseudo-progression was not scored as progressive disease for the primary end point. Treatment continued until confirmed radiographic progression, unacceptable toxic effects, investigator decision, patient withdrawal of consent or for a maximum of 12 months; extended to 24 months in September 2017 for alignment with other pembrolizumab trials. PD-L1 expression was assessed after the study was closed at our local laboratory by the PD-L1 IHC 22C3 LDT assay in formalin-fixed tumor samples from tumor tissue received at baseline. Expression was categorized according to a tumor proportion score (TPS), i.e. the percentage of tumor cells with membranous PD-L1 staining: 0%, 1-49% and ≥50% [13].

The primary end point was overall response rate (ORR) -complete response and partial response- at 12 weeks from randomization. Secondary end points included safety, progression-free survival (PFS), overall survival (OS) and disease control rate (DCR) at 12 weeks. End points were assessed in the intention-to-treat (ITT) population, including all patients that underwent randomization with the exception of 2 patients in the experimental arm, who both withdrew consent (Figure 1). Adverse events were graded according to the Common Toxicity Criteria, version 4.0, and were registered from the date of informed consent until discontinuation of trial treatment. Exploratory end points included the effect of PD-L1 expression and prior radiotherapy on efficacy.

Figure 1. Consort diagram.



Efficacy was assessed in the ITT population, and safety was assessed in the as-treated population, which included all patients who had undergone randomization and received at least 1 dose of the assigned therapy. A statistical analysis indicated that with a sample of 74 patients, 37 in each arm, the trial would have a power of 82% with an odds ratio of 4 to detect the difference between a response rate of 20% in the control arm and a response rate of 50% in the experimental arm at a 20sided significance level of P < .10. The Kaplan–Meier method was used to estimate OS and PFS. Data for patients who were alive or lost to follow-up were censored for OS at the time of last follow-up. Data for patients who were alive and did not have disease progression were censored for the analysis of PFS at the time of the last imaging assessment. Fisher's exact test was used to assess group differences in ORR at 12 weeks. DCR was compared using Fisher's test. PFS and OS were compared between arms using the log-rank test. The relation of patient and tumor characteristics to the effect of SBRT on PFS and OS were assessed using Cox proportional Hazard models. The relationship between PD-L1 expression and response at 12 weeks was assessed using the linear-by-linear association test.

RESULTS

Between July 1, 2015, and March 31, 2018, 92 patients were screened for enrollment, and 76 patients who met the eligibility criteria were randomly assigned to either the control arm (n = 40) or the experimental arm (n = 36). Of those, the median age was 62 years (range, 35-78 years), and 44 (58%) were men. Patient demographics, including previous radiotherapy, were well balanced between both arms. The percentage of PD-L1 negative tumors was slightly higher in the control arm (25 of 38 [66%]) than in the experimental arm (18 of 36 [50%]), and the number of patients with a TPS of 50% or higher was lower in the control arm than in the experimental arm (5 of 38 [13%] vs 10 of 36 [28%]) (P = .10) (Table 1). The tumor sites selected for SBRT were primarily lung lesions or lymph node metastases (Table S1).

Table 1. Baseline demographics and disease characteristics.

Total (n = 76)	Experimental arm n = 36	Control arm n = 40
Median age, years (range)	62 (35-78)	62 (38-78)
Men	20 (56%)	23 (57%)
Pack years ≥10	29 (81%)	32 (80%)
ECOG performance score		
0	17 (47%)	22 (55%)
1	19 (53%)	17 (43%)
2	0	1 (3%)
Histology		
Non-squamous	31 (86%)	36 (90%)
Squamous	5 (14%)	4 (10%)
Previous radiotherapy	15 (42%)	17 (43%)
Number of previous lines of systemic treatment		
1	26 (72%)	31 (78%)
2	6 (17%)	8 (20%)
3	4 (11%)	1 (3%)
PD-L1 TPS		
0%	18 (50%)	25 (66%)
1-49%	8 (22%)	8 (21%)
≥50%	10 (28%)	5 (13%)

Intention to treat population. Data are n (%), minimum - maximum range of age is given. ECOG = Eastern Cooperative Oncology Group. TPS = tumor proportion score.

Thirty-seven patients (92%) in the control arm and 35 patients (97%) in the experimental arm received at least 1 course of pembrolizumab. All patients who did not receive pembrolizumab were categorized as having progressive disease for further analyses. One patient received palliative radiotherapy before the primary end point but remained part of the ITT population. At the cutoff date of July 1, 2018, the median follow-up time was 23.6 months (range, 0.1-34.4 months). Seven patients (18%) in the control arm and 4 patients (11%) in the experimental arm were still receiving treatment. The median duration of treatment for patients with at least 1 dose of pembrolizumab was 2.1 months (95% CI, 1.2-5.6 months) in the control arm and 4.2 months (95% CI, 2.7-11.0 months) in the experimental arm (P = .30).

In the ITT population, the ORR at 12 weeks was 18% (95% CI, 7%-33%) in the control arm vs. 36% (95% CI, 21%-54%) in the experimental arm (P = .07) (Table 2). The increased ORR in the experimental arm (22%) compared with the control arm (4%) was largely influenced by ORR in the PD-L1-negative subgroup, although this ORR in the PD-L1-negative subgroup was not significant (P = .14). Response rates in the 2 PD-L1-positive subgroups were similar in both arms. There was 1 complete response (CR) in the control arm and 3 in the experimental arm. In the control arm, the majority of patients (21 of 40 [53%]) showed progressive disease (PD) as best ORR compared with the experimental arm, in which partial response (PR) was most common (14 of 36 [39%]). Stable disease (SD) as best response was identical in both arms (10 of 40 [25%] and 9 of 35 [25%], respectively). In the overall population, significant improvement (64% vs 40%; P = .04) was observed in the DCR at 12 weeks in the experimental arm. The effect of SBRT on response rates in patients who were previously treated with radiotherapy (ie, >6 months before randomization) and patients who never received any radiotherapy was similar (odds ratios, 3.1 [95% CI, 0.5-23.5] vs 2.4 [95% CI, 0.5-13.1], both in favor of the experimental arm; P = .81), suggesting that previous radiotherapy did not strongly affect study results (Table S2). The distribution of baseline PD-L1 expression did not differ between patients who received radiotherapy more than 6 months before inclusion (PD-L1 expression of 0%, 27 patients; 1%-49%, 7 patients; and ≥50%, 8 patients) and patients who did not receive radiotherapy before inclusion (PD-L1 expression of 0%, 16 patients; 1%-49%, 9 patients; and ≥50%, 7 patients) (P = .37) (Table S3). Two patients in the control arm had an initial increase in tumor burden of more than 20% at week 6 followed by PR at week 12, which was considered pseudoprogression.

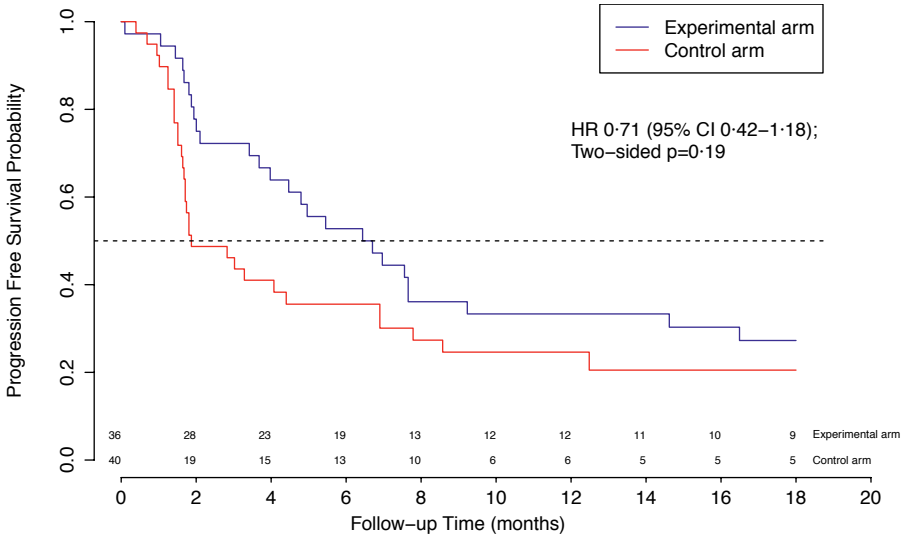
Table 2. Response to treatment.

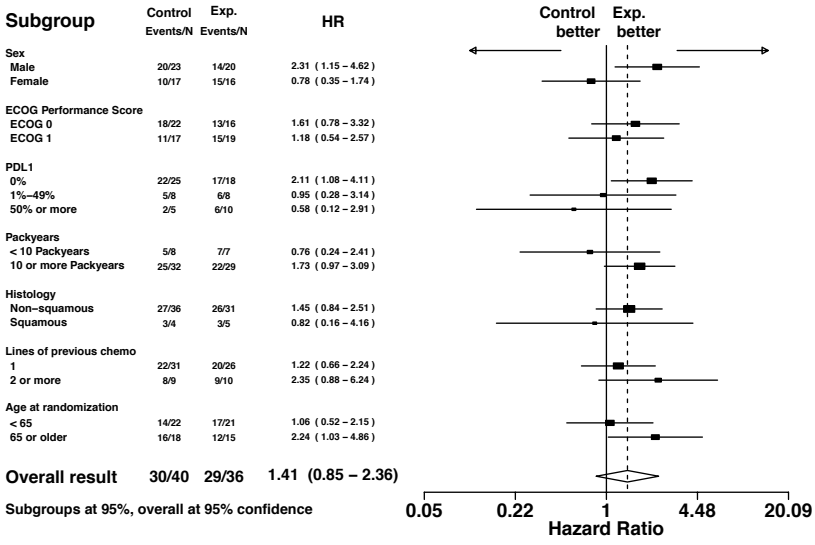
Response	Experimental arm n = 36	Control arm n = 40
Best overall response		
Complete response	3	1
Partial response	14	8
Stable disease	9	10
Progressive disease	10	21
Objective response rate (ORR) at 12 weeks		
Overall*	13/36 (36%)	7/40 (18%)
PD-L1 TPS 0%	4/18 (22%)	1/25 (4%)
PD-L1 TPS 1-49%	3/8 (38%)	3/8 (38%)
PD-L1 TPS ≥50%	6/10 (60%)	3/5 (60%)
Disease Control Rate (DCR) at 12 weeks**	23/36 (64%)	16/40 (40%)

Data are n/total n (%). TPS = tumor proportion score.
*P = 0.07; **P = 0.04

At the time of analysis, median PFS was 1.9 months (95% CI, 1.7-6.9 months) in the control arm and 6.6 months (95% CI, 4.0-14.6 months) in the experimental arm (Figure 2). The increased PFS in the experimental arm was not significant (hazard ratio [HR], 0.71; 95% CI, 0.42-1.18; P = .19). A significant benefit of SBRT with respect to PFS was seen in the PD-L1-negative subgroup (HR, 0.49; 95% CI, 0.26-0.94; P = .03); however, the limited number of responders must be taken into account. No benefit from the addition of SBRT was seen in the PD-L1-positive subgroups (HR, 1.14; 95% CI, 0.45-2.89; P = .79) (Figure 2).

Figure 2. Progression free survival in the ITT population.

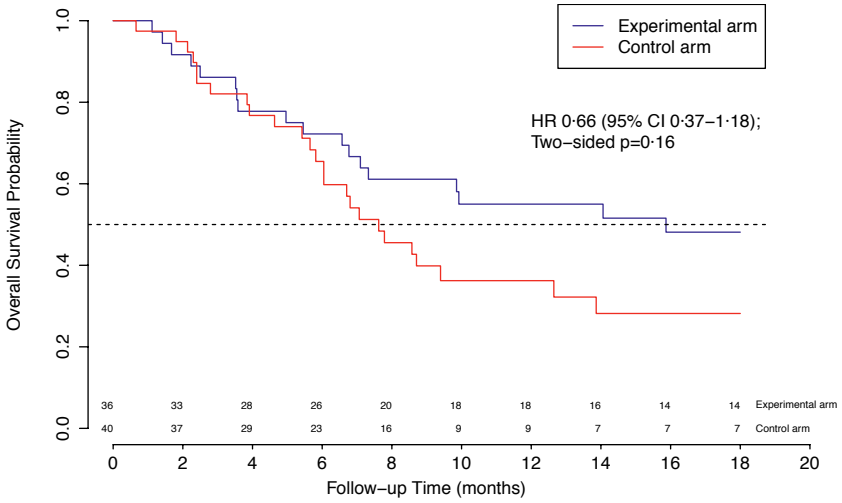


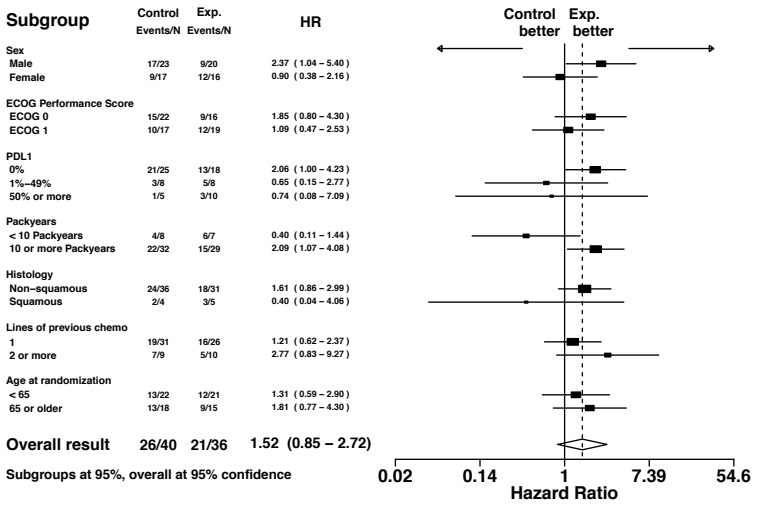


ITT = intention to treat. HR = hazard ratio. ECOG = Eastern Cooperative Oncology Group

At the time of analysis, 51 patients had died. A median OS of 7.6 months (95% CI, 6.0-13.9 months) in the control arm and 15.9 months (95% CI, 7.1 months to not reached) in the experimental arm was observed (Figure 3). This increased OS was not significant (HR, 0.66; 95% CI, 0.37-1.18; P = .16). The benefit of SBRT with respect to OS was observed only in the PD-L1-negative subgroup (HR, 0.48; 95% CI, 0.24-0.99; P = .046), and no benefit was seen in the combined PD-L1-positive subgroups (HR, 1.4; 95% CI, 0.42-4.66; P = .58). Male patients (HR, 0.42; 95% CI, 0.19-0.96; P = .04) and smokers (HR, 0.48; 95% CI, 0.25-0.93; P = .03) performed significantly better in the experimental arm compared with the control arm (Figure 3). After correction for other variables, only PD-L1 status remained a predictive factor for OS in the experimental arm.

Figure 3. Overall survival in the ITT population.





ITT = intention to treat. HR = hazard ratio. ECOG = Eastern Cooperative Oncology Group

The most common adverse events were fatigue (28 of 72 patients [39%]), flulike symptoms (23 of 72 [32%]), and cough (20 of 72 [28%]). Fatigue (10 of 37 patients [27%] vs 18 of 35 [51%]; P = .05) and pneumonia (3 of 37 [8%] vs 9 of 35 [26%]; P = .06) occurred more often in the experimental arm than in the control arm. Pembrolizumab-related toxic effects were primarily fatigue (18%), flulike symptoms (15%), and pruritus (14%). Grade 3 to 5 pembrolizumab-related toxic effects were reported in 12 patients (17%), with no significant differences between arms. Adverse events that appeared in more than 10% of patients and relevant pembrolizumab-related toxic effects are presented in Table 3. The number of patients that experienced an immune-related toxicity was similar in both arms (26 of 37 patients [70%] in the control arm vs 24 of 35 patients [69%] in the experimental arm; P = 1.0). The total number of immune-related toxicities showed a trend in favor of the control arm (68 vs 85 events; P = .08). One patient who received SBRT to a lung lesion developed a pneumonitis grade 2. Pembrolizumab was temporarily interrupted and the patient was retreated successfully, leading to a long-lasting PR. Five patients in the experimental arm experienced pneumonitis (n = 3) or grade 3 dyspnea (n = 2), but all 5 patients received SBRT on an extrathoracic lesion, therefore no SBRT-related toxicity was suspected. One patient developed a nephritis after 3 courses of pembrolizumab and SBRT to a retroperitoneal lesion in close relation to the kidney, which was deemed as related to the combination treatment, and immunotherapy was terminated. Eight patients stopped treatment due to grade 3 AEs: in the control arm, because of pneumonitis (n = 1), hepatitis (n = 1) and dyspnea (n = 1); in the experimental arm, because of nephritis (n = 1), duodenitis (n = 1) and a spinal fracture (n = 1). All except the spinal fracture were considered to be related to pembrolizumab administration. A cerebrovascular accident occurred in both arms (n = 2), but neither were related to study treatment. Both patients died because of complications several weeks to months afterwards. There were 2 grade 5 toxicities observed: an ileus in the experimental arm (considered not treatment-related) and 1 patient in the control arm died from multi-organ failure possibly related to the pembrolizumab treatment.

Table 3. AEs present in at least 10% of patients and immune-related toxicities related to pembrolizumab.

	All grades		Grades 3-5	
Adverse events	**Experimental arm n = 35**	**Control arm n = 37**	**Experimental arm n = 35**	**Control arm n = 37**
Fatigue	18 (51%)*	10 (27%)*	1 (3%)	0
Flu like symptoms	12 (34%)	11 (30%)	0	0
Cough	12 (34%)	8 (22%)	0	0
Dyspnea	9 (26%)	8 (22%)	4 (11%)	2 (5%)
Nausea	5 (14%)	10 (27%)	1 (3%)	2 (5%)
Pruritis	7 (20%)	5 (14%)	0	0
Pneumonia	9 (26%)*	3 (8%)*	4 (11%)	1 (3%)
Weight loss	5 (14%)	6 (16%)	2 (6%)	1 (3%)
Immune-related toxicities**				
All (n)	85	68	5	11
Pneumonitis	4 (11%)	2 (5%)	0	2 (5%)
Colitis	1 (3%)	2 (5%)	0	0
Duodenitis	1 (3%)	0	0	0
Hepatitis	0	1 (3%)	0	0
Hypothyroidism	2 (6%)	2 (5%)	0	0
Hyperthyroidism	1 (3%)	2 (5%)	0	0
Nephritis	1 (3%)	0	0	0
Nausea	0*	6 (16%)*	0	2 (5%)
Dyspnea	2 (6%)	1 (3%)	2 (6%)	1 (3%)
Skin rash	3 (9%)	1 (3%)	2 (6%)	0

Data are n (%). * There were no significant differences between the arms at the alpha = 0.1 level, except fatigue (p=0.052), pneumonia (p=0.060) and nausea (p=0.025). After applying the Holms-Bonferroni correction to compensate for the number of different adverse events categories compared, no significance differences between arms remained. ** Only the most clinical relevant immune-related toxicities are mentioned.

DISCUSSION

The PEMBRO-RT study is the first randomized trial, to our knowledge, to show an augmenting effect of SBRT on the response to PD-1 blockade in patients with metastatic NSCLC. The experimental arm showed an increase in ORR, DCR at 12 weeks, and median PFS and OS without an increase in toxic effects. The study did not meet its primary end point because the improvements did not meet the study's prespecified criteria -an increase of ORR from 20% in the control arm to 50% in the experimental arm at 12 weeks- for meaningful clinical benefit.

In recent trials, response rates of pembrolizumab-treated patients with advanced NSCLC were dependent of PD-L1 expression levels of the tumor [2, 4, 13, 14]. The response rates in the combined PD-L1-positive subgroups (PD-L1 ≥ 1%) in our study was much higher compared with other trials (52% [16 of 31] vs 18 to 27% [2, 13]. Patient and tumor characteristics in this study were comparable with previously reported studies. The reason for this study's high response rate remains unclear, but the excellent patient outcomes observed in both PD-L1-positive subgroups may have masked a potential augmenting effect of SBRT in this setting.

An imbalance of PD-L1 distribution in favor of the experimental arm has to be taken into account for the overall cohort; however, when data from the PD-L1-negative subgroups were evaluated, a significant benefit was observed from the experimental approach. Blood and tumor samples collected during this trial may assist in gaining better insight regarding whether this improvement can be attributed to an augmenting effect from SBRT in these PD-L1-negative patients.

LIMITATIONS

Little is known about the effects of radiotherapy dose, fractionation, and treatment site on the antitumor immune response. Several immunogenic mice studies reported that the immune-modulating effect of hypofractionated radiotherapy was more pronounced compared with single-dose radiotherapy [6, 15-17]. Thus, a dose of 3 × 8 Gy was chosen for SBRT preparation and delivery because of its high accuracy, which minimized the potential for toxic effects caused by the addition of radiotherapy. To further reduce the possibility of toxic effects, SBRT was administered to the experimental arm sequentially rather than concurrently, with no longer than 1 week between the last radiotherapy dose and the first pembrolizumab dose to minimize delay of systemic treatment. A study by Dovedi et al. reported a decrease in PD-L1 expression and anergy of tumor-reactive T-cells 7 days after the last dose of fractionated radiotherapy in mice models [8]. Further research is needed to explore whether the radiotherapy dose and schedule used in this clinical trial were optimal with respect to the immune-modulating potential of radiation in combination with immune checkpoint inhibition in patients with cancer.

The safety profile observed in this clinical trial was consistent with previous studies of pembrolizumab treatment for advanced NSCLC [2, 4, 13]. Most immune-mediated events were grade 1 or 2. No significant differences in toxic effects between arms were observed. Only 1 patient experienced an

immune-related adverse event that may have been augmented by SBRT. Nephritis developed in 1 patient after the administration of SBRT on a retroperitoneal lesion and the third course of pembrolizumab, resulting in discontinuation of treatment. Luke et al. reported safety data on 73 patients with solid tumors who were treated with pembrolizumab after SBRT to 2 to 4 tumor lesions [18]. The timing of SBRT was similar to this study, but doses varied from 30 to 50 Gy in 3 to 5 fractions, depending on the tumor site. They concluded that the administration of SBRT before pembrolizumab treatment was well tolerated. In a KEYNOTE-001 phase 1 clinical trial, Shaverdian et al analyzed the effects of previous radiotherapy on the efficacy and safety of pembrolizumab treatment in patients with NSCLC [19]. They reported that the safety profile was acceptable, with a longer PFS and OS in the subgroup that received previous radiotherapy. The effects of previous radiotherapy on the efficacy and safety of pembrolizumab could not be established in this study, but this possible bias should be further investigated.

CONCLUSIONS

The results of this study are encouraging, and further evaluation in a larger phase 2/3 trial is recommended to confirm the findings and elucidate the processes by which SBRT may activate noninflamed NSCLC tumors towards an inflamed tumor microenvironment, rendering them receptive to immune checkpoint inhibition.

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SUPPLEMENTARY DATA

Table S1. Tumor site selected for trail SBRT in experimental arm.

Radiated tumor site	n = 36
Lung, metastasis	11
Lymph node, intra thoracic	5
Lymph node, extra thoracic	4
Adrenal	4
Bone	4
Lung, primary tumor	4
Cutaneous	1
Liver	1
Pleural	1
Retroperitoneal	1

Table S2. Response rates previous vs. no previous radiotherapy.

	No previous RT		Previous RT	
	Experimental	Control	Experimental	Control
Response	n = 21	n = 23	n = 15	n = 17
CR/PR	7 (33%)	4 (17%)	6 (40%)	3 (18%)
SD	7 (33%)	7 (30%)	3 (20%)	2 (12%)
PD	7 (33%)	12 (52%)	6 (40%)	12 (71%)

When comparing responders (CR/PR) vs non-responders (SD/PD) we found an odds ratio of 2.3 in favor of the experimental arm in the patients that did not receive previous RT and an odds ratio of 3.1 in the same direction among the patients that did receive previous RT. These odds ratios are not significantly different from each other (P = .81). When comparing disease control (CR/PR/SD) vs progression (PD) we found an odds ratio of 2.2 in favor of the experimental arm in the patients that did not receive previous RT and an odds ratio of 3.6 in the same direction among the patients that did receive previous RT. These odds ratios are also not significantly different from each other (P = .61).

Table S3. PD-L1 expression previous vs. no previous radiotherapy.

	No previous RT	Previous RT
TPS	n = 42	n = 32
0%	27 (64%)	16 (50%)
1-49%	7 (17%)	9 (28%)
≥50%	8 (19%)	7 (22%)

The distribution of PD-L1 expression between patient receiving previous RT vs no previous was not significantly different (p=0.37).

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CHAPTER 6

Pembrolizumab with and without radiotherapy for metastatic non-small cell lung cancer: pooled analysis of two randomized trials

Accepted for publication in The Lancet Respiratory Medicine

RESEARCH IN CONTEXT

Evidence before this study

Immune checkpoint inhibition has a central role in the treatment of advanced NSCLC, but has only been beneficial in a minority of patients. We searched the scientific literature for a comparison of anti-PD-1 (e.g. pembrolizumab) with or without radiotherapy (RT) in the treatment of metastatic non-small cell lung cancer (mNSCLC). We used the search terms “pembrolizumab” AND “anti-PD-1” AND “non-small cell lung cancer” AND “response” AND “overall survival” to search for publications in PubMed from February 10, 2012 to June 17, 2020 and for abstracts presented at annual congresses of the American Association of Cancer Research (AACR), the American Society of Clinical Oncology (ASCO), American Society for Radiation Oncology (ASTRO), the European Society for Medical Oncology (ESMO), European Society for Radiotherapy and Oncology (ESTRO), and the World Conference on Lung Cancer (WCLC). We also searched the clinical trial registries of ClinicalTrials.gov and WHO International Clinical Trials Registry Platform. Only two randomized clinical trials evaluating the impact of combining pembrolizumab with RT on patient outcomes are currently published. The primary endpoint for both trials showed improvement in the combination therapy arm, but neither met the prespecified criteria for meaningful clinical benefit. This may indicate that a larger sample size is required to more accurately detect the effects of the addition of RT to immunotherapy on patient outcomes.

Added value of this study

Owing to their limited sample sizes, we hypothesized that the previous analyses of each individual trial lacked sufficient statistical power to detect practical, clinically attainable improvements in patient outcomes. This concern prompted re-evaluation with a pooled analysis to better evaluate this effect. We found that adding RT to pembrolizumab significantly increased response rates to unirradiated lesions, leading to a significant PFS and OS benefit.

Implications of all the available evidence

This pooled analysis shows that the combination of pembrolizumab with RT can be considered a treatment option for patients with mNSCLC, as it significantly increased treatment response and survival compared to pembrolizumab alone. These results warrant validation in a randomized phase III trial.

SUMMARY

Background

Radiation therapy (RT) may augment systemic antitumoral responses to immunotherapy. In metastatic non-small cell lung cancer (mNSCLC), several ongoing randomized studies are examining the addition of RT to various immunotherapy agents. However, the PEMBRO-RT and MDACC trials are the only known completed randomized comparisons of immunotherapy with or without radiation therapy (RT). When the trials were analyzed individually, a potential benefit was observed in the combination arms; however, the small sample size of each trial might have limited the detection of smaller than expected, but nevertheless clinically relevant, differences in response rates and outcomes. Hence, we performed a pooled analysis to infer whether RT improves responses to immunotherapy in mNSCLC patients.

Methods

Inclusion criteria for both completed trials were mNSCLC with at least one unirradiated lesion to monitor for out-of-field response. All patients were immunotherapy-naïve. The intention-to-treat population (ITTP) from both trials were included in this analysis. In the PEMBRO-RT trial (NCT02492568), patients were randomly assigned using a 1:1 ratio and stratified by smoking status (<10 vs ≥10 pack-years). In the MDACC trial (NCT02444741), patients were entered in one of two cohorts based on RT schema feasibility and subsequently randomized using a 1:1 ratio. Due to the nature of the intervention in the experimental arm (radiotherapy), blinding was not feasible in either trial. In both trials, pembrolizumab (200mg every 3 weeks) was administered with or without RT. In the PEMBRO-RT trial of previously chemotherapy-treated patients, the first dose of pembrolizumab was given sequentially <1 week after the last dose of RT (24Gy/3 fractions), while in the MDACC trial of both previously-treated and newly-diagnosed cases it was given concurrently with the first dose of RT (50Gy/4 fractions or 45Gy/15 fractions). Only unirradiated lesions were measured for response. The endpoints for this pooled analysis were out-of-field (abscopal) response rate (ARR), abscopal disease control rate (ACR), ARR at 12 weeks, ACR at 12 weeks, progression-free survival (PFS) and overall survival (OS).

Findings

Overall, 148 patients were analyzed (n=76 pembrolizumab; n=72 iRT). The median follow-up for all patients was 33 months. Most patients had non-squamous histology (84%) and received prior chemotherapy (75%). There were no differences between arms in terms of baseline variables, including PD-L1 status and metastatic disease volume. The most commonly irradiated sites were lung metastases (28/72, 39%), intrathoracic lymphatics (15/72, 21%), and lung primary disease (12/72, 17%). The ARR was 19.7% with pembrolizumab vs. 41.7% with iRT (odds ratio [OR] 2.96, 95% CI 1.42 to 6.20; p=0.004); ACR was 43.4% vs. 65.2% (OR 2.51, 1.28 to 4.91; p=0.009); median PFS was 4.4 months vs. 9.0 months (hazard ratio [HR] 0.67, 95% CI 0.45-0.99; p=0.026); and median OS was 8.7 months vs 19.2 months (HR 0.67, 0.54-0.84; p=0.006). No evidence for new safety concerns arose from this analysis.

Interpretation

Adding RT to immunotherapy significantly increased responses and outcomes in mNSCLC. These results warrant validation in a randomized phase III trial.

INTRODUCTION

The systemic treatment of metastatic non-small cell lung cancer (mNSCLC) continues to evolve rapidly, with immunotherapy (with or without chemotherapy) now being a cornerstone of first-line treatment [1-4]. However, the benefit of immunotherapy has been largely driven by a subset of patients with marked and durable responses to immunotherapeutic agents [5]. Just 17-48% of patients respond to immunotherapy-based approaches, leaving the need to explore further options for non-responders [1, 3, 4].

In order to improve outcomes for these patients, efforts have been aimed at increasing the response rate to immunotherapy, such as by combining immunotherapy with radiation therapy (RT). There is ample mechanistic evidence that RT can enhance the immune response in this setting [6-13]. Central to this notion is the concept of the abscopal effect, which refers to systemic (out of the RT field) anti-neoplastic effects caused by local RT. Biologically, RT enhances the systemic release of antigens from tumor tissue, which are then recognized by antigen-presenting cells and subsequently presented to T lymphocytes (especially CD8 cytotoxic T cells). Priming and activation of these cells causes a systemic immune response against tumor tissue both locally and systemically. Moreover, sublethal doses of RT have been mechanistically shown to more favorably modulate the tumor microenvironment so as to better attract T cells (e.g. potentially by means of reducing the inhibitory signal TGF-β), along with attenuating high-dose RT-induced immunosuppressive cell signaling (e.g. macrophage repolarization to the M1 subtype) [14-18].

Despite cumulative preclinical and clinical data, there remains relatively little randomized evidence of whether combining RT with immunotherapy (iRT) for mNSCLC improves response rates and/or outcomes over immunotherapy alone. The randomized PEMBRO-RT trial (n=78) conducted at the Netherlands Cancer Institute (NKI) suggested a trend towards improved response rates when pembrolizumab was combined with RT as compared to pembrolizumab alone, with a proportionally greater effect in PD-L1 negative patients [19]. A randomized study (n=80) from MD Anderson Cancer Center (MDACC) using either 50Gy/4 fractions or 45Gy/15 fractions did not discern outcome differences in the overall population, but did suggest proportionally greater effects on response rate and progression-free survival (PFS) when 50Gy/4 fractions was applied [20].

Analyzed individually, the relatively small sample size of both aforementioned clinical trials limited the detection of potentially significant differences in response rates and outcomes. While several ongoing randomized studies are examining the addition of RT to various immunotherapy agents, these are the only known completed randomized comparisons of immune checkpoint inhibition alone versus immune checkpoint inhibition combined with RT in mNSCLC. We therefore performed a pooled analysis of these two trials to better evaluate these clinical endpoints.

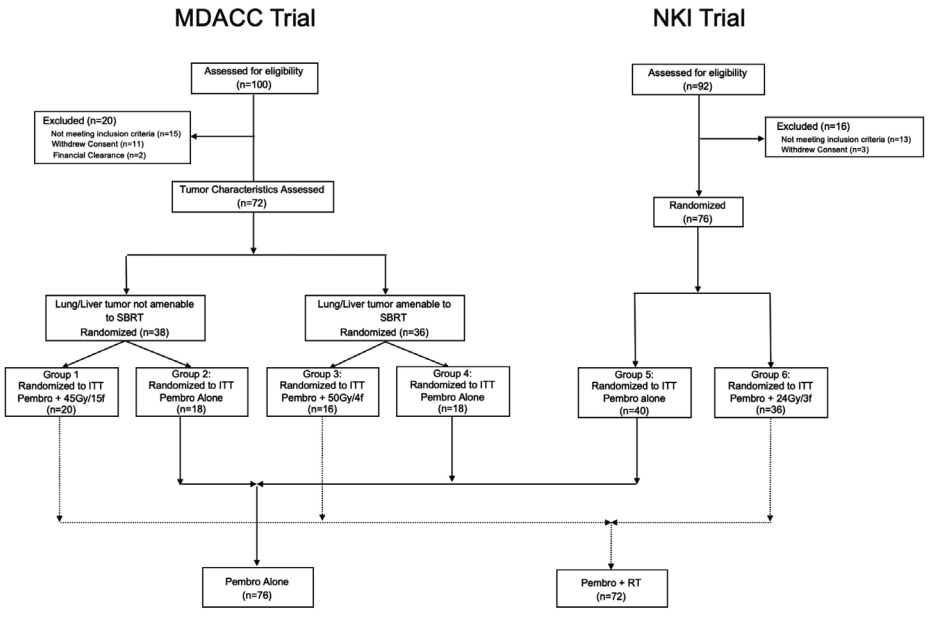
METHODS

Study design, participants and procedures

Both trials (NCT02492568 and NCT02444741) and this pooled post-hoc analysis were approved by the respective institutional review boards. Figure 1 shows a CONSORT diagram of the patient selection from both trials. This study analyzed outcomes for the overall intention-to-treat populations (ITTP), which were pooled based on receipt of pembrolizumab alone versus iRT, regardless of RT schema. Complete information regarding eligibility criteria, enrollment, randomization, and associated workup is included in the trial protocols (Supplemental file 1 and 2) and individual publications.

Of note, both studies required at least 1 unirradiated lesion to monitor the out-of-field response, and both trials administered 200 mg pembrolizumab every 3 weeks. In the PEMBRO-RT trial, the first dose of pembrolizumab was given sequentially <1 week after the last dose of RT, while in the MDACC trial it was given concurrently with the first dose of RT. All patients were immunotherapy-naïve. The Dutch PEMBRO-RT trial (2015-2018) examined only previously chemotherapy-treated patients, evaluated PD-L1 expression a(post-hoc) in nearly all patients, and utilized an RT dose of 24 Gy in 3 fractions (24Gy/3) for all patients in the RT arm [19]. The MDACC trial (2015-2018) encompassed both previously-treated and newly-diagnosed patients, did not mandate PD-L1 assessment, and utilized two fractionation schemas: 50 Gy in 4 fractions (50Gy/4) or (if 50 Gy was subjectively deemed unsafe owing to the size and/or location of the irradiated lesion) 45 Gy in 15 fractions (45Gy/15) with an optional simultaneous integrated boost (SIB) to gross disease of 60 Gy [20]. RT was delivered to 1 site in the PEMBRO-RT study and to a range of 1-4 sites concurrently and with the same dose/fractionation schema for each site in the MDACC study.

Figure 1. Consort diagram.



Randomization and masking

Both studies were open label; owing to the nature of the intervention in the experimental arms (radiotherapy), blinding was not feasible in either trial. In the NKI investigation, patients were randomly assigned using a 1:1 ratio carried out by Alea randomization software (FormsVision 2014) and stratified by smoking status (<10 vs ≥10 pack-years). In the MDACC study, patients were randomized using a 1:1 ratio by the MDACC Department of Biostatistics using the adaptive randomization method by Pocock and Simon with a minimization probability parameter of 0.90. The randomization process was controlled to ensure a balanced stratification by treatment arm.

Statistical analysis

For the PEMBRO-RT trial, the primary endpoint was overall response rate (ORR) at 12 weeks; for the MDACC trial, the primary endpoint was the best ORR. In this analysis, best out-of-field (abscopal) response rate (ARR), ARR at 12 weeks, best abscopal disease control rate (ACR), ACR at 12 weeks, progression free survival (PFS), and overall survival (OS) were evaluated as endpoints. ARR and ACR were defined in unirradiated lesions only based on the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 confirmed by independent radiologists with separate review at each center. PFS was calculated from the time of randomization to progression or death from any cause, or censored at the date of most recent imaging provided the lack of progressive disease. If the assigned treatment failed, and before patients switched treatment, PFS was censored on the date of the last on-study tumor assessment documenting absence of progressive disease for patients who were alive. OS was calculated from date of randomization to date of death from any cause.

Owing to the pooling of two separate trial populations, a fixed effect model was utilized in order to examine possible heterogeneity between trials; this was estimated by means of the I^2 statistic, which indicates heterogeneity caused by total variation across trials rather than chance. PFS and OS were estimated using the Kaplan-Meier method (KM). In exploratory subgroup analyses, the effect of RT on PFS and OS in predefined subgroups was evaluated using Cox proportional hazard models presented in a forest plot (the forest plot shows outcomes for all subgroup analyses, and findings reported in the Results are restricted to those with a 10% difference in effect size). Univariate and multivariable Cox analyses (covariates being the same variables as in the aforementioned forest plot) were performed to determine significant predictors for PFS and OS. Statistical analyses were performed with IBM SPSS v24 (Chicago, IL) and GraphPad Prism v8 (La Jolla, CA), and a p-value <0.05 was considered statistically significant. Power calculations are located in the original publications of each trial.

Role of the funding source

This analysis was designed by the principal and co-principal investigators of both trials. Both the MDACC and NKI trials were financially supported with an unrestricted grant by Merck Sharp & Dohme that included medication supply. The sponsors had no role in the analysis or interpretation of the data or in the writing of the report. The corresponding author had full access to all of the data and the final responsibility for the decision to submit for publication.

RESULTS

Altogether, 148 patients were analyzed, 72 from the MDACC trial and 76 from the PEMBRO-RT trial. The median follow-up for all patients was 33 months. Of these patients, 76 received pembrolizumab alone and 72 underwent iRT. Four of the twenty 45Gy/15 patients received SIB to 60Gy. Table 1 displays clinical characteristics of both cohorts and supplemental file 3 lists the details of unirradiated lesions.

Table 1. Characteristics between two treatment cohorts.

Parameter	Pembrolizumab	Pembrolizumab + RT	*P* Value
	76	72	
Age			
Median (range, y)	64 (33-82)	65 (33-91)	
≥65	36	35	0.88
<65	40	37	
Gender			
Male	43	42	0.83
Female	33	30	
Histology			
Squamous	13	11	0.76
Non-Squamous	63	61	
Lines of previous chemotherapy			
0	16	21	0.3
1	41	30	
≥2	19	21	
Smoking Status			
Current	13	18	
Former	49	41	0.48
Never smoker	14	13	
Sum of the baseline RECIST measurements			
≤median	42	40	0.97
>median	34	32	
Prior radiation therapy			
≤6 months	8	7	
>6 months	31	29	0.98
No	37	36	
PDL1 status			
Unknown	8	8	
<1%	36	31	0.79
1-49	16	20	
≥50%	16	13	
Radiated tumor site			
Lung, metastasis		28	
Lymph node, intra-thoracic		15	
Lung, primary tumor		12	
Lymph node, extra-thoracic		7	
Adrenal		7	
Bone		4	
Cutaneous		1	
Liver		2	
Retroperitoneal		2	
Pleural		1	

In MDA cohort, 2 patients received 2 lesions RT, 1 with 3 lesions RT and 1 with 4 lesions RT.

The response to treatment is shown in Table 2. For best overall response, the ARR (19.7% vs. 41.7% for pembrolizumab alone and iRT, respectively, p=0.004, odds ratio (OR) 2.96, 95% confidence interval (CI) 1.42-6.20; Supplemental Figure 1) and ACR (43.4% vs. 65.2%, p=0.007, OR 2.51, 95% CI 1.28-4.91; Supplemental Figure 2) were significantly higher in the iRT cohort. The ARR in the iRT arm was higher in each PD-L1 subgroup, but this was non- significant (p=0.08 for PD-L1<1%, OR 0.33, 95% CI 0.10-1.04; p=0.16 for PD-L1 1-49%, OR 0.30, 95% CI 0.06-1.44; p=0.70 for PD-L1 >50%, OR 0.58, 95% CI 0.13-2.69). For response at 12 weeks, ARR was 17.1% in the pembrolizumab alone group and 36.1% in the iRT group (p=0.09, OR 1.95, 95% CI 0.91-4.20; Supplemental Figure 3) and ACR was 38.1% in the pembrolizumab alone group and 62.5% in the iRT group (p=0.003, OR 2.71, 95% CI 1.39-5.28; Supplemental Figure 4).

Table 2. Response to Treatment

	Pembro alone	Pembro+RT	NTT	*P* Value	OR, 95% CI
Best overall response, No., %					
Best ARR	15/76 (19.7)	30/72 (41.7)	2	0.004	0.34 (0.16-0.72)
Best ACR	33/76 (43.4)	47/72 (65.2)	4.58	0.009	0.41 (0.21-0.79)
PD-L1 TPS, %					
<1%	6/36 (16.7)	11/29 (38)	4.69	0.08	0.33 (0.1-1.04)
1-49%	3/14 (21.4)	9/19 (47.4)	3.85	0.16	0.30 (0.06-1.44)
≥50%	5/15 (40)	6/13 (46.2)	16.13	0.70	0.58 (0.13-2.69)
Objective response at 12 wk, No., %					
Overall	14/76 (17.1)	25/72 (36.1)	5.26	0.03	0.42 (0.19-0.9)
Disease control	29/76 (38.1)	45/72 (62.5)	4.09	0.005	0.37(0.19-0.72)

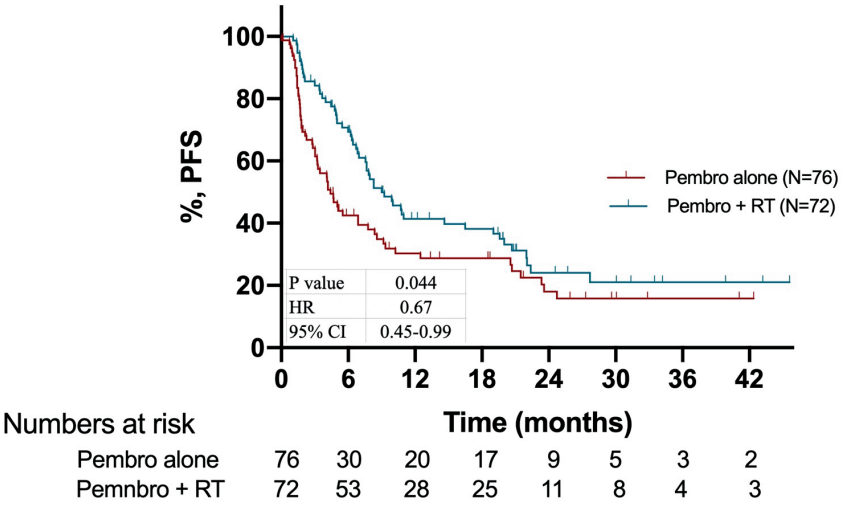
Pembro = pembrolizumab; RT = radiotherapy; NTT = number needed to treat; OR = odds ratio; CI = confidence interval; ARR = abscopal response rate; ACR = abscopal control rate; PD-L1 = programmed death–ligand 1; TPS, tumor proportion score; wk = week.

The median follow-up times were median follow-up times of 33.2 and 34.0 months in pembro alone and pembro+RT group.

Figures 2 and 3 shows comparative outcomes. The iRT cohort experienced a significantly higher median PFS compared to the pembrolizumab alone cohort (9.0 vs. 4.4 months, hazard ratio (HR) 0.67, 95% CI 0.45-0.99, p=0.044, Figure 2A; Supplemental Figure 5). Exploratory subgroup analyses (Figure 2B) suggested that the addition of RT was most beneficial in males (p=0.032), patients having received ≥2 lines of prior chemotherapy (p=0.016), or patients with low (1-49%) PD-L1 expression (p=0.012).

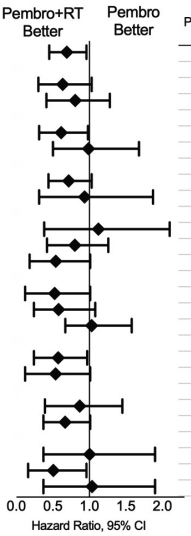
Figure 2. Kaplan-Meier curves for progression-free survival (A) between the pembrolizumab versus iRT cohorts, along with exploratory subgroup analysis (B) of associated factors.

A



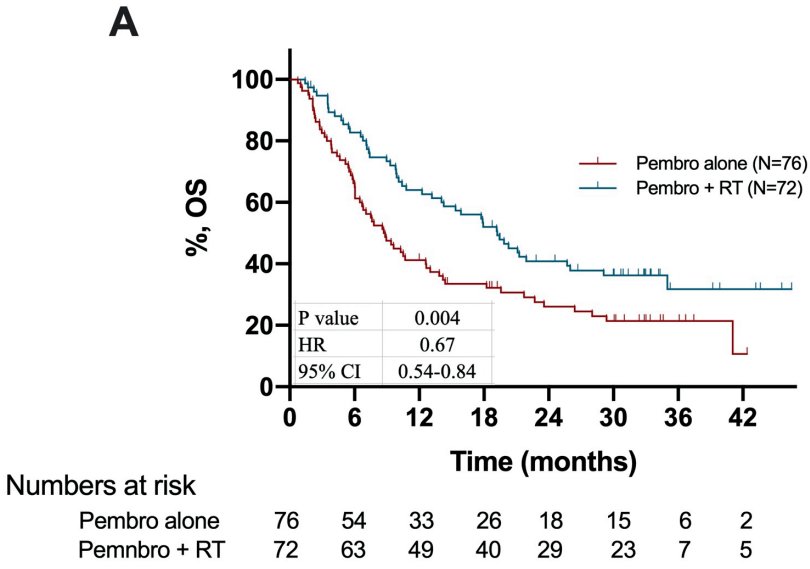
B

Subgroup	Pembro+RT Events, No. / Total No.	Pembro Events, No. / Total No.	HR, 95% CI	Pembro+RT Median PFS (months)	Pembro Median PFS (months)	*P*
Total	59/72	53/76	0.66 (0.45-0.96)	9.0	4.4	0.026
Age						
≥65	30/35	24/36	0.57 (0.30-1.03)	14.1	6.7	0.080
<65	29/37	29/40	0.73 (0.41-1.28)	4.6	6.7	0.330
Gender						
Male	31/42	32/43	0.55 (0.31-0.98)	10.2	6.1	0.032
Female	28/30	21/33	0.78 (0.50-1.68)	6.8	4.9	0.778
Histology						
Non-Squamous	7/11	5/13	0.68 (0.44-1.03)	7.9	4.5	0.054
Squamous	52/61	48/63	0.62 (0.31-1.87)	12.0	10.0	0.411
Previous chemotherapy						
0	15/21	11/16	0.89 (0.38-2.10)	12.3	6.5	0.700
1	28/30	30/41	0.72 (0.42-1.26)	7.4	5.0	0.231
≥2	16/21	12/19	0.43 (0.18-1.01)	8.3	1.6	0.016
Smoking Status						
Current	14/18	10/13	0.44 (0.12-1.01)	7.1	4.4	0.051
Former	37/41	36/49	0.41 (0.42-1.08)	8.4	4.4	0.060
Never	8/13	7/14	0.84 (0.67-1.58)	10.2	3.3	0.210
Sum of the baseline RECIST measurements						
≤median	31/40	26/42	0.51 (0.24-0.98)	11.7	6.5	0.048
>median	28/32	27/34	0.48 (0.12-1.01)	9.5	4.6	0.056
Prior RT						
Yes	23/36	21/39	0.76 (0.39-1.45)	7.4	4.4	0.390
No	36/40	32/37	0.62 (0.37-1.01)	9.6	4.7	0.053
PDL1 status						
<1%	27/31	22/36	0.74 (0.42-1.3)	6.8	4.7	0.290
1-49	14/20	12/16	0.4 (0.16-0.96)	10.4	3.3	0.012
≥50%	11/13	12/16	0.84 (0.37-1.9)	7.5	6.9	0.680

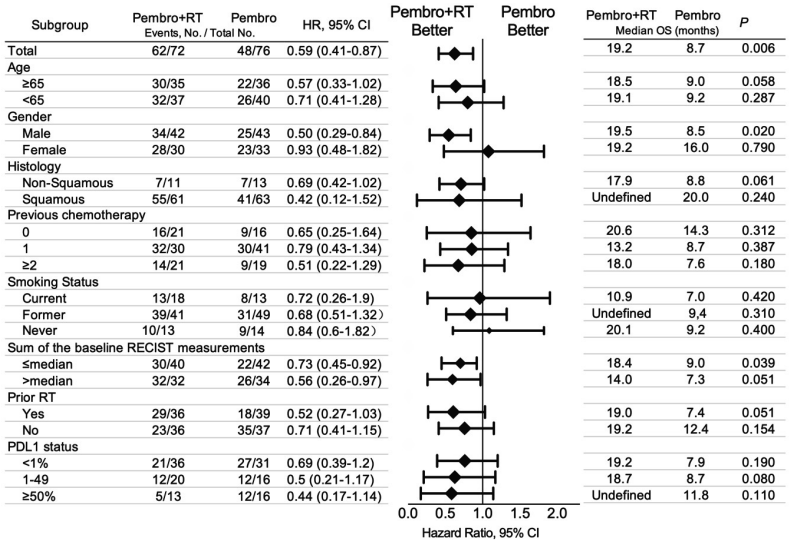


Patients who received iRT experienced significantly higher median OS compared to patients treated with pembrolizumab alone (19.2 vs. 8.7 months, HR 0.67, 95% CI 0.54-0.84, p=0.004, Figure 3A; Supplemental Figure 6). Exploratory subgroup analyses (Figure 3B) suggested a greater effect in males (p=0.02).

Figure 3. Kaplan-Meier curves for overall survival (A) between the pembrolizumab versus iRT cohorts, along with exploratory subgroup analysis (B) of associated factors.



B



A multivariable exploration of prognostic factors showed that never smokers and an RT schema of 50Gy/4 fractions were significantly associated with PFS (Table 3). There were no factors significantly associated with OS. Full details of adverse events are reported in the original publications of the two trials [19, 20]. Briefly, high-grade RT-related AEs were extremely uncommon and the pembrolizumab-related AEs were similar to those reported in other mNSCLC pembrolizumab monotherapy studies [3, 21, 22]; no new concerns regarding safety arose from this analysis.

Table 3. Univariate and multivariable Cox-analyses for intention-to-treat population.

	Univariate-	Multivariate-PFS			Univariate-OS			Multivariable-OS		
	P	HR	95% CI	*P*	HR	95%CI	*P*	HR	95% CI	*P*
Gender	0.39						0.26			
Male		0.85	0.57-1.25	0.44	0.78	0.50-1.21		0.77	0.50-1.21	0.26
Female		Ref			Ref			Ref		
Age	0.09						0.18			
≥65		1.33	0.89-1.98	0.17	0.97	0.64-1.48		0.97	0.64-1.48	0.90
<65		Ref			Ref			Ref		
Histology	0.16						0.07			
Non-Squamous		0.66	0.37-1.18	0.16	0.55	0.28-1.06		0.55	0.28-1.06	0.07
Squamous		Ref			Ref			Ref		
Smoking history	0.044						0.28			
Never		0.76	0.44-0.98	0.048	0.81	0.41-1.32		0.83	0.39-1.32	0.30
Former		0.91	0.37-1.33	0.097				1.03	0.80-1.76	0.40
Current		Ref			Ref			Ref		
Lines of previous chemotherapy	0.032						0.52			
0		0.81	0.46-1.22	0.28	1.03	0.61-1.81		1.03	0.61-1.81	0.52
1		0.87	0.54-1.12	0.071	Ref			0.81	0.56-1.53	0.62
≥2		Ref			0.81	0.56-1.53		Ref		
Previous radiotherapy	0.57						0.26			
Yes		1.36	0.90-2.04	0.14	1.28	0.83-1.96		1.28	0.83-1.96	0.26
No		Ref			Ref			Ref		
PD-L1, %	0.11						0			
0		Ref			Ref			Ref		
1-49		1.04	0.56-1.92	0.91	0.73	0.42-1.27		0.73	0.42-1.27	0.27
≥50		0.83	0.4-1.72	0.62	0.53	0.29-1.12		0.53	0.29-1.12	0.09
Treatment	0.03						0.038			
Pembrolizumab alone		Ref			1.57	1.03-2.40				
Pembrolizumab+45Gy/5f		0.98	0.43-1.53	0.57				1.169	0.73-2.40	0.34
Pembrolizumab+24Gy/3f		0.76	0.46-1.09	0.083				0.84	0.53-1.43	0.14
Pembrolizumab+50Gy/4f		0.67	0.36-0.98	0.047				0.82	0.34-1.87	0.23
Irradiated lesion	0.026						0.15			
Primary lung		Ref						Ref		
Metatistic lung		0.68	0.32-1.26	0.41				0.77	0.43-1.56	0.37
Lymph nodes		1.21	0.85-1.72	0.66				0.98	0.43-1.67	0.24
Others		0.83	0.43-1.39	0.34				1.07	0.72 -1.63	0.42

PFS = progression-free survival; OS = overall survival; HR=hazard ratio.
The four multi-RT patients were analyzed in the metastistic lung group as all of them had at least 1 lesion received RT in the lung. Progression-free survival was defined as the time from randomization to progression.

In the MDACC trial, RT scheme was chosen subjectively based on physicians' discretion and safety owing to the size and/or location of the irradiated lesion dose. Therefore, this pooled analysis is not suited to address the comparative efficacy of various RT schemas. However, it was notable that the ARR for the 45Gy/15 subgroup seemed similar to patients who received no RT, and that the ARR for both the 50Gy/4 and 24Gy/3 subgroups were similar as well, but over twice as high as the other 2 subgroups (Supplemental Figure 7). To further probe into this finding, we evaluated the difference in absolute lymphocyte count (ALC) before and after RT based on particular schema. As lymphocytes are important for an effective antitumor immune response, but are also very radiosensitive, specific RT schemas may also negatively influence the antitumor immune response induced by immunotherapy.

There was a significant drop in ALC for only the 45Gy/15 subgroup, whereas no effect on ALC was seen in both other schemas, suggesting a potentially detrimental effect with the 45Gy/15 schema (Supplementary Figure 8).

DISCUSSION

Despite the mounting pre-clinical and clinical data describing the augmenting effects of RT on immunotherapeutic treatment of mNSCLC, the only two existing randomized trials thus far were not able to show a significant improvement in patient outcomes, likely owing to limited sample size[19, 20]. This was the primary impetus to perform a pooled analysis of both studies, resulting in the largest prospectively collected cohort assembled to date. We found that adding RT to immunotherapy significantly increased the response rates of unirradiated lesions, which led to a significantly higher PFS and OS.

The abscopal effect is a relatively uncommon phenomenon, although it has been proposed that the addition of RT to immunotherapy could enhance the occurrence of abscopal responses and hence improve outcomes. To date, higher-volume randomized clinical data have been largely absent. This pooled analysis largely comprised irradiated intrathoracic disease; it shows that the abscopal effect was induced considerably more often with the addition of RT. The improved control of systemic disease likely drove the improved PFS and OS findings in the iRT arm.

Notably, both of the trials were powered to detect a 30% difference in response rate. While the primary endpoint for both trials showed improvement in the iRT arm, the results of neither study met the prespecified criteria for meaningful clinical benefit [19, 20]. A larger sample size would therefore likely be required to more accurately detect the effects of the addition of RT to immunotherapy on patient outcomes. Also, one of the major concerns in the PEMBRO-RT trial was the imbalance of PD-L1 distribution in favor of the iRT arm. Pooling the data of both trials eliminated this imbalance, strengthening the evidence that the observed improvement in patient outcomes was indeed due to the addition of RT.

Although this pooled analysis alleviates sample size concerns from each individual trial, it should be mentioned that the subgroup analyses are undoubtedly still limited by a low sample size and should thus be evaluated with caution. Notably, there was no correlation of PD-L1 expression with outcomes in our combined cohort. The improvements by iRT for the PD-L1 negative patients within the PEMBRO-RT study disappeared in this pooled analysis. This could be from bias due to the lack of PD-L1 scores for 19% (14/72) of the MDACC cases (as compared to only 3% (2/76) of PEMBRO-RT cases). Additionally, other data that may have a predictive role for response to immunotherapy, such as tumor mutational burden and baseline immune status, were not available. Taken together, it remains difficult to conclude whether a meaningful association between PD-L1 status and benefit from iRT exists, and larger-volume studies with mandatory PD-L1 assessment are required to address this unresolved question.

To date, many questions remain about the impact of different RT dose and fractionation schemas on the magnitude of the immune-boosting effect. RT schemas were variable in both trials largely because there is currently no consensus on optimal RT dosing in the mNSCLC setting. Because RT schemas were not applied randomly, but rather based on trial variability and/or physicians' discretion, statistical comparison between RT schemas was not feasible. Nevertheless, the large difference in ARR of the 50Gy/4 and 24Gy/3 subgroups compared to the 45Gy/15 and pembrolizumab alone subgroups remained striking. These results logically lead to inquiry regarding whether the findings were due to differences in ALC, unforeseen clinical factors associated with physician choice of RT schema, or both. With regard to ALC, the observation that 45Gy/15 fraction RT was associated with a more pronounced ALC decline, along with the similar ARR as pembrolizumab alone (20% for both) requires further investigation. With regard to unforeseen clinical factors, patients in the 24Gy/3 fraction cohort were more heavily pretreated, and were less likely to have received RT before study inclusion. There were also some important differences in trial design. The timing of RT and pembrolizumab was different between trials. Also, in the PEMBRO-RT study, only one lesion was treated with RT; in the MDACC study, up to 4 lesions were irradiated. Although only 4/16 (25%) patients in the 50Gy/4 cohort received concurrent multiple-site RT, these patients tended to perform better (data not shown) and could explain why the Cox multivariable analysis revealed that the 50Gy/4 fraction schema was significantly associated with PFS (but not OS). Additionally, this analysis did not show a differentially advantageous location or designated target lesion (e.g. primary vs metastasis) for application of RT owing to the similar PFS and OS in the corresponding subgroups. Taken together, owing to the multitude of aforementioned reasons, conclusions regarding the optimal dosing, timing or location of RT in order to induce an abscopal response cannot be drawn from this study.

In summary, this pooled analysis of two randomized trials examining pembrolizumab with or without RT in mNSCLC showed that the addition of RT to immunotherapy significantly increased the ARR, and was additionally associated with significant improvements in PFS and OS. These hypothesis-generating results should be corroborated in a dedicated, large-volume, randomized trial.

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SUPPLEMENTARY DATA

Figure S1. Results for the heterogeneity I2 test and the fixed effect model for best abscopal response rate (ARR).

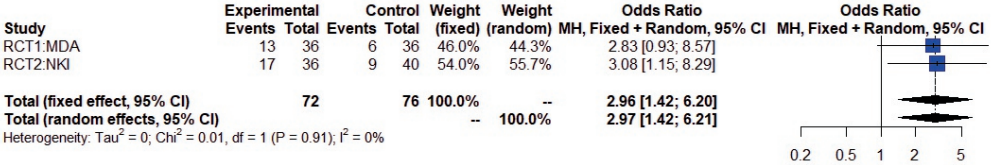


Figure S2. Results for the heterogeneity I2 test and the fixed effect model for best best abscopal control rate (ACR).

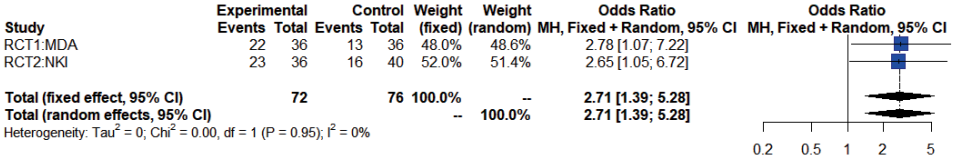


Figure S3. Results for the heterogeneity I2 test and the fixed effect mode for abscopal response rate (ARR) at 12 weeks.

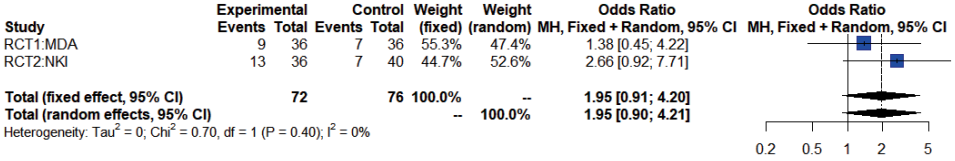


Figure S4. Results for the heterogeneity I2 test and the fixed effect modelfor abscopal control rate (ACR) at 12 weeks.

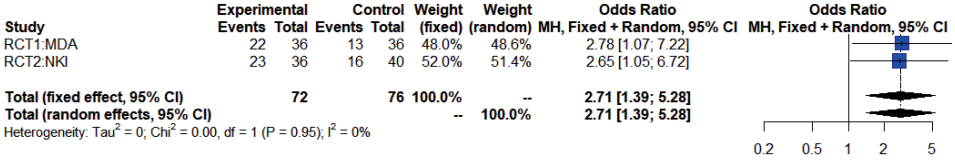


Figure S5. Results for the heterogeneity I2 test and the fixed effect model for abscopal control rate (ACR) at 12 weeks.

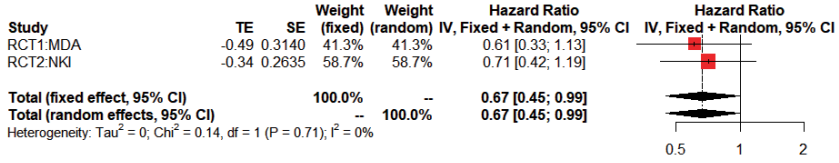


Figure S6. Results for the heterogeneity I2 test and the fixed effect model for median overall survival (OS).

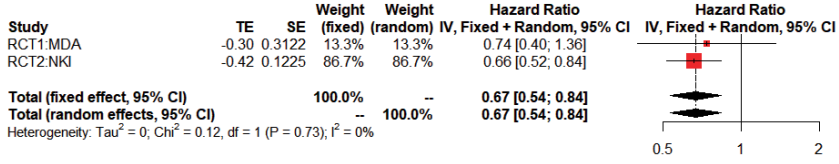


Figure S7. Best out-of-field (abscopal) response rates for various RT schemas and pembrolizumab alone.

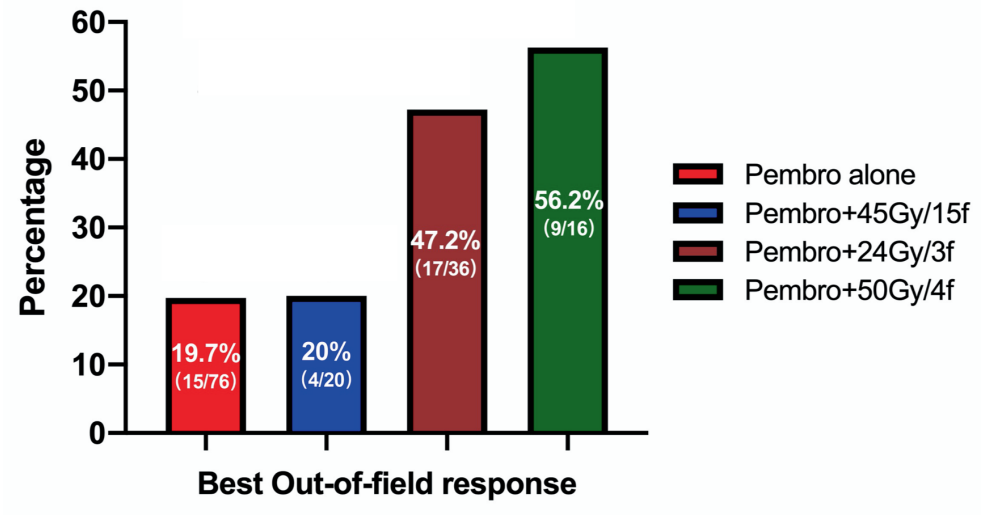
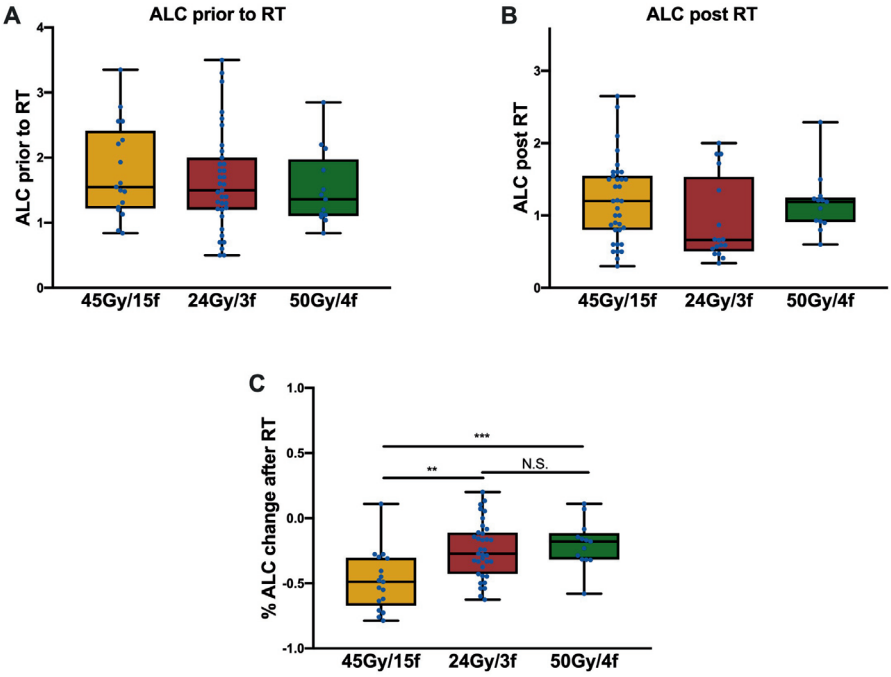


Figure S8. Absolute leucocyte count (ALC) change after RT for various RT schemes.



PART III.

General discussion and summary / samenvatting

CHAPTER 7

General discussion and future perspectives

The good news

During the course of this thesis, the landscape of systemic treatment options concerning non-small cell lung cancer (NSCLC) has kept on changing, which has led to some good news. In the United States, it was reported that the cancer-related death rate had declined over the past two decades. The largest single-year drop of 2.2% was established over the last-measured year, 2016-2017 [1]. This decline was driven by an accelerated drop in lung cancer deaths, which dropped around 5% from 2013-2017. The decrease is attributed at least in part to advances in treatment, like the introduction of immunotherapy. Although these results are encouraging, it must be stressed out that lung cancer is still the leading cause of cancer death even in the United States. Our work here is far from finished.

Recent developments in the systemic treatment of metastatic non-small cell lung cancer

After registration of programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) blockade in second-line treatment, several phase 3 trials in NSCLC have been performed to evaluate immunotherapy in first-line setting. Firstly, the KEYNOTE-024 study showed a convincing improvement of overall survival (OS) of the PD-1 inhibitor pembrolizumab over platinum-based chemotherapy in patients with previously untreated advanced NSCLC with a PD-L1 expression of ≥50% [2]. Subsequently, the KEYNOTE-189 and KEYNOTE-407 combined platinum-based chemotherapy with pembrolizumab in first-line setting and compared this regimen to platinum-based chemotherapy in all-comers, i.e. irrespective of PD-L1 expression [3, 4]. Progression free survival (PFS) and OS were in favor of the platinum-doublet chemotherapy and pembrolizumab combination, sometimes referred to as 'triple-therapy'. Also, the PD-L1 inhibitor atezolizumab was approved for treatment-naïve advanced NSCLC patients based on two studies that had met their co-primary endpoint of PFS and OS benefit for the addition of atezolizumab to carboplatin and nab-paclitaxel (IMpower 130) and to carboplatin, paclitaxel and bevacizumab (IMpower 150) both in nonsquamous NSCLC [5, 6]. Benefit was irrespective of PD-L1 expression measured as a combined score of expression on tumor cells (TC) as well as on tumor-infiltrating immune cells (IC). However, the Checkmate-026 study failed to show benefit in regard to OS of first-line PD-1 inhibitor nivolumab over platinum-based chemotherapy in advanced NSCLC with PD-L1 ≥5% measured on TC [7]. This discrepancy in benefit compared to the other PD-1 inhibitor pembrolizumab could possibly be attributed to differences in patient selection, but differences in binding ability of the drug to the PD-1 receptor has also been suggested.

Based on previous successes in melanoma patients, blockade of the immune checkpoint cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) has been investigated in NSCLC as well. No randomized trials with monotherapy of the two available anti-CTLA-4 drugs, ipilimumab and tremelimumab, have been performed in NSCLC. Combining PD-1/PD-L1 blockade with CTLA-4 antibodies has led to conflicting results when compared to platinum-doublet chemotherapy in treatment-naïve NSCLC setting. The combination of ipilimumab with nivolumab in the multi-arm CheckMate-227 study improved OS compared to platinum-based chemotherapy for both the PD-L1 positive and negative subgroup [8]. However, the combination of the PD-L1 antibody durvalumab with tremelimumab investigated in the MYSTIC trial showed no PFS or OS benefit over first-line chemotherapy in patients with advanced NSCLC [9]. The role of the addition of CTLA-4 antibodies in advanced NSCLC therefore remains

somewhat unclear, especially now that PD-1/PD-L1 blockade is (part of) the standard of care in metastatic NSCLC first-line setting, and no approval of anti-CTLA-4 treatment in NSCLC has been granted at time of writing. However, results of the CheckMate-227 have led to FDA approval of the ipilimumab plus nivolumab combination in first-line metastatic NSCLC in May 2020.

The recent results have established PD-1/PD-L1 blockade as the new cornerstone in first-line treatment of patients with metastatic NSCLC. However, the unanswered question whether patients with advanced NSCLC with PD-L1 expression ≥50% are better off with triple-therapy or if pembrolizumab monotherapy could suffice still remains. Also, is the addition of chemotherapy to immunotherapy in patients with PD-L1 expression <50% indispensable? The KEYNOTE-042 study compared pembrolizumab monotherapy to platinum-based chemotherapy in first-line setting in patients with metastatic NSCLC and a PD-L1 expression of ≥1% [10]. The authors stated that pembrolizumab monotherapy could be a treatment option for these patients based on the OS benefit in the pembrolizumab group. However, it should be mentioned that the OS benefit was driven by the PD-L1 ≥50% subgroup and that in a subgroup analysis, patients with PD-L1 expression between 1-49% derived no OS benefit from pembrolizumab over chemotherapy; in fact, the OS curves crossed, suggesting that for at least a part of these patient the need for a chemotherapy backbone remains.

As data matures, long-lasting anti-tumor immune responses in advanced NSCLC have been described on PD-1 blockade [11, 12]. PD-L1 expression on tumor cells remains the best and most accessible clinical biomarker for response to immune checkpoint inhibition to date and has been used to guide treatment choices in clinical trials. But based on the issues mentioned above regarding PD-L1 subgroups, PD-L1 expression seems far from perfect to predict the optimal treatment choice for NSCLC patients.

PART I. Exploring the tumor immune microenvironment

Because the aim of anti-PD-1/PD-L1 treatment is blocking the interaction between the PD-L1 receptor expressed by tumor cells and the PD-1 receptor on immune cells, or to be more specific on cytotoxic T cells, it seemed the most sensible choice to pick PD-L1 expression in tumors to explore as a biomarker for response. Unfortunately, in advanced NSCLC results were far from clean-cut: objective response rates (ORR) varied from 8% in PD-L1 negative tumors up to approximately 30% in tumors with PD-L1 expression of ≥50% [13-15]. These results prove that other mechanisms of tumor-immune interaction must be present (or are lacking) in order to induce tumor killing by the host immune system through PD-1/PD-L1 blockade. To enable exploration of the tumor immune environment in NSCLC, we built a database of over 600 tumor samples from resected patients and collected patient and tumor characteristics. Immunohistochemical (IHC) staining of PD-L1 on TC and IC separately as well as CD8 infiltration, mutational data, and gene expression mainly of genes related to immunologic function were obtained.

The role of PD-L1 expression on tumor cells vs tumor-infiltrating immune cells

Because PD-L1 expression on both TC and IC appeared to be independently associated with response to atezolizumab, this scoring approach may help us in understanding more about the discrepancy of the link between PD-L1 expression on TC and response on anti-PD-1/PD-L1 treatment.

In **chapter 2**, we sought to improve insights in the associations of PD-L1 expression and specific patient' or tumor characteristics. Only for PD-L1 expression on TC positive associations were found (*KRAS* mutations and smoking), but none were found for IC. We then explored overlap and differences between expression of PD-L1 on TC and IC. Our interest was raised by the fact that more than half of the cohort had a PD-L1 positive immune infiltrate, which was associated with other immune gene markers, but without upregulation of PD-L1 on TC. In a subsequent analysis, we found that in the subgroup of TC0/IC3 samples an impairment of IFNγ response in the TC might be responsible for the lack of upregulation of PD-L1 in these tumors. From clinical trials we know that this TC0/IC3 subgroup has a higher overall response rate (ORR) compared to the overall TC0 tumors (22% vs. 8%) [15, 16]. These findings may contribute to the understanding why patients with PD-L1 TC negative NSCLC may still (only) require immune checkpoint blockade to gain response and survival benefit.

Protein expression of PD-L1 was assessed by the IHC SP142 assay. In the Blueprint PD-L1 IHC Assay Comparison Project, this specific assay exhibited fewer stained TC overall when compared to the three other assays tested (22C3, 28-8 and SP263) [17]. Therefore, tumors may have been unjustly scored as PD-L1 negative. This may have resulted in an underestimation of the finding of a hampered IFNγ-PD-L1 axis in our TC0 subgroup and might explain why we did not find this impairment in the TC0/IC2 and TC0/IC1 subgroups.

Unfortunately, analyses performed within our cohort cannot be extrapolated towards prediction for response as these resected patients never received immunotherapy. The data of this database enables us to look for associations between different biomarkers, patient and tumor characteristics and prognostic features. So, whether patients with tumors with a hampered IFNγ response would benefit from anti-PD-1/PD-L1 treatment remains unknown. Also, other explanations and hypotheses have been formulated to explain the discrepancy between the level of PD-L1 expression on TC and response to immune checkpoint inhibition: variable PD-L1 antibody assays, different IHC cut-offs, differences in tissue preparation or processing variability, tumor heterogeneity, primary versus metastatic biopsies and oncogenic versus immune-induced PD-L1 expression [18].

Alternative biomarkers for response to immune checkpoint inhibition

As mentioned in the recent developments section, clinical decision-making about first-line treatment choices in advanced NSCLC is based on PD-L1 expression on TC as a sole biomarker for immune checkpoint inhibition to date. In recent years, several other biomarkers have been investigated. The tumor mutational burden (TMB) is probably the most prominent one as it has been evaluated in several phase III clinical trials next to PD-L1 expression. Previous studies have reported that PD-L1 and TMB are independent predictors of response to immunotherapy in patients with NSCLC [7, 19, 20]. As smoking is an important cause of DNA damage, TMB in NSCLC is one of the highest among solid cancers [21]. The improved anti-tumor response in the setting of checkpoint inhibitor therapy may be

due to a higher number of tumor mutations increasing the probability of generating a “high quality” immunogenic peptide. The interest of TMB as a predictive biomarker seemed risen especially in the combination of anti-PD-1/PD-L1 with anti-CTLA-4 treatment. In the multi-arm CheckMate-227 study, the combination of nivolumab with ipilimumab significantly improved PFS compared to platinum-based chemotherapy in first-line NSCLC with high TMB (≥10 mutations/megabase (mut/Mb)) and this was irrespective of PD-L1 expression [20]. CheckMate-568 successfully validated the TMB cutoff of ≥10 mut/Mb as a biomarker for improved ORR and PFS for the ipilimumab and nivolumab combination again irrespective of PD-L1 expression in a single arm study design [22]. Another immunotherapy combination of durvalumab with tremelimumab in the MYSTIC trial also showed benefit over first-line chemotherapy in patients with high TMB measured in blood (≥16 mut/Mb), but not for PD-L1 expression of >25% [9]. Unfortunately, in the updated analysis of the Checkmate-227 study, no difference in survival outcomes between patients whose tumors had high or low levels of TMB was observed and the application of frontline approval for the combination of nivolumab and ipilimumab for patients with advanced NSCLC with a TMB of ≥10 mut/Mb was withdrawn [8]. As the updated analysis of the MYSTIC trial showed a negative result for the durvalumab and tremelimumab combination over chemotherapy in the overall study cohort, further clinical development of this treatment option remains unsure. Also, several issues regarding methods of obtaining and reporting TMB in NSCLC need to be mentioned. Some studies report TMB in terms of the absolute number of mutations, while others assess mutations per DNA megabase, i.e. mut/Mb. Additionally, thresholds to establish high TMB vary greatly without a widely used standard currently existing [23]. Besides a relatively long lead time to obtain TMB, tumor purity may also have an effect on TMB measurements [24]. These issues raise questions of utility and reproducibility for TMB as a useful clinical tool for treatment decision making in metastatic setting.

As opposed to TMB, exhaled breath could provide a very accessible biomarker for prediction as its retrieval is rapid and noninvasive. The volatile organic compounds (VOCs) in exhaled breath may represent systemic and local metabolic processes, which can be recognized and analyzed through electronic nose (eNose) technology. In a cohort of 92 advanced NSCLC patients this technique was able to discriminate between responders and non-responders on PD-1 checkpoint blockade at baseline and this finding was validated in a separate cohort of 51 NSCLC patients [25]. The distinction by the exhaled breath analysis was more pronounced compared to assessment of PD-L1 expression and furthermore, with the right cut-off exhaled breath was also more effective in predicting non-response to immune checkpoint blockade compared to PD-L1 expression. However, this biomarker will still need further evaluation. Another field of interest for biomarker research was found in the microbiome of cancer patients. Recent research has characterized the microbiome of NSCLC patients in a Chinese population and found that larger microbiome diversity could serve as a potential biomarker in predicting a favorable response to PD-1 blockade [26]. Apart from being a potential biomarker, influencing the gut microbiome, for example by fecal transplantation, might also lead to overcoming host ‘immune-breaking’ characteristics and therefore serve as potential lead for treatment optimization.

Although promising, as of yet none of these biomarkers have proven themselves more accessible and/or reliable compared to PD-L1 expression on tumor cells by IHC and further research is needed to improve (first-line) treatment selection in advanced NSCLC patients.

Interaction between tumor and immune cells in adenocarcinoma vs squamous cell carcinoma

Activated $CD8^+$ T cells are the key players in the anti-cancer immune response that is generated or amplified by immune checkpoint inhibitors. However, this immune response and also the tumor immune microenvironment are heterogeneous entities, involving a whole range of immune cells together with a wide spectrum of soluble chemokines and cytokines. Gene expression analysis allows us to perform excessive immunoprofiling of all the different aspects of the tumor immune microenvironment. In **chapter 3**, we investigated immune gene expression by NanoString of the resection cohort mentioned in the previous chapter. We found that in NSCLC adenocarcinomas (AD) the level of overall inflammation as assessed by immune gene expression was significantly higher compared to squamous cell carcinomas (SCC). This seemed to be related to a higher infiltration rate of immune cells within the tumor bed of AD compared to SCC based on a significant difference in tumor cell percentage between both histologies, i.e. tumor cell percentage being higher in SCC. This may suggest a different interaction of immune cells and tumor cells between the different histologies. Interestingly, a cluster of 34 genes did not correlate with the general level of inflammation, the level of PD-L1 expression or $CD8^+$ T cell infiltration. Expression of this 34-gene cluster, identified by unsupervised clustering, did not differ between AD and SCC histology, but high expression of this signature showed a clear OS benefit in SCC, but not in AD. This finding was validated in two independent NSCLC cohorts. We then tried to allocate the nature of this 34-gene signature and found the strongest correlation with Natural Killer (NK) cell related gene expression. Cell surface genes involved in NK cell recognition and killing - *ULBP2* and *HLA-C* – were significantly different between SCC and AD histology in favor of our hypothesis, namely that SCC may be more susceptible to NK cell killing than AD. Unfortunately, there is no established gold standard for assessing NK cell infiltration and/or activation level in tumor samples. Also, these cells are generally scarce within the tumor microenvironment and our IHC NK cell double-staining was not able to differentiate the 34-gene high from the 34-gene low samples.

Many endeavors of gene expression-based exploration of the immune tumor microenvironment have been performed using RNA sequencing platforms. RNA sequencing allows for a broader number of genes to be assessed per sample compared to the NanoString technique used in our cohort, but this goes at the expense of sensitivity. Almost all genes within our 34-gene signature could not be adequately measured by RNA sequencing technique due to low expression levels of the genes in the signature. To further investigate the biological rationale of such low-level signatures newer techniques might come of aid. Single cell sequencing using NanoString or microarray-based techniques may be able to further dissect the different aspects of the tumor immune microenvironment with a higher sensitivity together with assessing the level of activation of these different immune cells. This information could bring new insights in the role of immune cells that are present within the tumor infiltrate in low quantities, like NK cells. Several clinical phase I/II trials in various solid tumors manipulating the anti-cancer immune response through activation of NK cells are currently ongoing. Trials within this field are investigating the safety and efficacy of i) infusing NK cells as monotherapy or in combination with immune checkpoint inhibitors, chemotherapy or targeted drugs; ii) new molecules that target NK cells and T-cell activation signals to specific receptors on cancer cells, like antibody-dependent cell-mediated cytotoxicity (ADCC);

iii) chimeric antigen receptor T (CAR-T) cells or CAR-NK cells to redirect and activate NK cells into the tumor bed; iv) hematopoietic stem cell transplantation; v) cytokines and immunostimulatory drugs to boost the anti-tumor activity of NK cells [27].

PART II. Modulating the tumor immune microenvironment

As mentioned earlier, long-lasting responses in patients with advanced NSCLC on PD-1 blockade have been established with a reported estimated 5-year OS ranging between 15 - 27% [11, 12]. Although these are numbers previously unheard of in advanced NSCLC, there is still an urgent need for further improvements, especially for those patients not responding to immune checkpoint blockade. Radiation therapy (RT) could be a potent modulator of the tumor microenvironment and could augment the antitumor immune response when combined with immune checkpoint inhibition. In **chapter 4**, we provided a review about the off-target effects of RT, the so-called abscopal effect. We describe how RT may counteract the mechanisms of failure of immunotherapy and an oversight of pre-clinical and clinical data supporting augmentation of abscopal events by RT when combined with immune checkpoint inhibition is presented.

Based on these biological principles and at that time mainly pre-clinical results, we set up the PEMBRO-RT trial. In this multicenter study, patients with advanced NSCLC that had received at least one prior line of chemotherapy but were immunotherapy-naïve, were randomized between pembrolizumab treatment (control arm) vs pembrolizumab treatment within one week after three doses of 8 Gy on a single tumor lesion (experimental arm). Stratification was based on smoking status: <10 pack years vs ≥10 pack years. The primary end point was ORR at 12 weeks from randomization according to Response Evaluation Criteria in Solid Tumors (RECIST). The results of the PEMBRO-RT trial are presented in **chapter 5** [28]. The intention-to-treat (ITT) population consisted of 76 patients. Although the ORR at 12 weeks doubled in the experimental arm compared to the control arm (36% vs 18%), this difference was not statistically significant (p=0.07). The PD-L1 negative subgroup experienced a significant PFS and OS benefit in the experimental arm compared to the control arm, but no differences were seen in the overall ITT population regarding these outcomes. No increase in treatment-related toxicity was observed in the experimental arm. So, although an augmenting effect of RT on the response to PD-1 blockade in patients with metastatic NSCLC was observed, the study did not meet its primary end point of prespecified criteria for meaningful clinical benefit.

At the time of publication of the PEMBRO-RT trial, the MD Anderson Cancer Center (MDACC) was analyzing results from a similar randomized trial of pembrolizumab alone vs pembrolizumab in combination with RT [29]. In this study, patients with advanced NSCLC that were treatment-naïve or who had received prior chemotherapy both were allowed to participate. Patients were randomized between pembrolizumab treatment (control arm) vs pembrolizumab treatment with concurrently applied RT with the first dose of immunotherapy (experimental arm). Stratification was based on amenability of a lung or liver lesion to one of two RT regimens: 50 Gy in 4 fractions (50Gy/4, stereotactic body radiotherapy (SBRT) vs 45 Gy in 15 fractions (45Gy/15, traditional RT). The primary end point was disease response according to immune related response criteria (irRC). Preliminary results were

presented at ASCO 2019 and although response rates and PFS were similar between the RT and the control arm in the overall cohort, the 50Gy/4 SBRT subgroup showed a non-significant improvement in response rate in the non-irradiated lesions with a significant improvement in PFS compared to the traditional fractionated RT. Based on these results, it was hypothesized that a possible augmentation of an antitumor immune response on immune checkpoint inhibition may only exist when a SBRT regimen, but not traditional fractionation, is applied.

In **chapter 6**, we present the results of the pooled analysis of these two randomized trials. By exploring the possible abscopal effect in a larger cohort of advanced NSCLC patients, we found not only a significant improvement of abscopal response rate (ARR) in the experimental arm compared to the control arm, but also a significant PFS and OS benefit was observed in the patients treated with pembrolizumab and RT. Because RT regimen was not applied randomly, but rather based on trial variability and/or physicians' discretion, statistical comparison between RT schemas was not feasible. However, the 45Gy/15 subgroup showed an ARR similar to the control group, both around 20%. However, the other two RT regimens produced an ARR over two times as high. Exploration of the absolute lymphocyte count (ALC) showed a more pronounced ALC decline in the 45Gy/15 subgroup, which provides a hypothesis of a detrimental effect on immune response by traditional fractionation that requires further investigation.

In the PEMBRO-RT study, expression of PD-L1 was assessed after termination of the trial. At the time of writing of the study protocol in 2014/2015, the role of PD-L1 expression on clinical decision making was still under debate and not yet accessible in the clinical setting. The distribution of PD-L1 expression between the control arm and the experimental arm was skewed, leading to a higher number of patients with high PD-L1 expression, i.e. ≥50%, in the experimental arm at the expense of PD-L1 negative tumors, i.e. 0%. Fortunately, in our pooled analysis this imbalance in PD-L1 distribution between arms was corrected. Subsequently, no association between PD-L1 expression or benefit of pembrolizumab combined with RT could be established. Although comparison between RT regimens was limited by confounding, the baseline characteristics between the control and experimental arm of the overall intention-to-treat (ITT) population were well balanced, making these interpretations statistically sound.

Although these data suggest that RT is able to augment systemic immunotherapy responses and improve outcomes for patients with advanced NSCLC, these results are not yet convincing enough to change clinical decision making. Not only will we need more data on the optimal timing of application of RT and start of immunotherapy, selection of number and location of RT lesions and ideal RT regimen, but also what tumor and/or patient characteristics are more prone to benefit from this combination. One of the special requirements of this study was the collection of tumor biopsies before and after 6 weeks of therapy of non-irradiated tumor lesion. Ongoing translational research of blood and tumor samples collected during the trial will hopefully bring insights in associations between tumor and/or patient characteristics and abscopal benefit. Whole exome sequencing (WES) of all baseline tumor samples is currently obtained for determination of TMB, identification of possible neoantigens involved in abscopal responses, enrichment in mutation pathways, clonal composition and other gene alterations. T cell receptor (TCR) sequencing will be performed of matched baseline and on-treatment samples collected from non-irradiated tumor lesions to evaluate broadening of the TCR repertoire during treatment to allow

identification of a true antitumor immune response compared to overall induction of inflammation by either treatment regimens, i.e. RT or pembrolizumab, and compare the amplitude between responders in the control arm to responders in the experimental arm. Also, may sufficient material remain, RNA sequencing will be performed of these matched samples to explore pathway activation and the evolution of the composition of the immune infiltrate during treatment. This can be compared to the changes in T cell subsets and up- or downregulation of several immune checkpoints within the tumor immune microenvironment as assessed by multiplex immunohistochemistry (mIHC) using the Vectra technology. Patterns of circulating tumor DNA will be analyzed to explore differences between arms and between responders and non-responders and relate these to the sequencing data. Peripheral blood mononuclear cells (PBMCs) have been collected during treatment and will be used to perform functional neoantigen screens by pulse autologous T cells based on WES and TMB. A proteomics profile in plasma able to predict response on anti-PD-1 treatment in advanced melanoma patients has been established [30]. This was later also shown in advanced NSCLC [31, 32]. This proteomics signature will be determined at baseline and after application of RT, but before first pembrolizumab dosage, to explore whether RT would be able to transform a previous 'resistant' tumor into a 'sensitive' one. Lastly, baseline and on-treatment imaging will be assessed through a radiomics analysis pipeline possible enhancing our understanding of response to treatment, tumor heterogeneity and tumor immune microenvironment additionally to the already mentioned analyses of more invasively obtained materials. Hopefully these results may guide us how to proceed clinical implementation of the abscopal phenomenon.

Future perspectives on the investigation of abscopal responses

Due to the success of PD-1/PD-L1 inhibition in advanced NSCLC, immunotherapy also found its way in the treatment of earlier and therefore curable stages of disease. In 2018, the PACIFIC-trial showed improvement of PFS and OS from one-year adjuvant durvalumab over placebo for patients that had not developed progression after treatment with concurrent chemoradiation (CCRT) for locally advanced irresectable NSCLC [33]. This adjuvant treatment is now the first application of immunotherapy as SoC in earlier stage NSCLC. Also, the concurrent administration of nivolumab with CCRT proved feasible and safe based on results from a formal interim safety analysis in the NICOLAS-trial [34]. Two courses of neo-adjuvant nivolumab in resectable NSCLC was also deemed safe and showed major pathological responses (MPR) grossly irrespective of pre-operative radiologic assessments [35]. Preliminary results of ongoing neo-adjuvant phase II trials with immunotherapy previous to resection also showed a beneficial safety profile, discordant radiological and pathological responses and a presumably higher MPR rate and memory TILs induction of the combination of ipilimumab with nivolumab over nivolumab alone [36, 37].

Many trials are currently ongoing exploring the safety and efficacy of adjuvant treatment with PD-1/PD-L1 blockade after resection, SBRT and CCRT. Clinical outcomes of trials investigating concurrent application of immune checkpoint inhibition with CCRT are awaited as well. The Induction trial, currently running at the Netherlands Cancer Institute (NKI) is the only study to date evaluating the safety of neo-adjuvant treatment of immunotherapy -dual checkpoint inhibition with durvalumab and tremelimumab- in locally advanced CCRT setting (NCT04287894). Also, further exploration of neo-adjuvant treatment

in resectable disease will provide us with improved insights in the antitumor effects of immunotherapy, but may provide improvements in patient outcomes as well. Combining immunotherapy -two doses of pembrolizumab- with SBRT and comparing this regimen to either treatment alone in neo-adjuvant resectable setting, as is performed in the NKI-based trial NCT03446911, will allow to investigate loco-regional pathological investigation as well as systemic immune responses.

In the current treatment landscape of advanced NSCLC, investigation of underlying mechanisms and tumor-immune interactions for abscopal responses by the addition of RT to immunotherapy has become challenging. PD-1/PD-L1 blockade is now combined with platinum-doublet chemotherapy and only in a specific already immunogenic subgroup of tumors with PD-L1 expression of ≥50% monotherapy with immune checkpoints remains an option. It is difficult, maybe even impossible to date, to establish whether response is attributable to chemotherapy alone, immunotherapy alone or the combination specifically. Advancements of the role of immunotherapy into earlier stages of disease might provide opportunities to further explore the combination of RT with checkpoint inhibition. A challenging factor in stage I-III disease is that all (known) tumor lesions receive ablative local therapy with either resection or RT, thereby disabling exploration of a possible off-target invigorated antitumor immune response. However, in resectable stage with more than one tumor location, i.e. a primary tumor with N1 or uni-level N2 disease, a neo-adjuvant design with a combination of immunotherapy and RT could be proposed. By only radiating the primary tumor, evaluating an off-target effect in lymph node metastases would remain possible and a subsequent resection would be yield material for elaborate translational research. Still, in advanced NSCLC, an interesting approach of investigating the RT-immunotherapy combination could be performed in second-line setting as a means to overcome primary or secondary resistance to immunotherapy. Re-invigoration of responses to immunotherapy through the addition of RT after development of secondary resistance have been described [38, 39]. In this setting, a multi-arm optimal dose/fractionation-finding study could be performed; also, a comparison of monotherapy or combinations of immune modulating systemic treatment with RT could be tested this way.

Hopefully, the translational endeavors from the PEMBRO-RT trial may bring useful insights and biomarkers to better identify the abscopal effect in a clinical setting. These may assist us in optimization further research protocols investigating the possible advantage of adding RT to systemic treatment like immunotherapy in particular in NSCLC.

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SUMMARY

Since long, the interaction between the host immune system and tumor growth/control has been of interest within the oncology research field. Unraveling the tumor immune microenvironment has led to significant new insights. One of the most studied tumor immune escape mechanisms is mediated through the inhibitory programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway. Binding of tumor-expressed PD-L1 to the PD-1 receptor on cytotoxic T cells activates an inhibitory signal leading to apoptosis or inactivation of the immune cells and thereby allowing the tumor to evade the host immune response. Development of immune checkpoint inhibitors (ICIs), e.g. PD-1/PD-L1 monoclonal antibodies, has led to long-lasting anti-tumor immune responses in patients with metastatic non-small cell lung carcinoma (NSCLC).

Overall response rates (ORR) and other patient outcomes are associated with the protein expression level of PD-L1 on tumors as assessed by immunohistochemistry (IHC) and is therefore widely used as a predictive clinical biomarker for response on PD-1/PD-L1 blockade. However, patients with PD-L1 negative tumors still have an 8% chance of response and even at the highest expression level, i.e. ≥ 50%, a 70% failure rate occurs. These outcomes show that the treatment option immuno-monotherapy as well as the biomarker PD-L1 expression both remain far from optimal.

This thesis sought to obtain a better understanding of the composition of the immune microenvironment and its interaction with NSCLC. Also, improvement in NSCLC patient outcomes was aspired by combining ICIs with a potential immune modulator: radiation therapy (RT). In **chapter 1**, a general introduction on the epidemiology, treatment options and possible biomarkers for immunotherapy in NSCLC are presented. Furthermore, a short description of the abscopal effect of RT, the out-of-field or systemic antitumor response after local radiation, and the biological rationale of augmented immune responses by combining RT with ICIs are given.

PART I. Exploring the tumor immune microenvironment

To enable exploration of the tumor immune environment in NSCLC, we built a database of over 600 tumor samples from resected patients and collected patient' and tumor characteristics. IHC staining of PD-L1 expression on tumor cells (TC) and tumor-infiltrating immune cells (IC) separately and of CD8 infiltration, mutational data, and gene expression mainly of genes related to immunologic function were obtained.

In **chapter 2**, we sought to improve insights in the associations of PD-L1 expression and specific patient' or tumor characteristics. Only for PD-L1 positive TC associations were found (*KRAS* mutations and smoking), but none were found for PD-L1 expression on IC. We then explored overlap and differences between expression of PD-L1 on TC and IC. Our interest was raised by the fact that more than half of the cohort had a PD-L1 positive immune infiltrate, which was associated with other immune gene markers, but without upregulation of PD-L1 on TC. In a subsequent analysis, we found that in the subgroup of TC0/IC3 samples an impairment of IFNγ response in the TC might be responsible for the lack of upregulation of PD-L1 in these tumors. These findings may contribute to the understanding why

patients with PD-L1 TC negative NSCLC may still (only) require immune checkpoint blockade to gain response and survival benefit.

In **chapter 3**, we investigated immune gene expression by NanoString using the same resection cohort of early-stage NSCLC. We found that in adenocarcinomas (AD) the level of overall inflammation as assessed by immune gene expression was significantly higher compared to squamous cell carcinomas (SCC). This seemed to be related to a higher infiltration rate of immune cells within the tumor bed of AD compared to SCC based on a significant difference in tumor cell percentage between both histologies, i.e. tumor cell percentage being higher in SCC. This may suggest a different interaction of immune cells and tumor cells between the different histologies. Interestingly, a cluster of 34 genes, identified by unsupervised clustering, did not correlate with the general level of inflammation, the level of PD-L1 expression or $CD8^+$ T cell infiltration. Expression of this 34-gene cluster did not differ between AD and SCC histology, but high expression of this signature showed a clear OS benefit in SCC, but not in AD. This finding was validated in two independent NSCLC cohorts. We then tried to allocate the nature of this 34-gene signature and found the strongest correlation with Natural Killer (NK) cell related gene expression. Cell surface genes involved in NK cell recognition and killing - *ULBP2* and *HLA-C* – were significantly different between SCC and AD histology in favor of our hypothesis, namely that SCC may be more susceptible to NK cell killing than AD. Unfortunately, there is no established gold standard for assessing NK cell infiltration and/or activation level in tumor samples. Also, these cells are generally scarce within the tumor microenvironment and our IHC NK cell double-staining was not able to differentiate the 34-gene high from the 34-gene low samples. Almost all genes within our 34-gene signature could not be adequately measured by RNA sequencing techniques due to low expression levels of the genes in the signature. The further investigation of the biological rationale of such low-level signatures could bring new insights in the role of immune cells that are present within the tumor infiltrate in low quantities, like NK cells.

PART II. Modulating the tumor immune microenvironment

Although the improvements of patient outcomes by the introduction of PD-1/PD-L1-antibodies in advanced NSCLC have been impressive, there is still an urgent need for further investigation, especially for those patients not responding to immune checkpoint blockade. RT could be a potent modulator of the tumor microenvironment and could augment the antitumor immune response when combined with immunotherapy.

In **chapter 4**, we provided a review about the off-target effects of RT, the so-called abscopal effect. We describe the biological rationale how RT may counteract the mechanisms of failure of immunotherapy. Also, an oversight of pre-clinical and clinical data supporting augmentation of abscopal events by RT when combined with immune checkpoint inhibition is presented.

To investigate this possible clinical impact of the abscopal phenomenon, the PEMBRO-RT trial was set up. In this multicenter study, patients with advanced NSCLC that had received at least one prior line of chemotherapy but were immunotherapy-naïve, were randomized between pembrolizumab treatment (control arm) vs pembrolizumab treatment within one week after three doses of 8 Gy (24Gy/3) on a

single tumor lesion (experimental arm). Stratification was based on smoking status: <10 pack years vs ≥10 pack years. The primary end point was ORR at 12 weeks from randomization according to Response Evaluation Criteria in Solid Tumors (RECIST). The results of the PEMBRO-RT trial are presented in **chapter 5**. The intention-to-treat (ITT) population consisted of 76 patients. Although the ORR at 12 weeks doubled in the experimental arm compared to the control arm, this difference was not statistically significant. The PD-L1 negative subgroup experienced a significant PFS and OS benefit in the experimental arm compared to the control arm, but no differences were seen in the overall ITT population regarding these outcomes. No increase in treatment-related toxicity was observed in the experimental arm. So, although an augmenting effect of RT on the response to PD-1 blockade in patients with metastatic NSCLC was observed, the study did not meet its primary end point of prespecified criteria for meaningful clinical benefit.

The MD Anderson Cancer Center (MDACC) was analyzing results from a similar randomized trial of pembrolizumab alone vs pembrolizumab in combination with RT (50Gy/4, stereotactic body radiotherapy (SBRT) or 45Gy/15, traditional RT). In **chapter 6**, we present the results of the pooled analysis of these two randomized trials. By exploring the possible abscopal effect in a larger cohort of advanced NSCLC patients, we found not only a significant improvement of abscopal response rate (ARR) in the experimental arm compared to the control arm, but also a significant PFS and OS benefit was observed in the patients treated with pembrolizumab and RT. Because RT regimen was not applied randomly, but rather based on trial variability and/or physicians' discretion, statistical comparison between RT regimens was not feasible. However, the 45Gy/15 subgroup showed an ARR similar to the control arm, where the other two RT regimens produced an ARR over two times as high. Exploration of the absolute lymphocyte count (ALC) showed a more pronounced ALC decline in the 45Gy/15 subgroup in comparison to both other RT regimens, which provides a hypothesis of a detrimental effect on immune response by traditional fractionation that may require further investigation.

Future perspectives

Finally, **chapter 7** provides a short summary of the recent developments in the systemic treatment of advanced NSCLC and a general discussion on the previously described findings in this thesis is given. Alternative predictive biomarkers for response to ICIs are currently under investigation, but although promising, as of yet none of these biomarkers have proven themselves more accessible and/or reliable compared to PD-L1 expression on tumor cells by IHC. Further research will be needed to improve (first-line) treatment selection in advanced NSCLC patients. Also, an oversight of ongoing translational research on the blood and tumor samples collected during the PEMBRO-RT trial is presented in this chapter. Hopefully, these will bring further insights in associations between tumor and/or patient characteristics and abscopal benefit and therefore guide us how to proceed clinical implementation of the abscopal phenomenon. Furthermore, endeavors implementing the use of immunotherapy within earlier stages of NSCLC are ongoing. In locally advanced stage III disease, where immunotherapy is combined with concurrent chemoradiation might provide opportunities to further explore the combination of RT with checkpoint inhibition. Also, neo-adjuvant treatment of immunotherapy in resectable disease will allow to investigate loco-regional pathological investigation as well as systemic immune responses

and may aid in biomarker development. And also applying neo-adjuvant RT in this setting may bring useful insights and biomarker assessment to better identify the abscopal effect in a clinical setting. These may assist us in optimization further research protocols investigating the possible advantage of adding RT to systemic treatment like immunotherapy in particular in NSCLC.

SAMENVATTING

De interactie tussen het immuunsysteem van de gastheer en de groei/controle van tumoren is sinds lange tijd een belangrijk focus van onderzoek binnen de oncologie. Het ontrafelen van het immunologisch micromilieu van tumoren heeft tot belangrijke nieuwe inzichten geleid. Een van de meest bestudeerde mechanismen van het ontsnappen van tumorcellen aan het afweersysteem wordt gemedieerd via de inhiberende programmed death-ligand 1 (PD-L1)/programmed death 1 (PD-1) pathway. Binding van de door de tumorcellen tot expressie gebrachte eiwit PD-L1 aan de PD-1-receptor op cytotoxische T-cellen activeert een remmend signaal leidend tot apoptose of inactivering in deze T-cellen. Hierdoor kan de tumor de immuunrespons van de gastheer ontwijken. Ontwikkeling van immuun checkpoint-remmers (ICI's), zoals monoklonale antilichamen tegen PD-1/PD-L1, hebben bij patiënten met gemetastaseerd niet-kleincellig longcarcinoom (NSCLC) geleid tot langdurige immuunresponsen tegen de tumor.

Algemene responspercentages (ORR) en andere patiëntgebonden uitkomsten op behandeling met PD-1/PD-L1-gerichte antilichamen zijn geassocieerd met het expressieniveau van het eiwit PD-L1 op tumoren beoordeeld door middel van immunohistochemie (IHC) en wordt daarom veel gebruikt als een voorspellende klinische biomarker voor respons op PD-1/PD-L1 blokkade. Patiënten met PD-L1-negatieve tumoren hebben echter nog steeds een kans van 8% op respons en zelfs bij het hoogste expressieniveau, namelijk ≥50%, is het percentage van falen van de therapie rond de 70%. Deze resultaten laten zien dat de behandelingsoptie immunotherapie als monotherapie en de biomarker PD-L1-expressie beiden verre van optimaal zijn.

In dit proefschrift werd getracht een beter begrip te krijgen van de samenstelling van het immunologisch micromilieu van tumoren en de interactie hiervan met NSCLC. Ook werd gestreefd naar verbetering van de patiëntgebonden uitkomsten bij gemetastaseerd NSCLC door ICI's te combineren met een potentiële immuunmodulator: radiotherapie (RT). In **hoofdstuk 1** wordt een algemene inleiding gegeven over de epidemiologie, behandelingsmogelijkheden en mogelijke biomarkers voor immunotherapie bij NSCLC. Verder wordt een korte beschrijving gegeven van het abscopal effect van RT (de out-of-field oftewel systemische antitumorrespons na lokale bestraling) en de biologische rationale van het versterken van de immuunrespons door RT te combineren met ICI's.

DEEL I. Het exploreren van het immunologisch micromilieu van tumoren

Om het exploreren van het immunologisch micromilieu van tumoren in NSCLC mogelijk te maken, hebben we een database met meer dan 600 tumormonsters van patiënten, die geopereerd zijn, met bijbehorende patiënt- en tumorkarakteristieken opgezet. IHC-kleuringen van afzonderlijk PD-L1 op tumorcellen (TC) en tumor-infiltrerende immuuncellen (IC) werd verkregen. Daarnaast werd CD8-infiltratie op basis van IHC-kleuring, mutatiegegevens en genexpressie van voornamelijk van immuungerelateerde genen verzameld.

In **hoofdstuk 2** hebben we getracht de inzichten in de associaties van PD-L1-expressie en specifieke patiënt- of tumorkarakteristieken te verbeteren. Alleen voor PD-L1-expressie op TC werden positieve

associaties gevonden (KRAS-mutaties en roken), maar geen voor PD-L1-expressie op IC. Vervolgens hebben we de overlap en verschillen tussen expressie van PD-L1 op TC en IC onderzocht. Opvallend was het feit dat meer dan de helft van het cohort een PD-L1-positief immuuninfiltraat bevatte, dat ook geassocieerd was met de expressie van andere immuun(gen)markers, maar zonder opregulatie van PD-L1 op TC. In een aanvullende analyse ontdekten we dat in de subgroep van TC0/IC3-monsters een verminderde IFNγ-respons in de TC verantwoordelijk zou kunnen zijn voor het gebrek aan opregulatie van PD-L1 in deze tumoren. Deze bevindingen kunnen bijdragen aan het begrip waarom patiënten met een PD-L1 TC-negatief NSCLC mogelijk nog steeds baat kunnen hebben van behandeling met (alleen) ICI's qua respons en overlevingsvoordeel.

In **hoofdstuk 3** hebben we genexpressie van immuungerelateerde genen door middel van NanoString onderzocht in hetzelfde vroegstadium NSCLC-resectiecohort. We ontdekten dat in het adenocarcinoom (AD) het niveau van algehele inflammatie op basis van immuungenexpressie significant hoger was in vergelijking met het plaveiselcelcarcinoom (SCC). Dit leek verband te houden met een hogere infiltratie van immuuncellen in het tumorbed van AD vergeleken met SCC gezien een significant verschil in tumorcelpercentage tussen beide histologieën, dus een hoger tumorcelpercentage in SCC. Dit kan een verschil in interactie suggereren tussen immuuncellen en tumorcellen tussen de verschillende histologieën. Opvallend was dat een cluster van 34 genen, geïdentificeerd door unsupervised clustering, niet correleerde met het algemene level van inflammatie, de mate van PD-L1-expressie of infiltratie van CD8+ T-cellen. De hoogte van expressie van dit 34-genencluster verschilde niet tussen AD en SCC, maar hoge expressie van deze genetische signature toonde een duidelijk OS-voordeel in SCC, maar niet in AD. Deze bevinding werd gevalideerd in twee onafhankelijke NSCLC-cohorten. Vervolgens probeerden we de aard van deze 34-genensignature te alloceren en vonden daarbij de sterkste correlatie met Natural Killer (NK) cel-gerelateerde genexpressie. Celoppervlakgenen die betrokken zijn bij NK-celherkenning en -doding - *ULBP2* en *HLA-C* - waren significant verschillend tussen SCC en AD ten gunste van onze hypothese, namelijk dat SCC gevoeliger is voor NK-celdoding dan AD. Helaas is er geen vastgestelde gouden standaard voor het beoordelen van NK-celinfiltratie en/of activeringsniveau in tumormonsters. Ook zijn deze cellen over het algemeen schaars binnen het immunologisch micromilieu van tumoren. Onze NK-cel-dubbelkleuring middels IHC was niet in staat om de '34-gen high' te onderscheiden van de '34-gen low' monsters. Bijna alle genen binnen onze 34-genensignature konden niet adequaat worden gemeten met RNA-sequencing technieken vanwege de lage expressieniveaus van de genen in de signature. Verder onderzoek naar de biologische rationale van dergelijke lage-expressie signatures kan nieuwe inzichten opleveren in de rol van immuuncellen, zoals NK-cellen, die in kleine hoeveelheden in het tumorinfiltraat aanwezig zijn.

DEEL II. Het moduleren van het immunologisch micromilieu van tumoren

Hoewel de verbeteringen van de patiëntgebonden resultaten door de introductie van PD-1/PD-L1-antilichamen in gemetastaseerd NSCLC indrukwekkend zijn, is er nog steeds dringend behoefte aan verder onderzoek, vooral voor patiënten die niet reageren op blokkade van immune checkpoints. RT zou een krachtige modulator van het immunologische micromilieu van tumoren kunnen zijn en zou de

immuunrespons tegen tumoren kunnen versterken wanneer deze behandeling gecombineerd wordt met immunotherapie.

In **hoofdstuk 4** wordt een overzicht gegeven van de off-target effecten van RT, het zogenaamde abscopal effect. We beschrijven de biologische rationale hoe RT de mechanismen van falen van immunotherapie kan tegengaan. Ook wordt een overzicht gegeven van preklinische en klinische data die de mogelijke augmentatie van het abscopal effect door RT ondersteunen met name in combinatie met remming van immune checkpoints.

Om de mogelijke klinische impact van het abscopal fenomeen te onderzoeken, werd de PEMBRO-RT-studie opgezet. In deze multicenter studie werden patiënten met gemetastaseerd NSCLC die ten minste één eerdere chemotherapiebehandeling hadden ondergaan maar die niet eerder waren behandeld met immunotherapie, gerandomiseerd tussen behandeling met pembrolizumab (controle-arm) versus behandeling met pembrolizumab binnen een week na drie doses van 8 Gy (24 Gy/3) bestraling op een enkele tumorlaesie (experimentele arm). Er werd gestratificeerd op rookstatus: <10 pakjaren versus ≥10 pakjaren. Het primaire eindpunt was ORR op 12 weken na randomisatie volgens Response Evaluation Criteria in Solid Tumors (RECIST). De resultaten van de PEMBRO-RT-studie worden gepresenteerd in **hoofdstuk 5**. De intent-to-treat (ITT)-populatie bestond uit 76 patiënten. Hoewel de ORR na 12 weken verdubbelde in de experimentele arm ten opzichte van de controlegroep, was dit verschil niet statistisch significant. De PD-L1-negatieve subgroep had een significant PFS- en OS-voordeel in de experimentele arm ten opzichte van de controlegroep, maar er werden geen verschillen gezien in de totale ITT-populatie met betrekking tot deze uitkomsten. Er werd geen toename in behandelgerelateerde toxiciteit waargenomen in de experimentele arm. Dus hoewel een versterkend effect van RT op de respons op PD-1-blokkade bij patiënten met gemetastaseerd NSCLC werd waargenomen, voldeed de studie niet aan het primaire eindpunt van vooraf gespecificeerde criteria voor zinvol klinisch voordeel.

Het MD Anderson Cancer Center (MDACC) analyseerde de resultaten van een vergelijkbare gerandomiseerde studie met alleen pembrolizumab versus pembrolizumab in combinatie met RT (50Gy/4, stereotactic body radiotherapy (SBRT) of 45Gy/15, traditionele RT). In **hoofdstuk 6** presenteren we de resultaten van de gepoolde analyse van deze twee gerandomiseerde studies. Door het mogelijke abscopal effect in een groter cohort van gemetastaseerd NSCLC-patiënten te onderzoeken, vonden we niet alleen een significante verbetering van het abscopal responspercentage (ARR) in de experimentele arm ten opzichte van de controle-arm, maar werd ook een significant PFS- en OS-voordeel waargenomen in de patiënten behandeld met pembrolizumab en RT. Omdat het gekozen RT-regime niet gerandomiseerd was toegepast, maar gebaseerd op de variabiliteit per studie en/of per discretie van de behandelend arts, was een statistische vergelijking tussen RT-regimes niet haalbaar. De 45Gy/15-subgroep vertoonde echter een ARR vergelijkbaar met de controle-arm, waar de andere twee RT-regimes een ARR van meer dan tweemaal zo hoog lieten zien. Exploratie van het absolute aantal lymfocyten (ALC) toonde een meer uitgesproken daling van de ALC in de 45Gy/15-subgroep ten opzichte van beide andere RT-regimes, wat een hypothese geeft van een nadelig effect op de immuunrespons door traditionele fractionering die mogelijk verder onderzoek behoeft.

Toekomstperspectieven

Hoofdstuk 7 geeft tenslotte een korte samenvatting van de recente ontwikkelingen in de systemische behandeling van gemetastaseerd NSCLC en daarnaast een algemene discussie over de eerder beschreven bevindingen in dit proefschrift. Alternatieve voorspellende biomarkers voor respons op ICI's, die momenteel worden onderzocht lijken veelbelovend, maar hebben tot nu toe niet bewezen toegankelijker en/of betrouwbaarder te zijn dan de biomarker PD-L1-expressie op tumorcellen middels IHC. Verder onderzoek zal nodig zijn om de (eerstelijns) behandelingsselectie bij gemetastaseerde NSCLC-patiënten te verbeteren. In dit hoofdstuk wordt ook een overzicht gegeven van lopende translationele onderzoeken van de bloed- en tumormonsters die tijdens de PEMBRO-RT-studie zijn verzameld. Hopelijk zullen deze verder inzicht opleveren in de associaties tussen tumor- en/of patiëntkarakteristieken en het voordeel van een abscopal effect en ons begeleiden bij verdere klinische implementatie van het abscopal fenomeen. Bovendien zijn er inspanningen gaande om het gebruik van immunotherapie in vroegere stadia van NSCLC toe te passen. Bij lokaal gevorderde stadium III-ziekte, waarbij immunotherapie wordt gecombineerd met gelijktijdige chemoradiatie, kunnen mogelijkheden worden geboden om de combinatie van RT met checkpoint-remming verder te onderzoeken. Ook zal neo-adjuvante behandeling van immunotherapie bij resectabele ziekte het mogelijk maken om loco-regionaal pathologisch onderzoek en systemische immuunreacties te onderzoeken en kan het ondersteunen bij de verdere ontwikkeling van biomarkers. En ook het toepassen van neo-adjuvante RT in deze setting kan nuttige inzichten opleveren om het abscopal effect in een klinische setting beter te identificeren. Deze ontwikkelingen kunnen ons helpen bij het optimaliseren van verder onderzoek naar het mogelijke voordeel van het toevoegen van RT aan systemische behandeling zoals immunotherapie in NSCLC.

PART IV.

Appendices

LIST OF PUBLICATIONS

This thesis

W.S.M.E. Theelen, D. Chen, V. Verma, B. Hobbs, H.M.U. Peulen, J.G.J.V. Aerts, I. Bahce, A.N. Niemeijer, J.Y. Chang, P.M. de Groot, Q. Nguyen, N.I. Comeaux, G.R. Simon, F. Skoulidis, S.H. Lin, K. He, R. Patel, J. Heymach, P. Baas, J.W. Welsh. Pembrolizumab with and without radiotherapy for metastatic non-small cell lung cancer: pooled analysis of two randomized trial. *Accepted for publication in The Lancet Respiratory Medicine.*

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CURRICULUM VITAE

Willemijn Theelen was born on July 20th 1981 in Leiden, The Netherlands. In 1999, she graduated cum laude at the Stedelijk Gymnasium in Leiden. In the same year, she started her medical studies at the Rijks Universiteit Groningen. In April 2006 she graduated as a medical doctor.

Subsequently, she worked as a resident at the pulmonary department of the Onze Lieve Vrouwe Gasthuis in Amsterdam, the pulmonary, cardiology and internal medicine department of the Zaans Medisch Centrum and at the pulmonary department of the Medisch Centrum Alkmaar. In the latter, she started her training as a chest physician in April 2008 and finished this training in September 2014. In October, she continued her work as a fellow thoracic oncology at the Antoni van Leeuwenhoek–Nederlands Kanker Instituut (AvL-NKI), combining her clinical work with a PhD trajectory. In April 2018, she gained a permanent position as a chest physician with a focus on immunotherapy in non-small cell lung cancer.

DANKWOORD

Heel hartelijk dank voor:

- de inhoudelijke input betreffende dit proefschrift
- de ondersteuning in mijn leercurve qua preklinische en translationele kennis
- de fraaie figuren in dit proefschrift
- de organisatorische ondersteuning met betrekking tot de (klinische) studies
- het plezier van multidsciplinair samenwerken
- het creëren van de gelegenheid om mijn proefschrift te mogen verdedigen
- het doorstaan van mijn eeuwige gemopper
- alle gezamelijke sociale activiteiten gedurende de afgelopen zeven jaar die niets met promoveren te maken hadden
- de steun om de motivatie te vinden om door te zetten
- de waardevolle gesprekken over niets en alles

In het bijzonder wil ik de patienten en hun naasten bedanken die bijgedragen hebben aan het totstandkomen van dit proefschrift; soms zelfs zonder direct voordeel hebben zij in een beladen levensperiode de moeite genomen om ons te helpen onze kennis over longkanker te vergroten.