Learning from the Past

Optimizing Future Use of Immune Checkpoint Inhibitors in Advanced Non-Small Cell Lung Cancer

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BY
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ENGLISH SUMMARY

In Denmark, approximately 5000 patients are diagnosed with lung cancer each year. The histopathological subgroup of non-small cell lung cancer (NSCLC) accounts for 80% of the cases, and half of the patients have incurable metastatic disease at diagnosis. Since 2015, immune checkpoint inhibitors (ICIs) have revolutionized the treatment of patients with advanced NSCLC, without targetable molecular alterations, based on improved response rates and overall survival (OS) in randomized controlled trials (RCTs). However, subgroups of patients with poor performance status (PS), high age and comorbidity are underrepresented in the RCTs but frequently treated in daily clinical cancer care. Therefore, study I and II investigated the ICI efficacy in patients with advanced NSCLC treated in daily clinical care. The selection of patients for ICI-treatment relies on the programmed death-ligand 1 (PD-L1) tumor proportion score, but the predictive value of PD-L1 is limited and complementary biomarkers are warranted. Therefore, study III investigated new predictive biomarkers of ICI efficacy.

Paper I describes study I. In the nationwide study I, the impact of ICI-implementation on OS and the efficacy of first-line (1L) ICI was assessed in Danish patients treated in daily clinical cancer care. The median OS (mOS) increased with 5.2 months and the 3-year OS rate increased from 6% to 18% after ICI-implementation. However, in ICI-treated patients, the 3-year OS rate was 29%. In a cohort of 579 patients treated with 1L ICI. The mOS of 18.3 months was lower than demonstrated in the RCTs. PS ≥1, bone metastases, and liver metastases were significantly associated with impaired OS. Around one fifth of the patients discontinued ICI due to early progressive disease (PD) within approximately 4 months.

Paper II describes study II. In the nationwide study II, the efficacy of second- or subsequent line ICI was assessed in 840 Danish patients treated in daily clinical cancer care. PS ≥1, male sex, bone metastases, and liver metastases were significantly associated with impaired OS. The mOS of 12.2 months was comparable to that demonstrated in the RCTs. As shown in study I, around one fifth of the patients discontinued ICI due to early PD within approximately 4 months.

Paper III describes study III. In study III, potential predictive baseline characteristics and gene expression profiling (GEP) of durable clinical benefit (DCB) was assessed. DCB was observed in half of the 123 included ICI-treated patients. Absence of liver metastases and high absolute lymphocyte count (ALC) were associated with DCB, and an ALC above 1.0 10^9/l may be predictive of DCB. GEP-assessed PD-L1 correlated strongly with PD-L1 assessed by immunohistochemistry and with treatment line. JAK/STAT loss signature scores were higher in patients with DCB and dendritic cell, myeloid, and TGF-β signature scores were higher in patients without DCB.

In conclusion, this dissertation provides information on ICI efficacy in a nationwide cohort of Danish patients with advanced NSCLC treated with ICI in daily clinical cancer care. The OS increased after the implementation of ICIs in Denmark. Furthermore, the survival was comparable to or slightly lower than the survival demonstrated in the RCTs, but subgroups of patients with poor PS, bone-, and liver metastases may not derive benefit. High ALC was associated with DCB, but the predictive value should be assessed in larger independent cohorts. GEP could be clinically relevant in PD-L1 assessment and four gene expression signatures were associated with DCB. However, the GEP-cohort was small, and the findings should be investigated in a larger prospective study.

DANSK RESUME

I Danmark bliver omkring 500 patienter diagnosticeret med lungekræft hvert år. Den histopatologiske subgruppe af ikke-småcellet lungekræft (NSCLC) udgår 80% af tilfældene og halvdelen af patienterne har metastatisk sygdom på diagnosetidspunktet. Siden 2015 har immune checkpoint hæmmere (ICI) revolutioneret behandlingen af patienter med avanceret NSCLC, uden targetérbare mutationer, baseret på forbedrede responsrater og overlevelse (OS) i randomiserede kontrollerede forsøg (RCT). Der er dog subgrupper af patienter med dårlig performance status (PS), høj alder og komorbiditet som ofte er underrepræsenterede i RCT'erne, men udgør en stor andel af de patienter som behandles i daglig klinisk praksis. Derfor har vi i studie I og II undersøgt effekten af ICI hos patienter med avanceret NSCLC som er behandlet i dagligt klinisk praksis. Udvælgelsen af patienter til behandling med ICI er baseret på programmed death-ligand 1 (PD-L1) tumor proportion score, men den prædiktive værdi af PD-L1 er begrænset og supplerende biomarkører er nødvendige. Derfor har vi i studie III undersøgt nye prædiktive biomarkører for effekten af ICI.

Artikel I beskriver studie I. Studie I var et nationalt studie hvor vi undersøgte betydningen af ICI-implementering for OS og undersøgte effekten af første linje (1L) ICI hos danske patienter behandlet i daglig klinisk praksis. Den mediane OS (mOS) steg med 5.2 måneder (mdr) og 3-års OS raten steg fra 6% til 18% efter ICI-implementering. Hos patienter behandlet med ICI var 3-års OS raten dog 29%. I en kohorte på 579 patienter behandlet med 1L ICI var PS ≥1, knogle- og lever metastaser signifikant associeret med forringet overlevelse. Den mediane OS på 18.3 mdr var kortere end demonstrereret i RCT'erne. Omkring en femtedel af patienterne stoppede ICI på grund af tidlig progression indenfor cirka 4 mdr.

Artikel II beskriver studie II. Studie II var et nationalt studie med 840 danske patienter hvor vi undersøgte effekten af ICI givet i anden eller senere behandlingslinje i daglig klinisk praksis. OS var signifikant forringet hos patienter med PS ≥1, knogle- og levermetastaser og hos mænd. Den mediane OS på 12.2 mdr var sammenlignelig med den som blev demonstreret i RCT'erne. Som vist i studie I, stoppede omkring en femtedel af patienterne deres ICI på grund af tidlig progression indenfor cirka 4 mdr.

Artikel III beskriver studie III. I studie III undersøgte vi potentielt prædiktive baseline karakteristika og gen ekspressions profilering (GEP) for durable clinical benefit (DCB). Halvdelen af de 123 inkluderede ICI-behandlede patienter havde DCB. Fravær af lever metastaser og højt absolut lymfocyttal (ALC) var associeret med DCB, og ALC over 1.0 10^9/l er muligvis prædiktivt for DCB. GEP-bestemt PD-L1 var stærkt korreleret med PD-L1 bestemt ved immunhistokemi og med behandlingslinje. JAK/STAT loss signatur scores var højere hos patienter med DCB og dendrit celle, myeloid og TGF-β signatur scores var højere hos patienter uden DCB.

Afslutningsvis bidrager denne afhandling med information om effekten af ICI i en national kohorte af danske patienter med avanceret NSCLC behandlet med ICI i daglig klinisk praksis. Den samlede overlevelse steg efter implementering af ICI-behandling. Desuden var overlevelsen i vores studier sammenlignelig med eller lidt dårligere end overlevelsen i RCT'erne, men subgrupper af patienter med dårlig PS, knogle- og lever metastaser har muligvis ikke glæde af ICI-behandling. Højt ALC var associeret med DCB, men den prædiktive værdi bør undersøges i større uafhængige kohorter. GEP kan være klinisk relevant til bestemmelse af PD-L1 og fire gen ekspressions-signaturer var associeret med DCB. GEP-kohorten var dog lille og resultaterne bør undersøges i et større prospektivt studie.

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ABBREVIATIONS

1L	First-line
2L/≥2L	Second-line/ second- or subsequent line
ALC	Absolute lymphocyte count
ALK	Anaplastic lymphoma kinase
ANC	Absolute neutrophil count
BMI	Body mass index
BSC	Best supportive care
CCIS	Charlson Comorbidity Index score
CI	Confidence interval
CPR	Danish Civil Registration
CR	Complete response
CRT	Chemoradiotherapy
CTCAE	Common terminology criteria for adverse events
CTx	Chemotherapy
DC	Dendritic cell
DCB	Durable clinical benefit
DLCR	Danish Lung Cancer Registry
DOLG	Danish Oncology Lung cancer Group
ECOG	Eastern Cooperative Oncology Group
EGFR	Epidermal growth factor receptor
EHRs	Electronic health records
FDA	U.S. Food and Drug Administration
FDR	False discovery rate
FFPE	Formalin-fixed paraffin-embedded
GEP	Gene expression profiling
HR	Hazard ratio
ICI	Immune checkpoint inhibitor
IHC	Immunohistochemistry
irAEs	Immune-related adverse events
KM	Kaplan-Meier
MDSCs	Myeloid-derived suppressor cells
mOS/OS	Median overall survival/overall survival
mPFS	Median progression-free survival
(m)TTD	(Median) time-to-treatment discontinuation
MSI	Microsatellite instability
NGS	Next generation sequencing
NLR	Neutrophil-to-lymphocyte ratio
NSCLC	Non-small cell lung cancer
OR	Odds ratio
ORR	Overall response rate
PD	Progressive disease
PD-1	Programmed cell death-1
PD-L1	Programmed death-ligand 1

PR/ltPR	Partial response/long-term partial response
PS	Performance status
QC	Quality control
RCT	Randomised controlled trial
RECIST	Response evaluation criteria in solid tumors
RKKP	Danish Clinical Quality Program
RT	Radiotherapy
RWS	Real-world studies
SBRT	Stereotactic body radiation therapy
SD/PrlSD	Stable disease/prolonged stable disease
TAMs	Tumor-associated macrophages
TILs	Tumor-infiltrating lymphocytes
TKIs	Tyrosine kinase inhibitors
TMB	Tumor mutational burden
TME	Tumor microenvironment
TNM	Tumor, node, metastasis
TPS	Tumor proportion score
UICC	Union for International Cancer Control

PAPERS

The dissertation is based on the following scientific papers:

PAPER I

Mouritzen, M.T.; Carus, A.; Ladekarl, M.; Meldgaard, P.; Nielsen, A.W.M.; Livbjerg, A.; Larsen, J.W.; Skuladottir, H.; Kristiansen, C.; Wedervang, K.; Schytte, T.; Hansen, K.H.; Østby, A.-C.; Frank, M.S.; Lauritsen, J.; Sørensen, J.B.; Langer, S.W.; Persson, G.F.; Andersen, J.L.; Frary, J.M.C.; Drivsholm, L.B.; Vesteghem, C.; Christensen, H.S.; Bjørnhart, B.; Pøhl, M. Nationwide Survival Benefit after Implementation of First-Line Immunotherapy for Patients with Advanced NSCLC—Real World Efficacy. *Cancers* **2021**, *13*, 4846.[1]
https://doi.org/10.3390/cancers13194846

PAPER II

Mouritzen, M.T.; Junker, K.F.; Carus, A.; Ladekarl, M; Meldgaard, P.; Nielsen, A.W.M.; Livbjerg, A.; Larsen, J.W.; Skuladottir, H.; Kristiansen, C.; Wedervang, K.; Schytte, T.; Hansen, K.H.; Østby, A.-C.; Frank, M.S.; Lauritsen, J.; Sørensen, J.B.; Langer, S.W.; Persson, G.F.; Andersen, J.L.; Homann, P.H.; Kristensen, E.B.; Drivsholm, L.B.; Bøgsted, M.; Christensen, H.S.; Pøhl, M. & Bjørnhart, B. Clinical features affecting efficacy of immune checkpoint inhibitors in pretreated patients with advanced NSCLC: a Danish nationwide real-world study, Acta Oncol. 2022 Jan 11;1-8.[2] https://doi.org/10.1080/0284186X.2021.2023213

PAPER III – IN PREPERATION

Mouritzen, M.T.; Ladekarl, M; Hager, H; Lippert, J.B.; Frank, M.S.; Mattesen, T.B.; Nøhr. A.K.; Egendal, I.B.; Carus, A. Gene expressions, high lymphocyte count, and absence of liver metastases may predict durable clinical benefit in patients with advanced NSCLC treated with immune checkpoint inhibitors [3].

TABLE OF CONTENTS

CHAPTER 1. INTRODUCTION

Worldwide, lung cancer is the second most common cancer diagnosis, and the leading cause of cancer death [4]. In Denmark, the number of lung cancer cases was 5,004 in 2021 [5]. In 2017-2019, the 5-year survival rate was 26% regardless of histopathology, disease stage, and treatment intention [6]. However, in patients with metastatic disease, the prognosis is poor with a 5-year survival rate of 8% [7]. The lung cancer diagnosis includes several histopathological subtypes, which are roughly categorized into two groups as non-small cell lung cancer (NSCLC) or small-cell lung cancer. The majority of patients are diagnosed with NSCLC (80-90%), with adenocarcinomas and squamous cell carcinomas being the most common subtypes [8, 9].

The main aetiological factor in lung cancer is tobacco consumption, and around 85% of lung cancer cases are attributable to smoking [10]. Currently, lung cancer incidence and mortality rates are higher in transitioned compared to transitioning countries [4]. However, the global trends in smoking prevalence change, and may reflect the future global distribution of lung cancer incidence and mortality rates [11]. Furthermore, the female-to-male lung cancer incidence ratio increases in most countries, which is primarily driven by an increase in female incidence of adenocarcinomas [9]. However, the change also reflects global smoking patterns according to sex [12]. Other factors that may contribute to lung cancer carcinogenesis include genetic susceptibility, poor diet, occupational exposures, and air pollution [13].

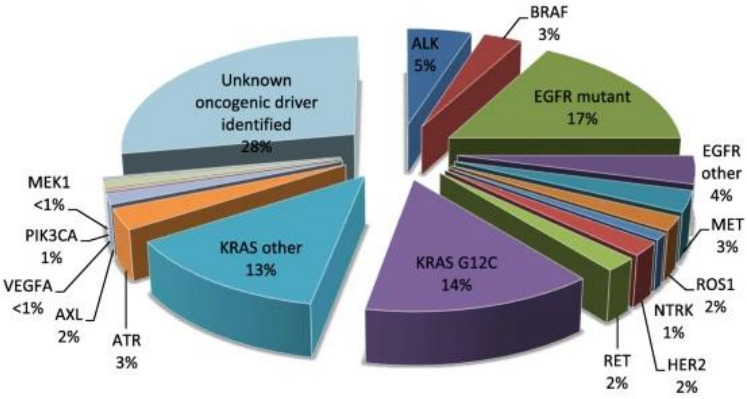
Symptoms of lung cancer can be directly and/or indirectly related to the tumor, but symptoms may not exist. The most frequent symptoms include persistent coughing, haemoptysis, dyspnoea, hoarseness, pain (typically chest, bone, shoulder, spine, and head), unintended weight loss, fatigue, finger clubbing, and superior vena cava syndrome. Rarely, lung cancer presents as Horner's syndrome (Pancoast tumors) or as paraneoplastic syndromes [14]. To improve the diagnostic process, the Danish Ministry of Health introduced the cancer patient pathways in 2007 [15]. Despite this initiative, around half of the patients have metastatic disease at time of diagnosis, and around 20% of all patients diagnosed with lung cancer do not receive any antineoplastic treatment [16].

Malignant tumors are classified according to the disease extent by the internationally accepted standard for cancer staging, the Union for International Cancer Control's (UICC) Tumor, Node, Metastasis (TNM) classification of Malignant Tumors. The T category describes the primary tumor site and size, the N category the regional lymph node involvement, and the M category the extent of distant metastatic spread [17]. The combination of the T, N, and M evaluation determine the final tumor stage. According to national NSCLC treatment guidelines, patients with localized disease (stage I, II, IIIa without N2 disease) may be offered surgery with or without adjuvant platinum-based chemotherapy (CTx) or stereotactic body radiotherapy (SBRT) for stage I-IIb

[18, 19]. The majority of patients with locally advanced disease (stage III) are offered curatively intended treatment; surgery and/or combined CTx and radiotherapy (CRT) [20]. However, some stage III patients are not suitable for curative treatment, and they may similarly to stage IV patients be offered palliative systemic antineoplastic treatment or best supportive care (BSC) [21]. This dissertation focuses exclusively on patients without curative treatment options. The single term 'advanced NSCLC' is used to classify these patients throughout this dissertation.

The histopathological classification of NSCLC, particularly adenocarcinomas, has changed considerably during the last decade [22]. These changes are caused by progress in molecular genetics and emerging targetable driver-mutations, translocations, gene-fusions and predictive biomarkers, which enable more personalized treatment with higher response rates, lower toxicity, and improved survival compared to empirical treatment without biomarker enrichment (Figure 1.1) [21, 23, 24].

Figure 1.1 Potential actionable molecular alterations in lung adenocarcinoma [25]



Modified version from 'Emerging therapeutic agents for non-small cell lung cancer' by R. Chen *et al*. [25]

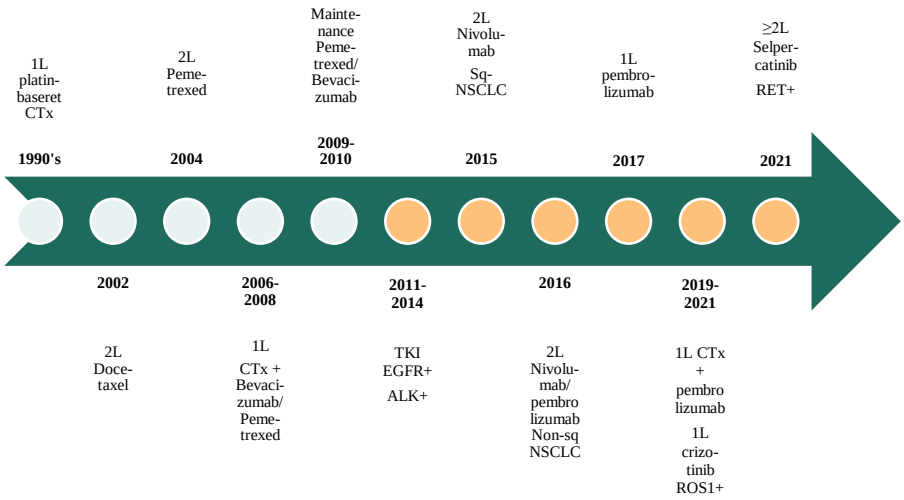
The terms 'prognostic' and 'predictive' are widely used to describe the relationship between a biomarker or clinical feature and the clinical outcome of interest. A prognostic factor or biomarker is associated with the clinical outcome in the absence of treatment or when standard treatment is applied [26, 27]. A predictive factor or biomarker is associated with response or lack of response to a particular treatment [26, 27]. For NSCLC, prognostic factors can be divided into patient characteristics, tumor features, and biological factors. The most significant factors include disease stage and number and location of metastatic sites, performance status (PS), weight loss, sex, age, comorbidity, and standard laboratory variables like haemoglobin, lactate dehydrogenase, albumin, and white blood cell count [28, 29]. Prognostic molecular characteristics have also been proposed during time, including *P53* and Ki-67, and new biological factors continue to emerge [29].

CHAPTER 2. BACKGROUND

2.1 PALLIATIVE TREATMENT EVOLUTION OF ADVANCED NSCLC IN DENMARK

Until the 1990's, no evidence-based systemic treatment options existed for patients with advanced lung cancer, and the median overall survival (mOS) was only 4-5 months [30]. With the option of palliative platinum-doublet CTx, the mOS improved to 8 months [30, 31]. For the following 20 years, only minor progress was seen, with the addition of second-line (2L) docetaxel and pemetrexed, first-line (1L) combination therapy with the vascular endothelial growth factor-inhibitor bevacizumab or pemetrexed, and maintenance pemetrexed or bevacizumab after 1L platinum-doublet CTx (Figure 2.1) [32-37].

Figure 2.1 Palliative treatment evolution of advanced NSCLC in Denmark [38, 39]



NSCLC, non-small cell lung cancer; 1L, first-line; 2L, second-line; ≥2L, second- or subsequent-line; CTx, chemotherapy; sq, squamous; *ALK*, anaplastic lymphoma kinase; *EGFR*, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor; *RET*, rearranged during transfection; *ROS1*, c-ros oncogene 1

Around year 2010, an era of personalized medicine commenced with the implementation of tyrosine kinase inhibitors (TKIs) for the treatment of patients harbouring epidermal growth factor receptor (*EGFR*)-mutations or anaplastic lymphoma kinase (*ALK*)-translocations [40-43]. However, only around 12% and 5% of patients harbour a targetable *EGFR*-mutation or *ALK*-translocation, respectively [44, 45]. Notably, *EGFR*-mutation frequency varies across different ethnicities and are more frequent in never smokers.

In the past six years, immune-checkpoint inhibitors (ICIs) have changed the treatment paradigm for patients with advanced NSCLC without specific druggable molecular alterations, owing to improved response rates and survival compared to CTx in randomized controlled trials (RCTs) (Table 2.2) [46-50].

Table 2.2 Clinical outcomes in the pivotal RCTs with nivolumab or pembrolizumab monotherapy [46-50].

	mOS months (95%CI)	**1-year OS rate, % (95% CI)**	**mPFS months (95%CI)**	**ORR % (95% CI)**	
				All patients	PD-L1 ≥50%
Checkmate 017 [47]					
Nivolumab	9.2 (7.3 – 13.3)	42 (34 – 50)	3.5 (2.1 – 4.9)	20 (14 – 28)	
Docetaxel	6.0 (5.1 – 7.3)	24 (17 – 31)	2.8 (2.1 – 3.5)	9 (5 – 15)	
Checkmate 057 [46]					
Nivolumab	12.2 (9.7 – 15.0)	51 (45 – 56)	2.3 (2.2 – 3.3)	19 (15 – 24)	
Docetaxel	9.4 (8.1 – 10.7)	39 (33 – 45)	4.2 (3.5 – 4.9)	12 (9 – 17)	
Keynote 010 [48]					
Pembro 2 mg/kg/3w	10.4 (9.4 – 11.9)	43 (NR)	3.9 (3.1 – 4.1)	18 (NR)	30 (NR)
Pembro 10 mg/kg/3w	12.7 (10.0 – 17.3)	52 (NR)	4.0 (2.7 – 4.3)	18 (NR)	29 (NR)
Docetaxel	8.5 (7.5 – 9.8)	35 (NR)	4.0 (3.1 – 4.2)	9 (NR)	8 (NR)
Keynote 024 [49, 50]					
Pembro 200 mg/3w	26.3 (18.3 – 40.4)	70 (62 – 77)	10.3 (6.7 – NR)	45 (37 – 53)	
Platinum CTx*	13.4 (9.4 – 18.3)	55 (46 – 62)	6.0 (4.2 – 6.2)	28 (21 – 36)	

* 66% crossover rate

Clinical outcomes in anti-PD-1 clinical trials of patients with advanced NSCLC.

Pembro, pembrolizumab; w, weeks; CTx, chemotherapy; n, number; PD-L1, programmed death-ligand 1; mOS, median overall survival; CI, confidence interval; OS, overall survival; mPFS, median progression-free survival; NR, not reported

The ICI-revolution in NSCLC was commenced by the approval of the anti-programmed cell death-1 (PD-1) antibody, nivolumab in previously treated, squamous NSCLC patients in September 2015 in Denmark, following the results from the Checkmate 017 trial [38, 47]. Subsequently nivolumab was approved by Danish authorities for previously treated non-squamous NSCLC patients with a programmed death-ligand 1 (PD-L1) tumor proportion score (TPS) >1% [38, 46]. The approval of another anti-PD-1 antibody, pembrolizumab, followed in NSCLC patients with PD-L1 >50% in the 1L regimen, and in NSCLC patients with PD-L1 >1% treated with ICIs in second- or subsequent-lines (≥2L) [38, 48, 49]. In 2019-2021, the combination regimens with pembrolizumab, carboplatin and pemetrexed or paclitaxel/Nab-

paclitaxel was approved by Danish authorities in the 1L treatment for patients with non-squamous NSCLC and PD-L1<50%, and for patients with squamous NSCLC and PD-L1 1%-49% [51-55] (Figure 2.1). Atezolizumab, an anti-PD-L1 antibody, was approved for a very short period in 2018 by Danish authorities, for unselected patients with previously treated advanced NSCLC [56, 57]. However, the Danish approval for patients with non-squamous NSCLC and PD-L1 TPS <1% was withdrawn by the authorities due to cost-benefit considerations[58].

2.2 RANDOMIZED CONTROLLED TRIALS

The approval of new drugs is typically based on results from RCTs. Due to the high internal validity in RCTs, subgroups of patients treated in routine clinical practice are often underrepresented. These subgroups include patients with PS ≥2, older age, and severe comorbidity. The median age of lung cancer patients included in the ICI-RCTs was around 61 years, which contrasts the median age of 70 years in Nordic NSCLC patients (Table 2.3) [59, 60]. Furthermore, the unequal sex distribution in international RCTs and real-world studies (RWS) reflects the low female-to-male NSCLC incidence ratio in most countries, which is less representative of the Nordic NSCLC population (Table 2.3) [61-63]. Thus, RWS may provide additional information on the effectiveness in these patients.

Table 2.3 Patient and treatment characteristics in the pivotal RCTs with nivolumab or pembrolizumab monotherapy [46-50].

Characteristics	**Checkmate 017** [47] (phase III)		**Checkmate 057** [46] (phase III)	
	Nivolumab 3 mg/kg/2w	**Docetaxel** 75 mg/m^2/3w	**Nivolumab** 3 mg/kg/2w	**Docetaxel** 75 mg /m^2/3w
All patients, n	135	137	292	290
Median age, years	62	64	61	64
Age, n (%)				
≥70 years	NR	NR	NR	NR
≥75 years	11 (8)	18 (13)	20 (7)	23 (8)
Sex, n (%)				
Male	111 (82)	97 (71)	151 (52)	168 (58)
Female	24 (18)	40 (29)	141 (48)	122 (42)
PS, n (%)				
0	27 (20)	37 (27)	84 (29)	95 (33)
1	106 (79)	100 (73)	208 /71)	194 (67)
≥2	0	0	0	0
NSCLC histopathology, n (%)				
Squamous	135 (100)	137 (100)	0	0
Non-squamous	0	0	292 (100)	290 (100)
Metastatic sites, n (%)				
Brain/CNS	9 (7)	8 (6)	34 (12)	34 (12)
Liver	NR	NR	NR	NR

Bone	NR	NR	NR	NR
PD-L1 TPS, n (%)				
Negative	54 (40)	52 (38)	108 (47)	101 (45)
≥1%	63 (47)	56 (41)	123 (53)	123 (55)
1-49%	NR	NR	NR	NR
≥50%	NR	NR	NR	NR
Not reported	NR	NR	NR	NR
Prior lines of palliative therapy, n (%)				
1	135 (100)	137 (100)	256 (88)	259 (89)
2	0	0	35 (12)	31 (11)
≥3	0	0	0	0
Treatment				
No. of doses, median	8	3	6	4
Median TTD, months	NR	NR	NR	NR

Characteristics	**Keynote 010** [48] (phase II/III)			**Keynote 024** [49, 50] (phase III)	
	Pembro 2 mg/kg/3w	**Pembro** 10 mg/kg/3w	**Docetaxel** 75 mg/m^2/3w	**Pembro** 200 mg/3w	**Platinum CTx***
All patients, n	344	346	343	154	151
Median age, years	63	63	62	65	66
Age, n (%)					
≥70 years	NR	NR	NR	NR	NR
≥75 years	NR	NR	NR	NR	NR
Sex, n (%)					
Male	212 (62)	213 (62)	209 (61)	92 (60)	95 (63)
Female	132 (38)	133 (38)	134 (39)	62 (40)	56 (37)
PS, n (%)					
0	112 (33)	120 (35)	116 (34)	54 (35)	53 (35)
1	229 (67)	225 (65)	224 (65)	99 (64)	98 (65)
≥2	3 (1)	1 (<1)	2 (1)	NR	NR
NSCLC histopathology, n (%)					
Squamous	76 (22)	80 (23)	66 (19)	29 (19)	27 (18)
Non-squamous	240 (70)	244 (71)	240 (70)	125 (81)	123 (82)
Metastatic sites, n (%)					
Brain/CNS	56 (16)	48 (14)	48 (14)	18 (12)	10 (7)
Liver	NR	NR	NR	NR	NR
Bone	NR	NR	NR	NR	NR
PD-L1 TPS, n (%)					
Negative	0	0	0	NR	NR
≥1%	344 (100)	346 (100)	343 (100)	NR	NR
1-49%	205 (60)	195 (56)	191 (56)	NR	NR
≥50%	139 (40)	151 (44)	152 (44)	154 (100)	151 (100)
Not reported	0	0	0	NR	NR
Prior lines of palliative therapy, n (%)					
1	243 (71)	235 (68)	235 (69)	NR	NR
2	66 (19)	69 (20)	75 (22)	NR	NR
≥3	27 (8)	34 (10)	29 (8)	NR	NR

Treatment					
Median doses, n	NR	NR	NR	11	4
Median TTD, months	4**	4**	2**	7	4

* 66% crossover rate

** For the safety population

Baseline characteristics in anti-PD-1 clinical trials of patients with advanced NSCLC.

Pembro, pembrolizumab; w, weeks; CTx, chemotherapy; n, number; PS, performance status; NSCLC, non-small cell lung cancer; CNS, central nervous system; PD-L1, programmed death-ligand 1; TPS, tumor proportion score; TTD, time-to-treatment discontinuation; NR, not reported

2.3 REAL-WORLD ICI TREATMENT

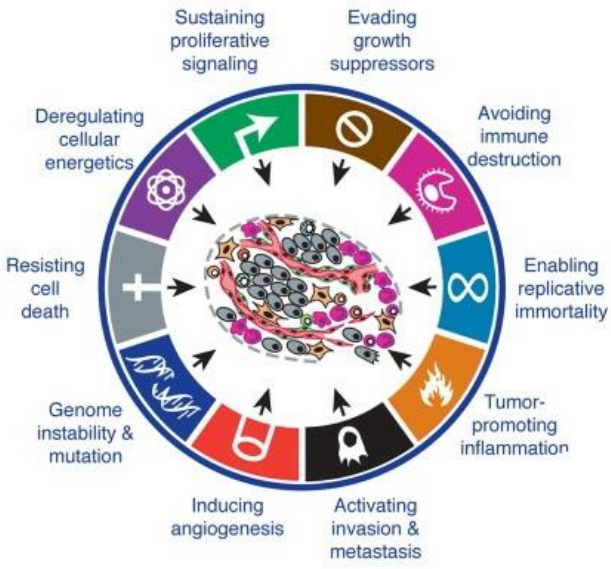
At PhD study initiation, minor real-world studies (RWS) with nivolumab demonstrated survival comparable to results from the RCTs, however, impaired survival was observed in subgroups of patients with poor PS and brain-, bone- and liver metastases [64-71]. The Italian expanded access program included pretreated patients receiving nivolumab, and suggested sustained efficacy in older patients, patients with brain metastases, and never-smokers [72-74]. Other RWS also concluded that older patients seemed to benefit from ICI-treatment but may be more vulnerable [73, 75, 76]. Lung cancer patients frequently have comorbidity, which may affect the treatment schedule and clinical outcome in ICI-treated patients [77, 78]. Despite the proposed prognostic impact of comorbidity and metastases at specific locations, these data were not reported in the RCTs, and scarcely assessed in RWS.

At PhD study initiation, no larger RWS of Nordic populations had been reported. Furthermore, the impact of real-world ICI-implementation on the OS of NSCLC patients had only been scarcely investigated [79, 80]. Additionally, the annual reports from the Danish Lung Cancer Registry (DLCR) revealed no remarkable increases in 1-year survival rates for lung cancer patients in the years post-approval ICI [81-83].

2.4 CANCER IMMUNOLOGY AND ICI

The importance of cancer immunology has become more and more apparent. Around year 2000 the hallmarks of cancer were described and 10 years later they were expanded to include immunological features of the tumor and tumor microenvironment (TME) (Figure 2.3) [84, 85].

Figure 2.3 The hallmarks of cancer [85].



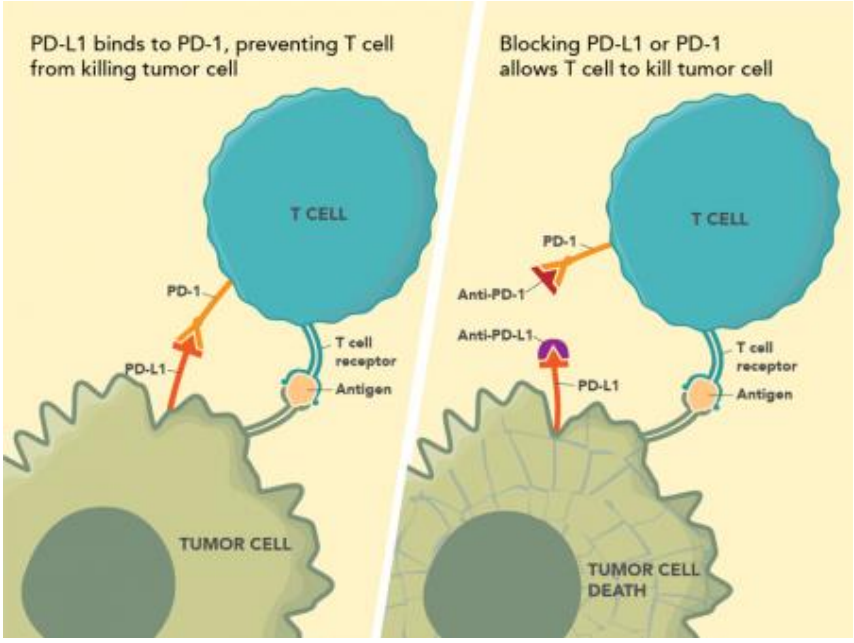
Modified version from 'Hallmarks of Cancer: The Next Generation' by D. Hanahan and R. Weinberg [85]. (With permission, RightsLink license 5424120442733)

Different immune cells are present in the TME, and both pro- and antitumorigenic effects coexist [86]. This paradoxical dual action of the immune system in cancer development is referred to as 'immunoediting', which proceed through three phases: elimination, equilibrium, and escape [87, 88]. These immunological mechanisms are elicited by the interplay of innate and adaptive immune mechanisms. Chronic inflammation is associated with a poor prognosis and impacts every step in the tumorigenesis [86, 89]. The innate immune mechanisms also play a role in NSCLC, and elevated baseline neutrophil count has been significantly associated with shorter OS [90]. However, the density of tumor-associated neutrophils, has not been significantly associated with disease-free survival or OS, whereas the intra-tumoral neutrophil-to-lymphocyte ratio (NLR) in resected NSCLC has been associated with increased risk of recurrence and poor OS [91, 92]. In recent years, the main focus has been on the adaptive immune system, and more precisely the distribution of tumor-infiltrating lymphocytes (TILs). High levels of $CD8^+$, $CD3^+$ og $CD4^+$ in tumor nest or tumor stroma have been associated with improved OS in lung cancer patients, whereas high level of $FOXP3^+$ T cells in tumor stroma is a poor prognostic factor [93]. With different immune-based metrics, tumors can be characterized as T-cell inflamed (hot) or non-T-cell inflamed (cold), which correlates with the clinical outcome [94, 95].

2.4.1 MECHANISMS OF IMMUNE CHECKPOINT INHIBITION

The function of the immune checkpoints CTLA-4 and PD-1 was discovered by Honjo and Allison in the mid-late 1990's [96-99]; discoveries that recently were honoured with the Nobel price award. Immune checkpoints can be stimulatory or inhibitory, and their function is to regulate the immune system which is crucial for self-tolerance[100]. The immunoinhibitory receptor PD-1, is expressed on activated t-cells, B-cells and myeloid cells [97]. Initially, the PD-L1 was reported to be a member of the B7 gene family, that was expressed on antigen-presenting cells and normal tissue [98]. The engagement of PD-1 by PD-L1 causes inhibition of T-cell receptor-mediated lymphocyte proliferation and cytokine release [98]. In 2002, the role of PD-1/PD-L1 interaction in tumor immunity was elucidated, and suggested that tumor expression of PD-L1 could be a potential mechanism of immune escape [99]. Furthermore, T-cell antitumor response was reactivated by in vivo administration of anti-PD-1 antibodies which was also demonstrated in phase I trials (Figure 2.4) [99, 101-103]. Due to the expression of the immune checkpoints on normal tissues, ICI-treatment may lead to immune-related adverse events (irAEs) [104].

Figure 2.4 Mechanisms of anti PD-1/PD-L1 immune-checkpoint inhibition [105]



Credit to the U.S. Food and Drug Administration (FDA) for the free copy license.
PD-1, programmed death-1; PD-L1, programmed death-ligand 1

2.5 BIOMARKERS FOR ICI IN NSCLC

A biomarker can be defined as:

"any substance, structure, or process that can be measured in the body or its products, and influence or predict the incidence of outcome or disease" [106].

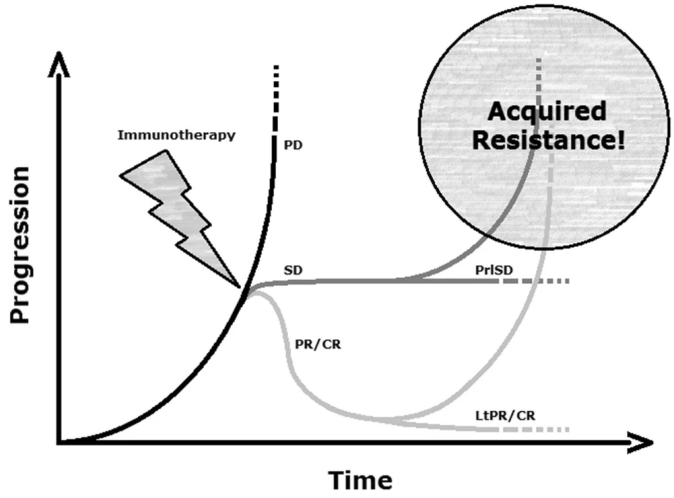
2.5.1 PD-L1 AS A BIOMARKER

Since the introduction of specific targeted therapies, predominantly TKIs, the clinical decision-making has become dependent upon evidencing addiction of the tumor to a given molecular pathway and/or oncogene. Expression of PD-L1 assessed through immunohistochemistry (IHC), has been the major initial molecular determinant of clinical benefit from the ICIs targeting PD-1 and PD-L1. In the pivotal RCTs, superior outcomes were demonstrated in ICI-treated populations across different levels of PD-L1 TPS compared to CTx [46-50, 56, 107, 108]. Concordance studies suggest that the different PD-L1 IHC assays associated with nivolumab, pembrolizumab and durvalumab (assays 28-8, 22C3 and SP263) produce comparable results [109]. In contrast, the assay used for atezolizumab (assay SP142) stains fewer tumor cells and the assay used for avelumab stains more tumor cells (assay 73-10) [109-112]. The expression of PD-L1 is considered induced in response to INFγ released by activated T cells, and consequently known to vary both spatially and temporally [111]. The nature of PD-L1 as a constitutive and adaptive biomarker, and the presence on both tumor and immune cells makes it a poor predictor of ICI efficacy [113-115]. Therefore, the use of PD-L1 as a predictive biomarker has some limitations

2.5.2 PROPOSED BIOMARKERS

Predictive ICI biomarkers identify patients as responders or non-responders pre-treatment. A potential underlying treatment resistance can be defined as primary or acquired/secondary and is defined by the best overall response, duration of drug exposure, and the time of progression (Figure 2.5) [116, 117]. However, the underlying resistance mechanisms may contribute and co-occur in both primary and acquired resistance [116].

Figure 2.5 Resistance to immune checkpoint inhibitors [116]



The definition of primary and acquired resistance to immune checkpoint inhibitors is based on the best overall response, duration of drug exposure, and the time of progressive disease (PD). Primary resistance exists when the best overall response is PD or stable disease (SD) for less than 6 months. Acquired or secondary resistance exists when PD occurs after initial prolonged (Prl) stable disease (SD) (> 6 months), long-term (Lt) partial response (PR), or complete response (CR) [117]. (With permission, RightsLink license 5424111006415)

Some of the factors that affect the efficacy of PD-1/PD-L1 ICIs include 1) the presence of non-synonymous mutations (which may differ between subclones) 2) the transcription of these genetic changes into potential neoantigens 3) the presentation of neoantigens to the immune system 4) the presence of a pro-inflammatory, permissive microenvironment 5) the dominant mechanism of immune evasion and 6) the degree of drug exposure and baseline resistance [118]. Different 'cancer immunograms' have been proposed as potential predictors of response to ICIs in different solid tumors [119, 120]. The cancer immunograms encompass known aspects of tumor, TME, the immune system, and host interactions such as tumor foreignness, general immune status, immune cell infiltration, absence of immune checkpoints, absence of soluble inhibitors, absence of inhibitory tumor metabolism, environmental (host-related) factors, and tumor sensitivity to immune effectors [119, 120]. Other predictive tumor classifications and models include the T cell inflamed 'hot' or non-T cell inflamed 'cold' tumor classification [95, 121], the 'immunoscore' defining lymphocyte population and their location in relation to tumor [122], and the 'response score' including tumor mutational burden (TMB) and RNA sequencing [123]. Some of the host-related factors which have been associated with ICI efficacy include body mass index (BMI) and gut microbiome [124, 125]. The background for this comprehensive approach is the unsatisfying predictive value of the currently FDA-approved ICI-biomarkers PD-L1, microsatellite instability (MSI) , and TMB [126, 127]. ICI biomarkers are investigated in different biological compartments such as histological or

cytological tissue samples, blood, and faeces, which require application of a variety of companion diagnostics with different degrees of invasiveness. Despite the comprehensive biomarker research, no biomarker or model exist, with a sufficient potential to predict ICI efficacy on an individual level. This implies administration of ineffective and toxic treatment to a large proportion of patients.

2.6 DIAGNOSTIC BIOPSIES AND BIOMARKER ANALYSES IN ADVANCED NSCLC

The histopathological subtype of NSCLC in patients with primarily advanced disease, is typically determined by immunohistochemical staining of fine- or core-needle biopsies from primary or metastatic lesions [128]. It is well known, that only scarce material is available from these small NSCLC samples, and the increased need for multiple molecular testing is challenging [129, 130].

Besides subtyping of NSCLC, IHC is routinely performed for the assessment of PD-L1 TPS and *ALK*- and *ROS1* rearrangements. In situations with faint or doubtful *ALK*- or *ROS1* staining by IHC, fluorescence in situ hybridization (FISH) analysis is performed to confirm the result [130]. However, these multiple IHC-analyses and potential FISH-confirmations are tissue-consuming, despite the use of initial reflex block cutting to avoid recutting the block [130].

Previously, *EGFR*-mutations were detected by PCR-based technologies. However, next generation sequencing (NGS) is established as a standard diagnostic method, in order to obtain information on targetable driver mutations, TMB and MSI [131]. NGS enables comprehensive testing of multiple DNA variants at the same time, and panel sizes vary according to the number of genes included. NGS also covers RNA sequencing, which enables quantification of gene expression, detection of gene fusions, and measurement of allele-specific expression at the same time. Therefore, RNA sequencing is currently being implemented as a routine method to obtain information on *ALK*-, *ROS1*, and *RET* rearrangements.

Due to the growing knowledge of the interaction between the tumor cells, TME, immune system and host-related factors, new methods emerge in the pursuit of predictive ICI biomarkers. One of the emerging methods, the nCounter® PanCancer IO 360™ panel is a 770 gene expression panel covering the interplay between the tumor, TME and immune response [132]. The panel provides potentially predictive biological signatures for ICI, can be performed with RNA derived from formalin-fixed paraffin-embedded (FFPE), and requires a sample input of only 50 ng [132, 133]. Therefore, the ability to use FFPE-specimens and small sample inputs indicates feasibility in routine clinical diagnostics of patients with advanced NSCLC.

CHAPTER 3. AIMS OF THE DISSERTATION

The overall aim of the dissertation was to analyse the effectiveness of ICIs in a Danish nationwide real-world cohort of patients with advanced NSCLC, and the potential survival changes after the implementation of ICIs in Denmark. This included a special attention to patients underrepresented in the RCTs. Furthermore, in a prospective study, we aimed to assess the feasibility of applying multiple methods on diagnostic tissue samples, to uncover possible biomarkers predictive of ICI efficacy. The three studies should bridge clinical and basic research and exploit the experience from previously treated patients to improve the outcome of future patients.

The specific aims of the studies were:

3.1 STUDY I-II

1. To report on OS and PFS in a consecutive population of patients with incurable, advanced or metastatic NSCLC treated with ICIs in any treatment line.
2. To uncover prognostic clinical factors for OS, with a special attention to the subgroups of patients who were underrepresented in the RCTs.
3. To report on treatment data, including treatment line, reasons for ICI discontinuation, treatment duration, irAEs, and hospitalization due to irAEs.
4. Additionally, in study I, to compare the OS of patients receiving first line, palliative, systemic, antineoplastic treatment before and after the implementation of ICIs in Denmark.

3.2 STUDY III

1. To assess the impact of baseline characteristics on durable clinical benefit (DCB) in patients with advanced NSCLC treated with ICIs in routine clinical cancer care.
2. To investigate the association between DCB and peripheral absolute lymphocyte count (ALC), absolute neutrophil count (ANC), and NLR.
3. To assess the feasibility of gene expression profiling (GEP) in routine clinical cancer care.
4. uncover predictive gene expressions for DCB in patients with advanced NSCLC treated with ICIs in routine clinical cancer care.

CHAPTER 4. MATERIAL AND METHODS

4.1 STUDY I-II

4.1.1 PATIENTS

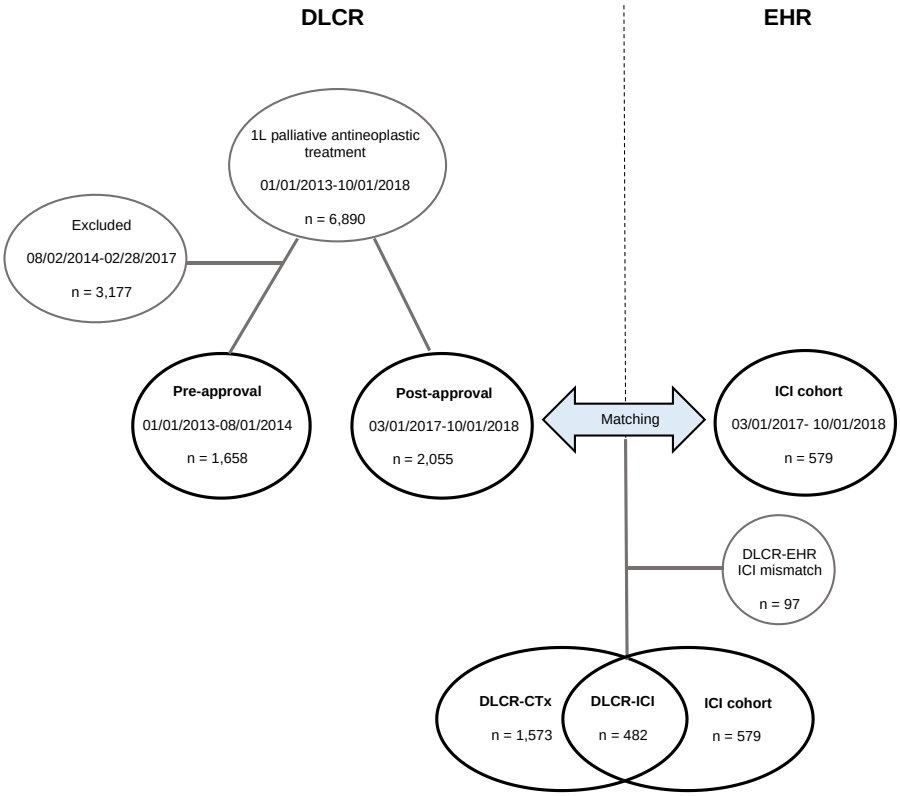
4.1.1.1 Study I: First-line treatment

Study I included patients with incurable stage III-IV NSCLC, without EGFR mutation or ALK translocation, who started 1L systemic, antineoplastic treatment from 1 January 2013 to 1 October 2018. The data was extracted from the DLCR and from electronic health records (EHRs) [1].

The DLCR is a national clinical registry and a part of the Danish Clinical Quality Program (RKKP) [134, 135]. The DLCR gathers data from the participating departments (the departments of lung medicine, thoracic surgery, and clinical oncology), the National Patient Registry, the Danish Civil Registration System (CPR), and the Danish Pathology Registry [134]. Baseline demographics and clinical data are included in the DLCR, however, data on PS and metastatic sites, and details regarding systemic antineoplastic treatment is lacking.

The DLCR dataset was separated into a *DLCR pre-approval cohort* and a *DLCR post-approval cohort* (Figure 4.1) [1]. The in-between cohort of 3,177 patients were excluded to minimize the impact of second-line ICI implementation in September 2015. From institutional records, the DLCR data set was supplemented with retrospectively identified patients who started 1L ICI treatment from 1 March 2017 to 1 October 2018 (named the *ICI cohort*). In order to stratify the *DLCR post-approval cohort* by type of antineoplastic treatment, the *DLCR post-approval cohort* was matched with the *ICI cohort* (Figure 4.1) [1].

Figure 4.1 Flowchart showing the generation of the Danish Lung Cancer Registry (DLCR) cohorts before and after the approval of immune checkpoint inhibitors (ICIs) [1]



Treatment data from the electronic health records (EHRs) were applied on the DLCR post-approval cohort to divide patients into the DLCR-chemotherapy (CTx) and DLCR-ICI cohorts. Due to missing and inaccurate data in the DLCR, 97 ICI-treated patients identified from institutional records were not registered in the DLCR

4.1.1.2 Study II: Second- or subsequent-line treatment

Study II included patients with incurable stage III-IV NSCLC who started second- or subsequent-line ICI treatment from 1 September 2015 to 1 October 2018. The patients were retrospectively identified from institutional records [2].

4.1.2 DATA MANAGEMENT

4.1.2.1 Definitions, covariates, and clinical endpoints

In Danish Oncological Lung cancer Group (DOLG) a working group defined the included variables and endpoints. First-line treatment was defined as the first palliative, systemic antineoplastic treatment administered after 1) the initial NSCLC diagnosis without any curative treatment option or 2) at relapse ≥6 months after the

end of curatively intended treatment for NSCLC [1]. ICI doses were either a fixed pembrolizumab dose of 200 mg or 2 mg/kg every three weeks in any treatment line, or a nivolumab dose of 3 mg/kg every two weeks in ≥2L [1, 2]. ICIs were administered for a maximum of two years. The index date was the first ICI administration date, and the censoring date for patients still alive was 1 March 2020. The date of progressive disease (PD) was the date of radiologically verified PD. In the absence of radiologically verified PD, the date of the first clinical evidence of PD was used. For patients still alive, the last follow-up date was defined as the date of the last EHR-documented patient activity [1, 2]. Baseline patient characteristics at the initiation of ICI treatment were obtained, and included sex, age, Eastern Cooperative Oncology Group (ECOG) PS, smoking status, and comorbidity according to Charlson Comorbidity Index Score (CCIS). In cases where PS were described as a range, such as PS 0-1, the highest value was recorded. Baseline disease characteristics included disease stage according to the American Joint Committee on Cancer and the UICC TNM classification, metastatic sites, NSCLC histopathological subtype, *EGFR*-mutation status, *ALK* rearrangement status, and PD-L1 TPS. PD-L1 TPS was categorized as negative (<1%), 1-49%, and ≥50% [1, 2]. Treatment data included ICI drug name, number of treatment line, treatment duration, reasons for ICI discontinuation, types of immune-related adverse events (irAEs), and irAEs leading to hospitalization or death. The types of irAEs were categorized as pneumonitis, hepatitis, skin toxicity, endocrinopathy, diarrhoea/colitis, and 'others'. The categorized irAEs were recorded as individual covariates. Furthermore, data on antineoplastic treatment administered prior to or after ICI treatment was obtained [1, 2]. The clinical endpoints were OS, PFS, time-to-treatment discontinuation (TTD), and reasons for ICI discontinuation. TTD was defined as the time from the index date to the date of last ICI administration [1, 2].

4.1.2.2 Data collection from Electronic Health Records

Clinical and treatment data of ICI-treated patients were manually collected from EHRs and stored in local databases at every department of oncology [1, 2]. Afterwards, the local data sets were extracted, covariates were aligned, and the data was gathered into one nationwide data set [1, 2]. The nationwide data set was completed by ensuring that the inclusion criteria were met. Quality control was performed on each covariate according to duplets, order of dates, missing values, and concordance between related covariates, such as disease stage and metastatic sites. In Denmark, four different EHR-systems were used at the time of data collection. The date of death was automatically and immediately referred from the CPR, likewise with data from the Danish Pathology Registry.

4.1.3 STATISTICAL METHODS

Descriptive statistics were performed [1, 2]. Among subgroups, differences in baseline categorical variables were tested with the chi-square test, while the Wilcoxon rank-

sum test was used to compare median age differences [1, 2]. In study I, the TNM stage was not considered in the comparison of DLCR cohorts due to a large proportion of missing values. Correction for multiple testing was not performed. A *p*-value of 0.05 was considered the threshold of statistical significance, and a confidence interval (CI) of 95% was used [1, 2].

OS, PFS and TTD were analysed using Kaplan-Meier (KM) estimates, and the log-rank test was used to test for differences according to baseline characteristics. Age-related background mortality was not considered in the survival analyses. The reverse KM estimate was used to calculate the median follow-up time [1, 2].

Multivariable Cox regression analyses were performed to adjust for multiple covariates and possible confounders and were extended with an interaction between histopathology and sex. TNM stage was excluded from the survival analyses of ICI-treated patients due to the interaction with metastatic sites [1, 2].

For each of the baseline categorical variables, the assumption of proportional hazard functions was assessed using visual inspection of the log-minus-log survival curves, and formally tested by the Grambsch-Therneau proportional hazard test with survival times transformed by the KM estimate. Average hazard ratios were estimated using weighted univariable and multivariable Cox regressions, due to the violation of the assumption by PS in study I and by PS, bone-, liver-, adrenal- and distant lymph node metastases, histopathology, and *EGFR* mutation status in study II [1, 2].

For KM estimates and Cox regressions, age was categorized as <75 years and ≥75 years, and CCIS was categorized as CCIS 0–1 and CCIS ≥2 [136]. However, in study II, comorbidities that were present in more than 5% of the cases, were included in the weighted univariable Cox regression analysis, to assess the survival impact of each comorbidity [1, 2].

All analyses were carried out using R version 4.0.2 (R Core Team, Vienna, Austria) [137]. The survival package was used to assess the assumption of proportional hazard functions, the ggsurvplot package for the visualization of KM estimates, and the coxphw package for the weighted Cox regression analyses [1, 2].

4.2 STUDY III

4.2.1 STUDY DESIGN AND PATIENT POPULATION

The study was a clinical, prospective, observational, and explorative study. The study population included patients with advanced NSCLC, that were considered candidates for palliative ICI treatment in any treatment line and started ICI treatment from August 2018 to September 2019. The patients were included at the Department of Oncology, Aalborg University Hospital (UH) (ClinicalTrials.gov NCT03658460) [3].

A complementary cohort of 65 patients included from July 2018 to June 2020 at Zealand University Hospital, Næstved, fulfilled the inclusion criteria, and were added to increase the amount of baseline tissue samples (ClinicalTrials.gov NCT03512847) [3].

Inclusion criteria:

- Confirmed diagnosis of NSCLC in core needle biopsy (CNB) or fine needle aspiration
- Age ≥18 years
- ECOG PS ≤2
- Candidate for ICI treatment
- Understand and accept oral and written information
- Signed written informed consent.

Exclusion criteria:

- Candidate for treatment with curative intend (surgical/oncological)
- Other synchronous cancer
- Positive *EGFR* mutation status or *ALK*-rearrangement.

4.2.2 DATA COLLECTION AND FOLLOW-UP

Baseline characteristics were prospectively registered and included sex, age, PS, weight, height, synchronous cancer, and smoking status. Additionally, disease characteristics included TNM stage (International Association for the Study of Lung Cancer 8th edition), with metastatic sites recorded in stage IV disease, NSCLC histopathological subtype, and PD-L1 tumor TPS. Treatment data was also collected and included ICI treatment line, ICI start- and stop date, reason(s) for ICI discontinuation, and subsequent systemic antineoplastic treatment. Baseline ALC and ANC was recorded and the NLR was derived [3].

The primary endpoint was DCB defined as PFS >6 months. PFS was calculated from the date of the first ICI-administration (index date) to the date of PD, death, or last follow-up or censoring. Response evaluation was described according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The date of the last radiological response evaluation was used as the last follow-up date. No patients were lost to follow up. Furthermore, OS was calculated from the index date to the date of death or data cut-off [3].

4.2.3 DATA STORAGE

Personal data was processed according to the Danish Health Act, the Committee Act, sections 10 and 11 of the Data Protection Act, as well as the General Data Protection

Regulation [138-141]. The database and research biobanks were registered on the North Denmark Region record to the Danish Data Protection Agency [142]. All clinical study data was collected and managed using REDCap electronic data capture tools hosted at the North Denmark Region [143, 144]. Diagnostic FFPE-samples were stored in a research biobank at the Departments of Pathology at Aalborg UH and Vejle Hospital, University Hospital of Southern Denmark.

4.2.4 ROUTINE DIAGNOSTICS AND STUDY METHODS

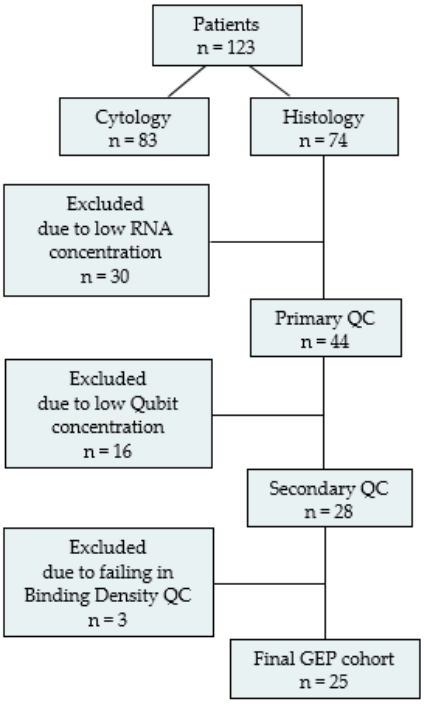
4.2.4.1 Routine diagnostics

Diagnostic tissue samples were used as baseline samples. Morphological examination and immunohistochemistry (IHC) were performed to establish the cancer diagnosis and histopathological subtype. PD-L1 TPS was assessed by IHC with the 22C3 pharmDx antibody stained on the Dako Omnis platform and was categorized as <1%, 1-49%, and ≥50% [3]. NGS was routinely performed with the TruSight® Tumor 15 assay (Illumina) for patients included at Aalborg University Hospital and GeneRead QIAact AIT Panel for patients included at Zealand University Hospital, Næstved. NGS assessed *EGFR*, *BRAF*, *KRAS* and *ERBB2* status. *ALK* rearrangements were also assessed by IHC, and additional fluorescence in situ hybridization (FISH) was performed to confirm the presence/absence of *ALK* rearrangements [3].

4.2.4.2 Gene expression profiling

Prior to GEP, the average tumor percentage on haematoxylin-eosin stained slices of 5µm thickness, was evaluated by a pathologist. Additionally, RNA quality control was performed leading to a final cohort of 25 patients with samples suitable for GEP (Figure 4.2) [3].

Figure 4.2 Flowchart of baseline diagnostic tissue samples prior to gene expression profiling [3]



n, number of patients; QC, quality control; GEP, gene expression profiling

GEP was performed using the nCounter® PanCancer IO360™ panel (NanoString Technologies, Inc.). Total ribonucleic acid (RNA) was extracted manually from 10x5 µm sections from FFPE samples using the miRNeasy FFPE kit (Qiagen). An input amount of 300 ng RNA was used for each sample during NanoString analysis. Prior to hybridization, the extracted RNA was eluted in 13 µl RNAase-free water and RNA concentrations was determined by using the Qubit 3 Flourometer (Invitrogen™). The purified RNA was stored at -80°C and only samples with RNA concentrations ≥60ng/ul were included in the final GEP cohort [3, 145]. The technical integrity of the nCounter® profiling assay underwent further quality control (QC). The sample input and reaction efficiency were assessed in each sample by using the geometric mean of housekeeper genes. Furthermore, QC according to imaging, binding density, positive control linearity, and limit of detection was performed. The final GEP cohort included data from samples that passed all QCs [3].

4.2.4.3 Next generation sequencing

The TruSight® Oncology 500 (TSO500; Illumina) gene panel was used for sequencing analysis. DNA was extracted from 10x5 µm sections from FFPE samples with the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (AS1135) [3]. The extraction was performed on the Maxwell® 16 MDx instrument according to the manufacturer's protocol, and the extracted DNA was eluted in 35 µl nuclease-free water and stored at -20°C. DNA concentrations were determined by using the Qubit 3 Flourometer (Invitrogen™) [3]. DNA concentrations ≥3,33ng/ul qualified for inclusion into the final GEP cohort. Library preparation was performed using the TruSight® Oncology 500 reagent kit according to the manufacturer's protocol and the samples were run on the NextSeq™ 550 instrument (Illumina®) [146 3]. Passing of all sequencing QCs qualified for further analysis. The TSO500 Local Run Manager TruSight® Oncology 500 v2.2 Analysis Module was used to generate TMB and MSI scores [147 3]. The TSO500 TMB algorithm was used to calculate TMB, which was defined as eligible variants/effective panel size. Eligible variants were defined as the total number of somatic synonymous and non-synonymous coding variants (variant frequency ≥5%, coverage >50X). The effective panel size was defined as the total coding region successfully sequenced (coverage >50X). The TMB-high cut-off was 10 mutations/Mb [3]. The TSO500 MSI algorithm, which assesses microsatellite sites for evidence of instability, was used to calculate the MSI score. The MSI score was defined as the number of unstable MSI sites/total number of assessed MSI sites [147]. The MSI-high cut-off was 20% [3].

Due to differences in primary study aims, GEP was the first study method applied on samples from patients enrolled at Aalborg UH, whereas NGS with TSO500 gene panel was the first method applied on the Næstved cohort.

4.2.5 STATISTICAL METHODS

4.2.5.1 Descriptive statistics, logistic regression and survival analyses

Comparisons of patients receiving 1L or ≥2L ICI was performed with ANOVA tests (continuous variables) and Fisher's exact tests (categorical variables). Median peripheral lymphocyte counts were used for the comparisons [3].

Logistic regression analysis was used to assess the association between baseline characteristics and DCB. Brain-, bone-, and liver metastases were included as the only metastatic sites due to the known prognostic impact on survival in NSCLC. Secondly, multivariable logistic regression analysis included age, sex, PS, PD-L1, and factors significantly associated with DCB in the univariable logistic regression analysis. Wald test p-values and profile likelihood confidence limits were reported [3].

OS analysis was performed with a Cox proportional hazards model. Only patients receiving 1L ICI treatment (n=96) were included, due to significant differences in selection criteria for ICI and prognostic baseline characteristics according to treatment line. Univariable and multivariable Cox regression analyses were performed, and age, sex, PS, and factors significantly associated with OS in the univariable analyses were included in the multivariable Cox regression analysis. One patient with missing ALC was excluded [3].

ALC as a predictor for DCB was used to draw a ROC curve. The optimal ALC cut-off for predicting DCB was found by using a Two-sample Kolmogorov–Smirnov plot [148]. This optimal cut-off was defined as the ALC cut-off value that yielded the maximal difference between the cumulative density of ALC in the DCB negative/DCB positive group. Subsequently, the optimal ALC cut-off was used to dichotomize the ALC [3].

P-values <0.05 were considered statistically significant. No adjustments for multiple testing were performed. Statistical analyses were performed with R version 4.2.1 [137].

4.2.5.2 Differential expression of genes

Gene expression analyses were performed to identify differentially expressed genes for response (DCB vs. no DCB). Gene counts were normalised to log2 counts per million using the function Voom (Limma R package) and the trimmed mean of M-values (TMM) method from the R package edgeR [149, 150]. A linear model was fit to each gene adjusting for biological factors associated with DCB using the R package limma [149]. The Benjamini–Hochberg false discovery rate (FDR) was used to correct for multiple testing. An FDR <0.05 was considered statistically significant. The gene expression patterns of genes with a p-value <0.05 were further explored using the ComplexHeatmap package [151]. The package was applied to cluster the patients and the genes using hierarchical clustering based on euclidean distance. ANOVA test was used to assess to the association between the categorical IHC-derived PD-L1 TPS and the continuous GEP-derived PD-L1 (CD274) [3].

4.2.5.3 Gene expression signatures

Differences in gene expression signature scores according to DCB were evaluated. Gene expression signature scores were calculated as a weighted linear combination of the included genes' expression values normalized to stable housekeeper gene expression as described by the manufacturer [152] [3]. A linear model was fit to each gene adjusting for NSCLC histopathological subtype and ALC using the R package limma [149]. FDRs <0.05 were considered statistically significant [3].

CHAPTER 5. ETHICS

5.1 STUDY I-II

Due to the retrospective nature of the studies and the use of routinely collected data, informed patient consent was waived by the Danish Patient Safety Authority (ID 3-3013-2162/1). The studies were reported to the Danish Data Protection Agency (ID 2017-80).

5.2 STUDY III

The study was approved by the Regional Committees on Health Research Ethics of the North Denmark Region (N-20180010) and Region Zealand (SJ-662) and reported to the Danish Data Protection Agency (ID 2017-80 and REG-006-2018)). The Ethics Committees considered that the applied study methods did fulfil the criteria of extensive mapping of the human genome. Before enrolment, written informed consent was obtained from all the participants. The study was conducted according to the principles of Good Clinical Practice, Good Laboratory Practice and the Declaration of Helsinki I and II [153, 154].

CHAPTER 6. RESULTS

6.1 STUDY I-II

6.1.1 BASELINE CHARACTERISTICS

6.1.1.1 DLCR cohorts in study I

The baseline characteristics for the DLCR pre- and post-approval cohorts were compared. Over time, a significant increase in median age from 68 to 70 years, in proportion of female patients from 46.9% to 50.2%, and in proportion of adenocarcinomas from 53.3% to 58.8% was observed [1]. TNM stage was missing in 69 and 246 patients (4.2% and 12.0%, respectively) in the pre- and post-approval cohort, respectively.
The baseline characteristics for the post-approval DLCR-CTx and DLCR-ICI cohorts were also compared. The DLCR-ICI cohort included a significantly higher proportion of females than the DLCR-CTx cohort (58.3% vs. 47.7%) [1]. Additionally, significant differences in the distribution of the NSCLC histopathological types were found, with higher proportions of adenocarcinomas and "other" in the DLCR-ICI cohort, and higher proportions of squamous cell carcinomas in the DLCR-CTx cohort [1]. TNM stage was missing in 206 and 40 patients in the DLCR-CTx and DLCR-ICI cohort, respectively.

6.1.1.2 Patients treated with ICI

Baseline characteristics for ICI-treated patients identified from institutional records were obtained [1, 2]. Information on prior treatment with curative intention and palliative RT was recorded for 1L ICI-treated patients[1]. Information on EGFR mutation status was recorded for ≥2L ICI-treated patients [2].

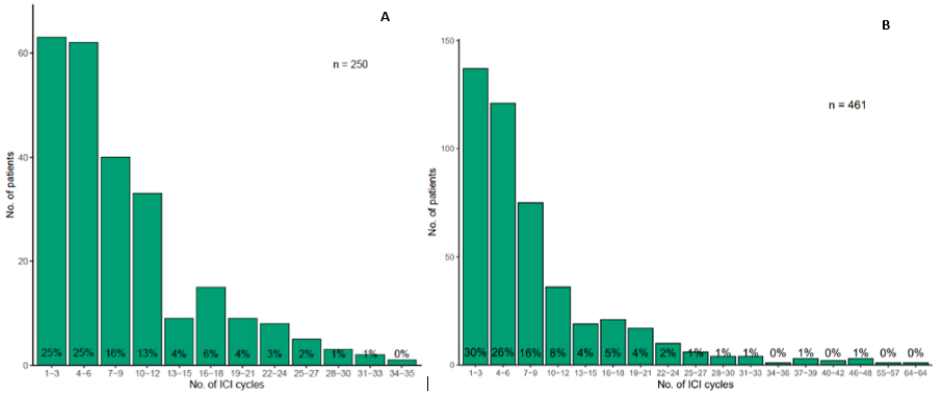
For patients treated with 1L ICI, the median age was 70 years and 58% were females [1]. Small subgroups existed for smoking status (never smokers n=26 (4%), unknown smoking status n=21 (4%)), PD-L1 status (<50% n=23 (4%), unknown n=4 (0.7%), and prior treatment with curative intention (surgery and CRT n=16 (3%)) [1].

For patients treated with ≥2L ICI the median age was 68 years and 49% were females [2]. Small subgroups existed for PS (missing n=21 (2%)), smoking status (unknown n=21 (2%)), and EGFR mutation (yes n=25 (3%))[2]. Furthermore, PD-L1 and EGFR mutation status was unknown in 29% and 33%, respectively [2]. Male patients had a significantly higher age, more comorbidities and more frequently had squamous cell carcinomas compared to female patients [2]. Specific comorbidities according to CCI were registered [2].

6.1.2 ICI TREATMENT CHARACTERISTICS

Due to the study definition of 1L systemic palliative treatment, 12 patients (2%) received nivolumab in 1L [1]. Of patients who received ≥2L ICI, one forth (24%) received ICI-treatment in third line, and 12% in fourth or subsequent line [2].
For patients treated with ICI in 1L or ≥2L, the mTTD was 4.8 months and 3.2 months, respectively [1, 2]. Regardless of treatment-line, around half of the patients ended ICI due to PD; 50-56% within six cycles and 79-80% within 12 cycles (Figure 6.1). Of alle patients, 10-15% discontinued ICI due to poor PS [1, 2].

Figure 6.1 Treatment-discontinuation due to progressive disease in patients treated with first-line (A) or second- or subsequent-line (B) ICI [1].



Proportion of patients who discontinued ICI due to progressive disease according to number of ICI cycles received. A) 1L ICI treatment (n = 250) B) ≥2L ICI treatment (n = 461). Patients with ongoing ICI-treatment were not included in this analysis. At time of analysis, 12-36 ICI cycles were administered to these patients. No., number; ICI, immune checkpoint inhibitor

ICI discontinuation due to irAEs was observed in around one fourth of all patients [1, 2]. Of the patients who received 1L ICI, 67% received no systemic anticancer therapy after ICI-discontinuation [1].

6.1.3 SURVIVAL

6.1.3.1 Survival before and after the implementation of ICIs

The survival comparison of patients who received 1L systemic antineoplastic treatment before (pre-approval cohort) and after (post-approval cohort) the implementation of ICIs showed an increase in mOS with 3.2 months, and a two- and

three-fold increase in 2- and 3-year OS rates, respectively (Table 6.1)[1]. The mOS of patients who received 1L CTx or ICI in the post-approval increased with 1.7 months and 11.2 months, respectively, compared to the mOS in the pre-approval cohort (Table 6.1) [1]. Compared to the pre-approval cohort, the 1-, 2- and 3-year OS rates in the post-approval DLCR-ICI cohort increased from 31% to 64%, 12% to 42%, and 6% to 29%, respectively (Table 6.1)[1].

Table 6.1 Survival of patients with advanced NSCLC treated with first-line systemic antineoplastic treatment before and after the introduction of ICIs [1]

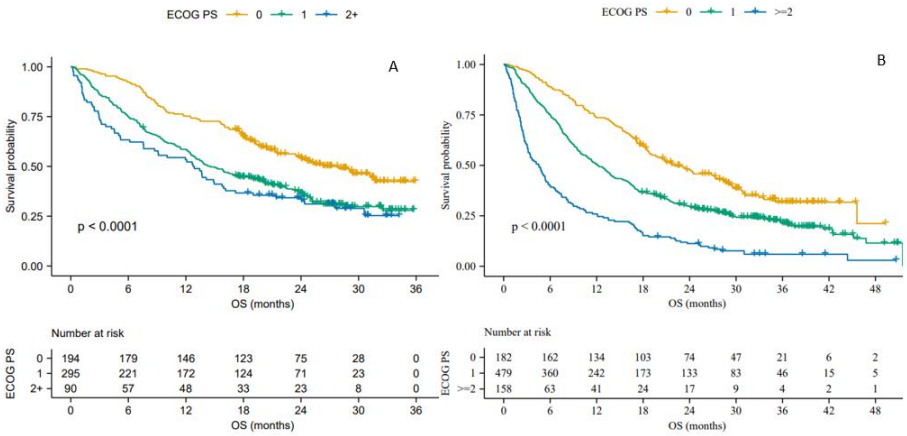
DLCR cohorts	n (%)	mOS, months (95% CI)	1-year OS % (95% CI)	2-year OS % (95% CI)	3-year OS % (95% CI)
Pre-approval cohort	1,658 (100)	7.8 (7.4 – 8.2)	31 (29 – 33)	12 (10 – 14)	6 (5 – 7)
Post-approval cohort	2,055 (100)	11.0 (10.2 – 11.9)	48 (46 – 50)	27 (25 – 29)	18 (16 – 20)
CTx	1,573 (77)	9.5 (8.9 – 10.3)	43 (40 – 45)	22 (21 – 25)	14 (12 – 17)
ICI	482 (23)	19.0 (16.0 – 22.0)	64 (60 – 68)	42 (38 – 47)	29 (24 – 35)

Median overall survival (mOS), 1-, 2-, and 3-year overall survival (OS) rates with 95% confidence interval (CI) before and after the approval of ICI treatment (the pre-approval cohort 01/01/2013 – 08/01/2014 and the post-approval cohort 03/01/2017 – 10/01/2018).
NSCLC; non-small cell lung cancer; DLCR, Danish Lung Cancer Registry; n, number of patients; CTx, chemotherapy; ICI, immune checkpoint inhibitor

6.1.3.2 Kaplan-Meier analyses of ICI-treated patients

The mOS for patients treated with 1L and ≥2L ICI was 18.3 months (95% CI; 16.0 – 21.3) and 12.2 months (95% CI; 10.8 – 13.8), respectively (Figure 6.3) [1, 2]. In 1L, male sex, PS 1 and PS ≥2 (Figure 6.2), never smoking, presence of bone- and liver metastases, and prior palliative radiotherapy (RT) were significantly associated with shorter mOS (Figure 6.3) [1]. In ≥2L, male sex, PS 1 and PS ≥2 (Figure 6.2), presence of bone- and liver metastases, non-adenocarcinoma histopathology, PD-L1 <50% or unknown, and positive EGFR-mutation status were significantly associated with shorter mOS (Figure 6.3) [2].

Figure 6.2 Overall survival for patients treated with 1L or ≥2L according to performance status [1, 2]



A) Patients treated with 1L ICI B) Patients treated with ≥2L ICI

ECOG, Eastern cooperative oncology group; PS, performance status; OS, overall survival

Figure 6.3 Median overall survival in months according to baseline characteristics of ICI-treated patients

ICI cohort	1L	Log-rank test	≥ 2L	Log-rank test
All patients	18.3 (16.0 – 21.3)		12.2 (10.8 – 13.8)	
Age				
<75 years	19.6 (16.5 – 23.1)	0.13	12.9 (11.3 – 14.3)	0.08
≥75 years	15.6 (12.8 – 23.6)		9.9 (8.2 – 14.0)	
Sex				
Male	15.2 (13.0 – 18.3)	0.03	10.0 (9.0 – 11.7)	< 0.0001
Female	21.5 (18.0 – 25.1)		15.1 (13.4 – 17.2)	
ECOG PS				
0	28.0 (21.5 – NR)		22.1 (18.8 – 28.5)	
1	14.6 (12.7 – 19.0)	< 0.0001	12.2 (10.7 – 13.8)	< 0.0001
≥2	12.8 (7.6 – 16.1)		4.5 (3.2 – 5.7)	
CCIS				
0–1	19.0 (15.9 – 23.1)	0.85	13.1 (11.0 – 14.4)	0.52
2+	17.2 (15.3 – 23.5)		11.3 (9.5 – 14.2)	
Smoking status				
Never	10.6 (7.8 – 19.6)		8.3 (6.2 – 13.7)	
Current/former	19.3 (16.6 – 23.4)	0.01	12.8 (11.0 – 14.2)	0.32
Unknown	-		-	
TNM stage				
III	20.2 (14.6 – 29.0)	0.39	15.7 (12.9 – 17.9)	0.07
IV	17.7 (15.8 – 21.4)		11.6 (10.3 – 13.5)	
Brain metastases				
Yes	17.1 (8.2 – 24.1)	0.16	12.3 (10.8 – 14.3)	0.53
No	19.0 (16.0 – 21.7)		12.0 (7.6 – 14.2)	
Bone metastases				
Yes	12.0 (9.5 – 14.9)	< 0.0001	13.7 (12.0 – 16.0)	0.00
No	21.5 (19.0 – 24.9)		9.0 (7.2 – 11.0)	
Liver metastases	13.4 (6.0 – 21.4)	0.00	13.8 (12.3 – 16.1)	< 0.0001
Yes	19.0 (16.1 – 22.5)		6.8 (4.3 – 8.3)	

No				
Adrenal metastases				
Yes	15.8 (12.5 – 24.1)	0.23	12.9 (11.2 – 14.3)	0.65
No	19.0 (16.4 – 21.5)		10.3 (8.1 – 13.7)	
Distant lymph node metastases				
Yes	19.6 (15.7 – 25.0)	0.65	12.0 (10.6 – 13.8)	0.18
No	17.6 (15.3 – 20.5)		13.1 (9.8 – 16.7)	
NSCLC histopathology				
Adenocarcinoma	19.6 (16.4 – 24.0)		13.7 (11.5 – 16.7)	
Squamous cell carcinoma	16.0 (12.1 – 20.2)	0.33	11.0 (9.6 – 13.2)	0.01
Other	19.1 (9.33 – NR)		10.4 (6.5 – 16.9)	
PD–L1				
Negative	NA		9.3 (7.7 – 12.9)	
≥ 1% and < 50%	NA		12.3 (10.0 – 15.4)	
<50 %	14.9 (9.3 – NR)	0.80	NA	0.00
≥50 %	18.3 (16.0 – 21.5)		16.7 (12.8 – 19.9)	
Unknown	NA		11.0 (9.0 – 13.4)	
EGFR mutation				
No	NA		13.2 (11.0 – 16.2)	
Yes	NA	NA	8.2 (6.1 – 13.5)	0.02
Unknown	NA		11.8 (9.9 – 14.3)	
Treatment line				
2	NA		12.1 (10.5 – 14.0)	
3	NA	NA	14.0 (11.0 – 16.9)	0.66
4	NA		8.8 (7.5 – 16.3)	
≥5	NA		10.6 (6.0 – NR)	
Prior treatment with curative intention				
Surgery +/- adj. CTx	19.4 (13.8 – NR)		NA	
CRT	18.3 (13.7 – NR)	0.72	NA	NA
Surgery and CRT	24.4 (9.8 – NR)		NA	
None	18.0 (15.8 – 21.3)		NA	
Prior palliative RT				
Yes	13.8 (10.1 – 21.8)	0.03	NA	NA
No	19.0 (16.1 – 22.5)		NA	

Median overall survival (mOS) in months with 95% confidence interval (CI) according to baseline characteristics. Log rank tests for Kaplan-Meier OS estimates are added.

ICI, immune checkpoint inhibitor; n, number of patients; ECOG PS, Eastern Cooperative Oncology Group performance status; CCIS, Charlson Comorbidity Index Score; TNM, tumor-node-metastasis classification of malignant tumors; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; EGFR, epidermal growth factor receptor; adj. CTx, adjuvant chemotherapy; CRT, chemoradiotherapy; RT, radiotherapy; NR, not reached; NA, not available.

The mPFS for patients treated with 1L and ≥2L ICI was 8.2 months (95% CI; 7.2 – 9.3) and 5.2 months (95% CI; 4.5 – 5.9), respectively [1, 2]. In 1L, PS 1 and PS ≥2, never smoking, and the presence of bone metastases were significantly associated with shorter PFS [1]. In ≥2L, male sex, PS 1 and PS ≥2, never smoking, presence of liver metastases, PD-L1 <50% or unknown status, and positive *EGFR*-mutation status were significantly associated with shorter PFS [2].

The mTTD for patients treated with 1L and ≥2L ICI was 4.8 months (95% CI; 4.1 – 5.5) and 3.2 months (95% CI; 2.8 – 3.6), respectively [1, 2]. In 1L, PS 1 and PS ≥2, and the presence of bone metastases were significantly associated with a shorter TTD [1]. In ≥2L, male sex, PS 1 and PS ≥2, presence of bone- and liver metastases, absence of distant lymph node metastases, PD-L1 <50% or unknown, and positive EGFR-mutation status were significantly associated with a shorter TTD [2].

The only statistically significant factor associated with both TTD, PFS and OS across all treatment lines was PS. The mTTD, mPFS, and mOS according to ICI treatment-line and PS are shown in Table 6.2.

Table 6.2 Median TTD, mPFS and mOS according to ICI treatment-line and PS

	mTTD months (95% CI)	**mPFS months (95% CI)**	**mOS months (95% CI)**
1L			
PS 0	6.9 (5.1 – 9.7)	11.0 (8.5 – 13.9)	28.0 (21.5 – NR)
PS 1	4.3 (3.5 – 5.5)	7.7 (6.4 – 8.8)	14.6 (12.7 – 19.0)
PS ≥2	2.8 (1.4 – 4.2)	6.0 (3.3 – 8.7)	12.8 (7.6 – 16.1)
≥ 2L			
PS 0	6.0 (5.1 – 7.8)	8.9 (7.0 – 11.1)	22.1 (18.8 – 28.5)
PS 1	3.3 (2.8 – 3.8)	5.4 (4.7 – 6.5)	12.2 (10.7 – 13.8)
PS ≥2	1.1 (0.7 – 1.4)	2.0 (1.7 – 2.6)	4.5 (3.2 – 5.7)

mTTD; median time-to-treatment discontinuation; mPFS, median progression-free survival; mOS, median overall survival; CI, confidence interval; 1L, first-line; ≥2L, second- or subsequent-line; PS, performance status

The 2-year OS rates for patients with PS ≥2 was 34% and 11% in 1L and ≥2L, respectively.

For patients treated with ≥2L ICI, univariable Cox regression analysis included specific comorbidities present in >5% of the cases, and they had no significant association with OS.

6.1.3.3 Multivariable Cox regression analyses of ICI-treated patients

In 1L, PS 1 (HR=1.88, 95% CI; 1.45 – 2.42) and PS ≥2 (HR=2.21, 95% CI; 1.52 – 3.21) compared to PS 0, liver metastases (HR=1.45, 95% CI; 1.01 – 2.08), and bone metastases (HR=1.75, 95% CI; 1.37 – 2.24) were significantly associated with poorer OS [1]. Interaction analysis between sex and histopathology showed that male patients with squamous cell carcinomas had a significantly poorer OS, compared to male patients with adenocarcinomas. Furthermore, in patients with squamous cell carcinomas, males had a significantly poorer OS than females [1].

In ≥2L, male sex (HR=1.35, 95% CI; 1.11 – 1.62), PS 1 (HR= 1.88, 95% CI; 1.52 – 2.33) and PS ≥2 (HR=4.15, 95% CI; 3.13 – 5.5) compared to PS 0, liver metastases

(HR= 1.72, 95% CI; 1.34 – 2.22), and bone metastases (HR=1.27, 95% CI; 1.03 – 1.58) were associated with poorer OS, while PD-L1 ≥50% compared to negative PD-L1 status (HR=0.69, 95% CI; 0.48 – 0.98) was associated with improved OS [2]. The interaction analysis between sex and histopathology showed a significantly poorer OS in male patients with adenocarcinoma, compared to female patients with adenocarcinoma [2].

6.2 STUDY III

6.2.1 BASELINE AND TREATMENT CHARACTERISTICS

A total of 123 patients were included. The median age was 67 years, and 56% of the patients were males. NSCLC subtypes were squamous cell carcinoma in 24% and adenocarcinoma in 68%, and 80% (n=98) had PD-L1 ≥50% [3]. PS, PD-L1 TPS, NSCLC subtype, lung and peripheral lymph node metastases were significantly different between patients receiving ICI in 1L and ≥2L [3]. ICI was administered in 1L (n=96) or ≥2L (n=27), and was primarily discontinued due to PD (55%) or toxicity (27%). Around half of the patients (49%) received post-ICI systemic antineoplastic treatment [3].

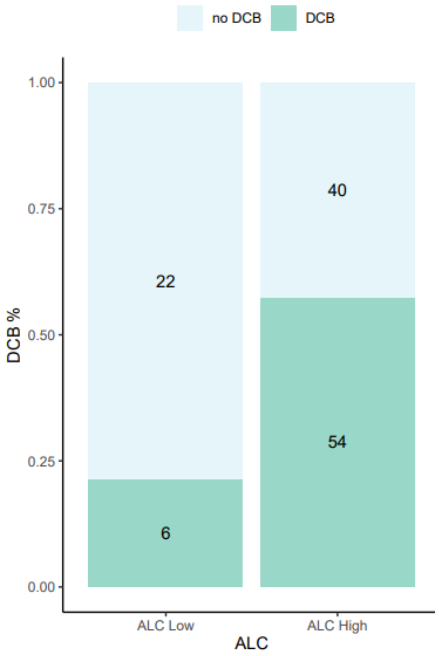
GEP was performed in 25 (33%) of all patients with a baseline histological biopsy (Figure 4.2). Significantly more patients with GEP had squamous cell carcinomas and received ≥2L ICI compared to those without GEP [3].

6.2.2 PREDICTIVE FACTORS OF DURABLE CLINICAL BENEFIT

DCB was observed in 49% (n=60) and did not significantly differ in 1L compared to ≥2L (51% vs. 41%, p=0.40). The presence of liver metastases was significantly associated with not achieving DCB (30% vs. 12%, p=0.02) and ALC above median was significantly associated with DCB (p=0.01) [3]. In univariable logistic regression analyses liver metastases (OR 0.31, p=0.01) and ALC (OR 2.05, p=0.02) were significantly associated with DCB. In multivariable logistic regression analysis liver metastases (OR 0.36, p=0.046) and ALC (OR 1.95, p=0.038) remained significantly associated with DCB [3].

A ROC curve analysis was made to investigate the predictive potential of ALC as a single biomarker for DCB, and this yielded an AUC of 0.63 (Supplementary Figure S4). An optimal cut-point of 1.0 10^9/l was found, corresponding to the 25% quartile, and using ALC dichotomised at this cut-point as a predictive biomarker for DCB resulted in a false positive rate of 0.64 and true positive rate of 0.90. DCB was observed in 21% of all patients with an ALC below the optimal cut-point of 1.0 10^9/l, and in 57% of all patients with an ALC above the optimal cut-point (Figure 6.3) [3].

Figure 6.3 Bar chart presenting the relationship between peripheral lymphocyte counts and durable clinical benefit [3]

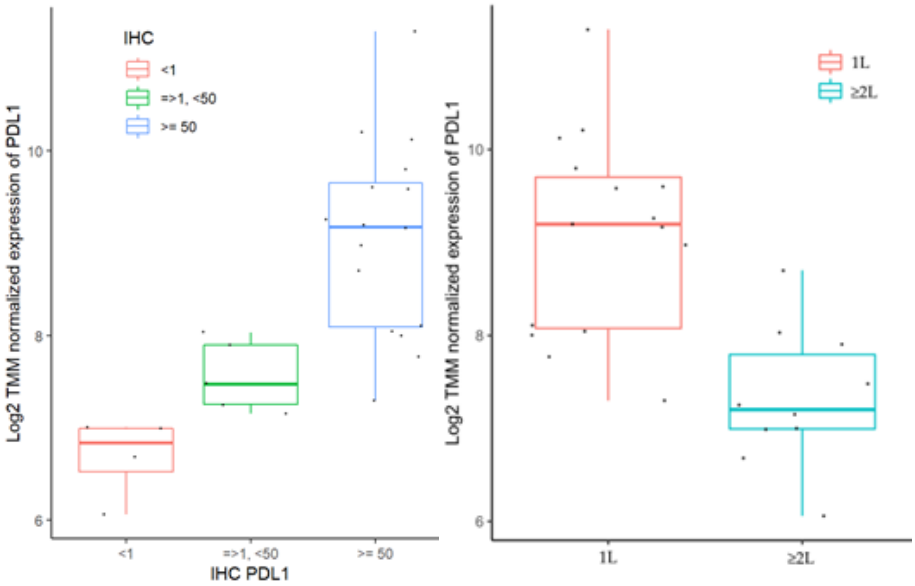


The patients (n=122) were categorized as ALC low or ALC high, separated by the optimal ALC cut-point of 1.0 10^9/l. The values in the bars represent the absolute number of patients in each group. ALC was missing in one patient.
DCB, durable clinical benefit; ALC, absolute lymphocyte count

6.2.3 GENE EXPRESSION ANALYSES

Comparison of gene expressions between patients with and without DCB revealed 53 genes with a p-value <0.05, including PD-L1 (CD274) (p=0.03). However, no genes were significant after adjustment for multiple testing (no FDR <0.05). A strong association between the categorical PD-L1 TPS assessed by IHC and the continuous PD-L1 (CD274) derived by GEP was identified (p=0.00013). Furthermore, PD-L1 (CD274) was differentially expressed between patients receiving 1L and ≥2L ICI (p=0.0017) (Figure 6.4) [3].

Figure 6.4 The association between PD-L1 (CD274) derived by gene expression profiling and A) PD-L1 assessed by immunohistochemistry and B) treatment line [3]

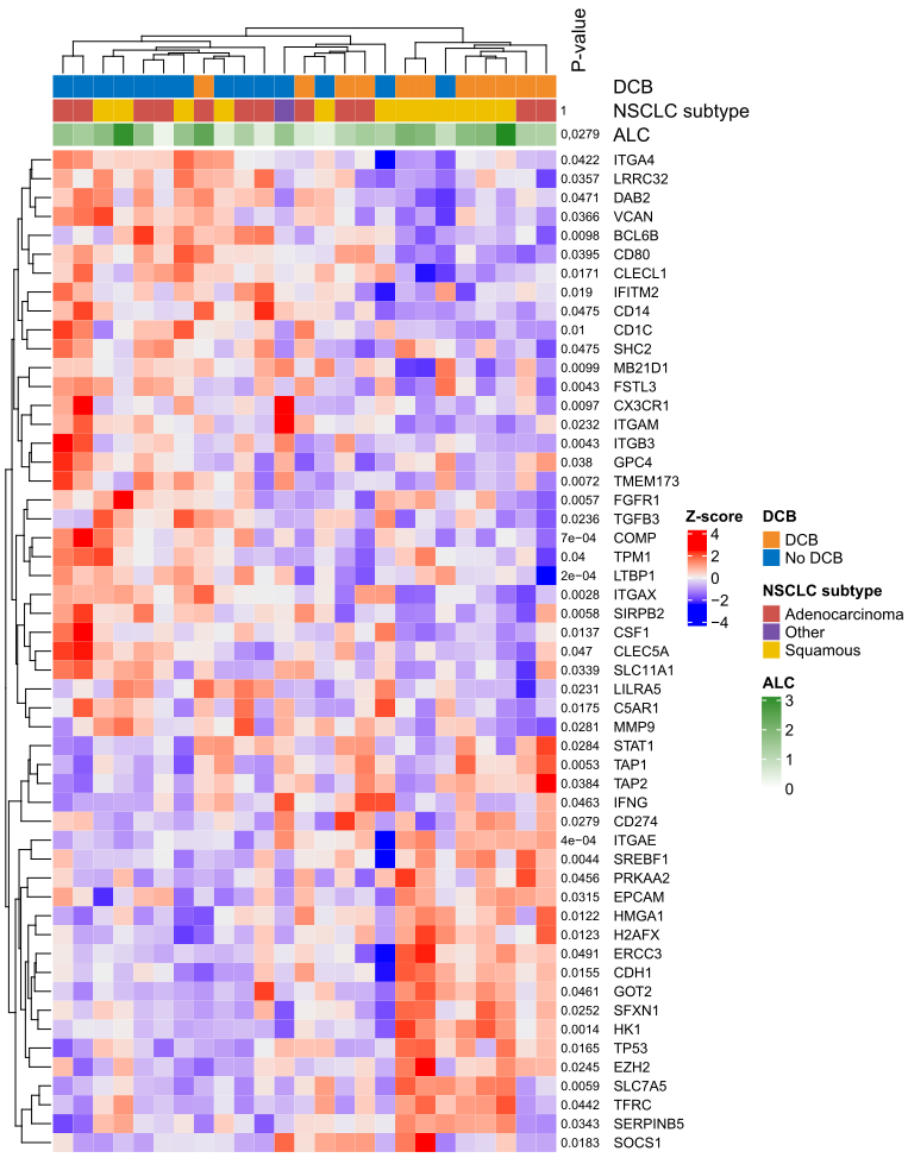


Boxplots of log2 normalized expression of PD-L1 for A) three levels of PD-L1 assessed by IHC (p=0.00013) and B) treatment line (p=0.00017).

PDL1, programmed death-ligand 1; IHC, immunohistochemistry; 1L, first-line treatment; ≥2L, treatment in second- or subsequent line

Hierarchical clustering of the 53 differentially expressed genes with a p-value <0.05 showed that two clusters separated the patients with and without DCB except for two patients, and an intermediary heterogeneous cluster consisted of patients with or without DCB [3].

Figure 6.5 Hierarchical clustering of the 53 differentially expressed genes with a p-value <0.05 [3]



Heatmap of gene expression z-scores for genes with a p-value <0.05 and patients with DCB compared to those without DCB. The patients (columns) (n=25) and the genes (rows) are clustered using hierarchical clustering based on euclidean distance. The dendrograms visualize the order of the clustering. In the top, three annotation rows are added to indicate each patients DCB status, NSCLC histopathological subtype, and ALC. A p-value is listed for each row. The p-values for NSCLC subtype and ALC compare DCB vs. no DCB using a Fisher's exact test and unpaired t-test, respectively. The p-value in front of the genes derive from the gene expression test [3].

The gene expression signature scores in patients with and without DCB were compared. No signature scores had an FDR <0.05. However, four signatures had unadjusted p-values <0.05. The dendritic cell (DC) (p=0.025, log2FC= -0.92), myeloid (p=0.024, log2FC= -0.80), and TGF-β (p=0.047, log2FC= -0.92) signature scores were higher in patients without DCB and the JAK/STAT loss signature scores (p=0.005, log2FC= 1.41) were higher in patients deriving DCB [3].

6.2.4 NEXT GENERATION SEQUENCING

Data from the TSO500 gene panel was available in only 42% (n=51) of the patients (n=123), and in only 24% (n=6) in the final GEP cohort. In the total cohort, 20% (n=24) had TMB-high tumor samples, and TMB status was not associated with DCB in the univariable logistic regression analysis. No patients had MSI-high tumor samples, and MSI status was not included in the statistical analyses [3].

CHAPTER 7. DISCUSSION

7.1 SURVIVAL BEFORE AND AFTER ICI IMPLEMENTATION

Study I demonstrated increased long-term survival after the implementation of ICIs, for patients receiving 1L systemic antineoplastic treatment (Table 6.1) [1]. The magnitude of the survival-improvement was most likely driven by the implementation of ICIs, since no other large changes in diagnostics, treatment or palliative care was introduced in this time period. The TNM stage was missing in a larger proportion of patients in the DLCR post-approval cohort, and according to the annual reports from the DLCR, the proportion of patients with TNM stage IV decreased from 2015 to 2017-2018 [1, 5]. However, the missing TNM stages were primarily in the DLCR-CTx cohort and would not impact the OS in the DLCR-ICI cohort [1]. Furthermore, the proportion of female patients and adenocarcinomas increased [1, 5]. Though, it should be noticed that the proportion of patients without a TNM-classification and with the pathology classification 'not otherwise specified' decreased in the same time period indicating an improvement in TNM-classification and pathology diagnostics [5]. Recently, Italian and Canadian studies also demonstrated improved survival in patients with advanced NSCLC treated with 1L systemic antineoplastic treatment after ICI implementation [155, 156]. Patients who did not receive any treatment were not included in this dissertation, and still accounts for approximately 20% of patients diagnosed with lung cancer [5].

7.2 PATIENT POPULATIONS, PROGNOSTIC AND PREDICTIVE CLINICAL FACTORS

The approval of new drugs is based on results from the RCTs, however, only around 30% of patients with advanced NSCLC treated with ICIs in daily clinical practice meet the in- and exclusion criteria in the pivotal ICI-RCTs [157]. In study I and II, around 20% of the patients aged ≥75 years, 20% were PS ≥2, and around one third had moderate-to-severe comorbidity according to CCIS [1, 2]. Those subgroups are typically underrepresented in RCTs. A review of clinical features affecting survival in ICI-RCTs suggested that OS could be affected by sex, PS, bone- and liver metastases, and smoking status [158]. Additionally, a meta-analysis of ICI-RWS including previously treated patients, demonstrated that the ORR, PFS, and mOS were comparable to results from the RCTs after adjustment for PS, age, liver- and CNS-metastases [159]. In both study I and II, PS 1 and PS ≥2, bone metastases, and liver metastases were associated with a significantly shorter OS [1, 2].

7.2.1 PERFORMANCE STATUS

PS ≥2 was associated with a significantly shorter mTTD and mPFS in both 1L and ≥2L, with a mPFS of only 2.0 months in ≥2L [1, 2]. Furthermore, the mOS in ≥2L was

significantly poorer; only 4.5 months [1, 2]. Therefore, the clinical benefit in patients with PS ≥2 is questionable, particularly in patients receiving ≥2L ICI. However, some patients with PS ≥2 became long-term responders (2-year OS rates of 34% and 11% in 1L and ≥2L, respectively), and the upfront identification of those patients who derive benefit remains a challenge. If patients with PS ≥2 are considered unsuitable for ICI treatment by the physician, standard CTx or BSC could be a better solution for the patient. No recent data on survival in CTx-treated patients with PS ≥2 is available; hence it is difficult directly to compare ICI- and CTx treatment in this patient population. In 2002, Schiller *et al*. demonstrated a mOS of 3.9 months in patients with PS ≥2 treated with a 1L platinum-doublet CTx regimen [31]. In 2004, Hanna *et al*. randomized patients for 2L pemetrexed or docetaxel and demonstrated a mOS of 3.6 months and 2.2 months, respectively, in patients with PS ≥2 [33]. Compared to these old studies, ICI-treatment may clinically benefit patients with PS ≥2.

We showed that PS 0 was associated with a high mOS in patients treated with ICI in both 1L (28.0 months) and ≥2L (22.1 months) compared to the pivotal RCTs [1, 2, 46-50]. However, the RCTs did not report on ICI-efficacy and survival in patients with PS 0 compared to PS 1. In our studies, the mOS difference between PS 0 and PS 1 was around 13 months in 1L and 10 months in ≥2L, which emphasizes the prognostic importance of PS in daily clinical practice, and the potential introduction of bias when patients with PS 0 and PS 1 are categorized and analysed as one cohort in RWS, and potentially in RCTs. In the multivariable Cox regressions PS 0 was the reference covariate, hence the statistical difference between PS 1 and PS ≥2 was not assessed. However, the KM curves showed significant differences in OS according to PS (Figure 6.2). In 1L, the KM curve of PS 1 approximated that of PS ≥2, which could indicate a clinical misclassification of PS ≥2 as PS 1 in patients treated with 1L ICI. This could be explained by the approval of 1L ICI for patients with PS 0-1 only. This migration in PS classification has not previously been reported, and the usual comparison of efficacy and survival between patients with PS 0-1 and PS ≥2 may be affected by this phenomenon. This issue could also exist in RWS of other 1L treatments approved only for patients with PS 0-1 and would decrease the true endpoint differences between PS 0-1 and PS ≥2.

7.2.2 METASTASES

In study I and II, disease stage was not included in the analyses due to the interaction with metastatic sites. Bone- and liver metastases were associated with a significantly impaired survival [1, 2]. In study I and II, 28% and 26% had bone metastases, respectively; however, the extent of bone tumor burden was not recorded. Bone metastases in patients with advanced NSCLC have been associated with a 'cold' tumor immune phenotype and attenuated ICI efficacy [160]. However, improved outcomes have been observed with the addition of bisphosphonates and/or combination therapy of ICI and CTx [161]. The presence of liver metastases has also been negatively associated with survival in other RWS and RCTs [2, 158, 162]. This may

be explained by lower CD8+ T-cell infiltration in liver metastases compared to other metastatic lesions, and increased PFS has been observed in liver metastases with combined PD-L1 TPS ≥1% and CD8+ T-cell infiltration [163].

Other metastatic sites such as brain metastases did not significantly impact OS [1, 2]. Similar results have been demonstrated in other RWS [164]. However, impaired PFS has been observed in patients with brain metastases which may be explained by downregulation of an immune gene expression signature in brain metastases compared to primary biopsies [165, 166]. Local treatment of identified and/or symptomatic brain metastases with surgery, whole brain RT or SBRT before ICI treatment could explain the insignificant OS impact of brain metastases. Furthermore, it has been demonstrated that age <70 years, adenocarcinoma histopathology, previous cranial radiation therapy (≥3 months prior to ICI initiation), and brain metastases present at diagnosis were associated with increased intracranial disease control [167]. Despite the large incidence and prognostic impact of metastatic burden and specific metastatic sites, particularly bone metastases, they have rarely been reported in the RCTs (Table 2.3) [161, 168]. However, the Checkmate 9LA RCT, which included patients with stage IV or recurrent NSCLC randomized for either nivolumab plus ipilimumab and platinum-based CTx or CTx alone, have reported on bone metastases which may pave the way for reporting on metastatic sites in future RCTs [169].

7.2.3 SEX AND NSCLC HISTOPATHOLOGICAL SUBTYPE

In both study I and II, KM estimates showed impaired OS in male compared to female patients [1, 2]. However, in multivariable Cox regression analyses the impaired OS in males only remained significant in those receiving ICI as ≥2L treatment. Additionally, interaction between sex and NSCLC histopathological subtype was observed in both 1L and ≥2L [1, 2]. Improved OS in females with adenocarcinomas was observed already in the pre-ICI era [170]. At the same time, the excess risk for male patients was reduced by 80% when adjusting for known prognostic factors (treatment-related factors, lifestyle- and tumor characteristics) [171]. Subsequently, sex-associated differences in immune responses, including immune features associated with ICI efficacy, have been described; however, divergent results have been observed in NSCLC [172, 173].

7.2.4 PERIPHERAL IMMUNE CELL COUNTS

In study III, high baseline ALC was significantly associated with DCB regardless of treatment line and with OS in patients treated with 1L ICI [3]. Additionally, the optimal cut-point of 1.0 10^9/l defined in study III is easily applicable in daily cancer care. However, study III was hypothesis-generating and the predictive value of an ALC cut-point of 1.0 10^9/l should be verified in independent cohorts. Peripheral immune cells, including ALC, have also been associated with ICI efficacy in other studies. High pre- and post-ICI peripheral lymphocyte counts, the distribution of lymphocyte subsets combined with PD-1 expression on T-cells before ICI treatment, and the dynamics of

exhausted T cells during ICI treatment have been associated with improved survival in patients with NSCLC [174, 175]. The most extensively investigated peripheral immune cell biomarker is NLR, which was not significantly associated with DCB in study III [3]. Low baseline NLR and early dynamics in NLR or derived NLR have been associated with improved survival in ICI-treated patients with NSCLC [176-178]. A post hoc analysis of the phase III OAK trial showed that NLR was more strongly associated with OS in patients treated with ICI compared to CTx, indicating a predictive potential [179].

7.3 ICI TREATMENT CHARACTERISTICS

ICI treatment discontinuation due to progressive disease was observed in half of the patients in both study I and II. Notably, half of those patients discontinued the treatment within 6 cycles, corresponding to approximately 4 months (Figure 6.1) [1, 2]. Hence, more than 25% of all the ICI-treated patients derived no DCB when defined as PFS <6 months. A recent real-world multicentre study of patients with NSCLC treated with nivolumab found BMI <25, ECOG PS >1, NLR >2.91, and concomitant treatment with antibiotics and glucocorticoids to be associated with early treatment discontinuation defined as less than 6 ICI cycles [180]. Additionally, study I showed that only around one third of the patients received post-ICI systemic antineoplastic treatment [1]. Another RWS showed that 22% of the patients receiving 1L ICI received subsequent line systemic antineoplastic treatment during the study period [181]. This likely reflects some cases of long-term response to ICI, but for the majority of patients ineligibility for further systemic treatment. The high proportion of patients with early progressive disease underline the importance of choosing the right treatment for the right patient at the right time. In study I and II, information on irAEs were retrospectively obtained by manual data extraction from EHRs. In other previous RWS, data on irAEs have been inconsistently collected and managed. In a few RWS, irAEs have been retrospectively graded according to the Common Terminology Criteria for Adverse Events (CTCAE) based on EHRs [182]. However, this approach is associated with data collection bias, and should not be directly compared with prospectively CTCAE-graded irAEs in the RCTs. Unfortunately, no international consensus on reporting of irAEs in RWS exists.

7.4 RESPONSE EVALUATION AND ENDPOINT DEFINITIONS

The comparison of studies remains challenging. RWS are heterogenous according to patient populations, but also according to definitions of covariates and clinical endpoints. In study I and II, the clinical endpoints were OS, PFS, and TTD with PD definition based on both CT-scans and the physician's decision. The definitions were chosen due to the inconsistent use of the RECIST in routine cancer care. Furthermore, Griffith *et al.* demonstrated that real-world progression-based endpoints correlated with OS, and that a clinician-anchored approach combined with radiology reports were more optimal than RECIST for characterizing progression from EHR-data [183,

[184]. Due to the heterogeneity in endpoint-definitions used in the RWS, a redefinition of real-world endpoints in ICI-trials has been suggested [184, 185]. In particular the definition of SD is ambiguous in both RWS, RCTs, and biomarker studies, and the definition in ICI trials may include both a survival- and a tumor growth parameter [186]. Additionally, terms like DCB, early progressive disease, long-term responders and long-term survivors are widely used, but no scientific guidelines describe those definitions, which complicate direct comparisons of study results.

7.5 RANDOMIZED CONTROLLED TRIALS VS REAL-WORLD STUDIES

The RCTs have high internal validity and provide ideal conditions to measure efficacy; the true biological effect of a treatment [187, 188]. On the other hand, the phase IV or RWS have high external validity and measure effectiveness; the beneficial effect observed, when the treatment is used for patients treated in daily cancer care [187, 188]. Increased emphasis has been put on treatment effectiveness in typical RCT-ineligible patients, and at the ESMO congress in September 2022, the IPSOS study late-breaking abstract was presented [189]. The IPSOS study included patients, ineligible for 1L platinum-based CTx and 1L clinical trials, and the participants were randomized for atezolizumab (an anti-PD-L1 antibody) or single agent CTx (2:1). The median age was 75 years (31% ≥80 years), 72% were male, and 82% had ECOG PS ≥2 [189]. Compared to single agent CTx, atezolizumab significantly improved the 2-year OS rate (24.3% vs 12.4%), ORR, median duration of response, and OS across PD-L1 levels, PS and histopathology [189]. These results are comparable to our results from study I and II, showing that more male patients had a poor PS and more comorbidity, and that some 1L ICI treated patients with PS ≥2 achieved long term responses [1, 2]. The data confirms the advantages of both RCTs and RWS and the synergy between the two study designs.

7.6 BIOMARKER ANALYSES AND FEASIBILITY

The focus on clinical applicability of RCT results, also accounts for biomarker studies. In biomarker research, the participants and the investigated samples must reflect the target population and the intended use of the biomarker(s) [190]. Currently, ICIs are mainly used in advanced or metastatic NSCLC and therefore, biomarker studies should include patients with advanced/metastatic disease and the biological material used for the biomarker assessment should resemble the samples used in the routine diagnostic framework.

The demand for multiple biomarker-testing in NSCLC is rapidly increasing owing to the continued discovery of new druggable alterations. Different predictive models for ICI efficacy have been proposed, however, the application of multiple detection techniques on scarce tissue samples remains a challenge in routine clinical settings[129, 191]. Despite the improved response rates and OS with biomarker-driven therapy, the MYLUNG consortium pragmatic study demonstrated that less than 50% of diagnostic

non-squamous NSCLC tissue samples are analysed for both *EGFR*, *ALK*, *ROS-1*, PD-L1 and *BRAF* status [192]. Additionally, NGS testing occurred in less than 50% of the cases [192]. Another study demonstrated that 27% of NSCLC tissue samples were analysed simultaneously for both *EGFR*, *ALK*, *ROS-1* and PD-L1 status, and sufficient tissue was available in only 25% of the included cases [193]. In study III, the proportion of FFPE samples suitable for GEP was 33% (25/74). Furthermore, only 50% of the patients had histological tissue samples. The low GEP success rate in study III, was caused by low RNA concentrations and poor RNA quality, probably due to thin tissue sections with low proportions of intact cells [3, 145]. Despite mimicking the target population and intended use of the biomarker, study III was a small non-randomized and explorative study, and careful interpretation of the GEP results is highly encouraged. Validation cohorts from the Cancer Genome Atlas is typically used in NSCLC biomarker studies; however, primarily resections from patients with early-stage disease are included. Currently no large-scale GEP of advanced-stage NSCLC exists [194].

Recently, a more efficient and tissue-sparing approach to cancer diagnosis and biomarker testing has been proposed called the "Combiome" [195]. This diagnostic framework aimed at reducing sequential biomarker testing, and thereby increase the proportion of patients with complete biomarker-testing leading to improved treatment-selection, response rates and survival for all cancer patients. However, this 'Combiome' setup is expensive, may not be necessary for all patients, and patient eligibility criteria for the 'Combiome' framework should be discussed before clinical implementation.

7.7 GENE EXPRESSION PROFILING

In study III, PD-L1 (CD274) was negatively correlated with LTBP1 ($p<0.05$). LTBP1, latent transforming growth factor beta binding protein, maintain TGFβ (TGFB1) in a latent state and release TGFβ to the TME mediated by integrins or cleavage [196, 197]. TGFB1 is a pleotropic cytokine which inhibits anti-tumor immune activity and promotes tumor growth and survival when present in the TME [198]. A new TGFβ-dependent signalling pathway, the MRTF-A-NF-κB/p65 axis, mediates PD-L1 transcription leading to tumor immune evasion [199]. The inverse correlation between LTBP1 and PD-L1 found in our study, could be explained by this signalling pathway. A pre-clinical study in anti-PD-1 refractory mice showed profound anti-tumor response and improved survival when combining an anti-PD-1 antibody with a selective inhibitor of latent TGFB1 [200]. In the current study, TGFβ signatures were also higher in patients without DCB. Clinical investigation of dual inhibition of TGFβ and PD-(L)1 is ongoing in many solid tumors including NSCLC [201].

TAP1, transporter associated with antigen processing 1, correlated positively with PD-L1 (CD274) in study III ($p<0.05$). TAP1 and PD-L1 are controlled by the JAK/STAT pathway upon interferon gamma (INF-γ) exposure [202]. Additionally,

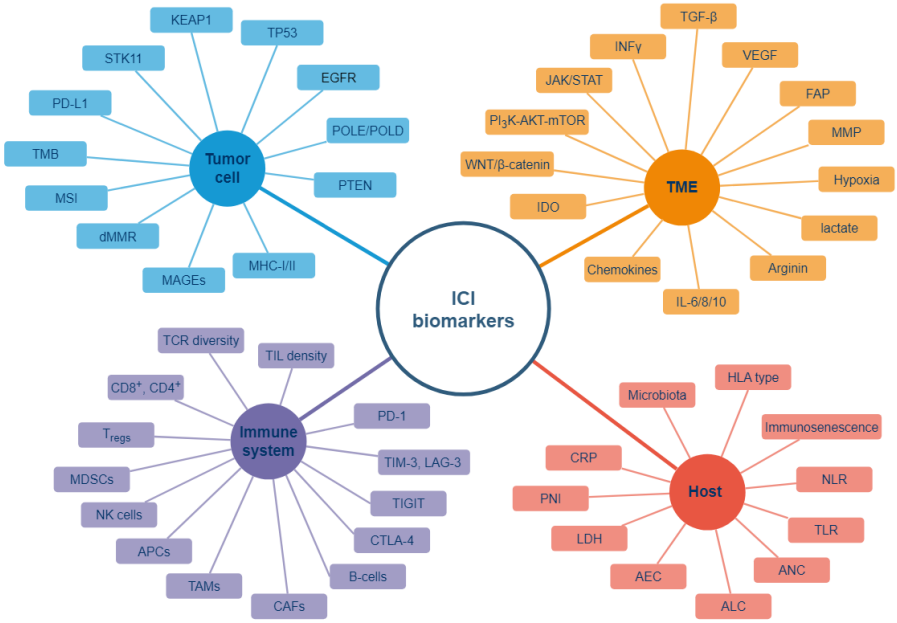
JAK/STAT loss signature scores were found to be higher in patients with DCB in study III. Previously, impaired INF-γ signalling pathways and JAK/STAT mutations in tumor cells have been associated with impaired ICI efficacy in patients with malignant melanoma [203, 204]. On the other hand, JAK/STAT-mediated chronic inflammation in pancreatic cancer impaired cytotoxic T-cell activation and decreased anti-PD-1 efficacy, and inhibition of STAT3-mediated immunosuppression in the TME may be a complementary immunotherapy target [205, 206]. The JAK/STAT pathway plays an essential role in the differentiation of T-helper cells, and JAK/STAT inhibition in Tregs has shown downregulation of Foxp3 [207, 208]. Hence, the JAK/STAT function is cell specific and the impact of JAK/STAT loss on ICI-efficacy seems to be cell-dependent. In study III, the JAK/STAT loss signature, defined by the manufacturer, was not restricted to a specific cell type, and could represent JAK/STAT pathways in both tumor cells and immune cells in the TME. INF-γ has pleiotropic impact on ICI efficacy, and recent GEP of ICI-sensitive and ICI-resistant tumor cells revealed a strong association between INF-induced ICI resistance and expression of the TNF-receptor regulating gene *Ripk1*; hence, this may become a future treatment target in ICI-resistant patients [209].

In study III, DC signature scores were higher in patients without DCB, which may reflect a primary resistance mechanism consisting of abundant but inactivated DC cells in those patients. DCs are key antigen presenting cells that play an essential role in initiation of T-cell responses against tumor [210]. However, STAT3 inhibits DC maturation, immature DCs generally induce immune tolerance, and tumors may disrupt normal DC function leading to tumor immune evasion [206, 210]. The myeloid signature measure key marker and effector genes of myeloid lineage immune cells, and in study III these signature scores were higher in patients without DCB. Myeloid cells in the TME include tumor-associated macrophages (TAMs), tumor-associated neutrophils, and myeloid-derived suppressor cells (MDSCs) which all facilitate tumor cell growth and invasion and suppress adaptive immune responses [211, 212]. ITGAE also known as CD103 correlated positively with PD-L1 (CD274) in study III. ITGAE interacts with E-cadherin and promotes cytolytic T-cell responses against tumor, and ITGAE-expressing $CD8^+$ T-cells have been associated with improved response to ICIs [213, 214].

7.8 OTHER PROPOSED BIOMARKERS OF ICI EFFICACY

A wide range of biomarkers have been proposed to predict ICI efficacy. The interplay between many factors related to the tumor cells, immune system, TME, and host is complex as roughly illustrated in Figure 7.1. Therefore, multi-omics approaches for the prediction of clinical endpoints in ICI-treated patients with NSCLC have been suggested [215].

Figure 7.1 Overview of proposed biomarkers of ICI efficacy



Tumor-intrinsic factors such as NSCLC driver mutations and gene rearrangements including *EGFR*, *HER2*, *ALK*, *ROS1*, *RET* and *MET* are predominantly associated with impaired response to ICIs [216]. However, *KRAS* and *BRAF V600E* mutations are associated with improved OS, whereas the co-occurrence *of KRAS* and *STK11* or *KEAP1* mutations impairs the ICI efficacy [216-219]. This mechanism could be explained by the association of *STK11* and *KEAP1* mutations with distinct immunophenotypes in *KRAS* mutant but not in *KRAS* wild type lung adenocarcinomas [219]. However, a large RWS found *STK11* and *KEAP1* to be poor prognostic factors regardless of treatment type [220]. Additionally, the expression of co-inhibitory receptors such as TIM-3, LAG-3, and TIGIT may contribute to ICI resistance, and clinical trials targeting those checkpoints are ongoing (such as NCT04140500 and NCT03708328 on ClinicalTrials.gov) [221-223]. However, double ICI therapy has not yet been implemented as a routine treatment of patients with NSCLC in Denmark.

Most recently, an artificial-intelligence-powered spatial analysis of TILs defined three immune phenotypes; inflamed, immune-excluded, and immune-dessert based on TIL-density in cancer epithelium and cancer stroma [224]. The immune phenotypes were significantly associated with ICI response and not with chemotherapy response which supports their predictive value in relation to immunotherapy [91, 224]. Additional GEP revealed enrichment of $CD8^+$ T cells, memory T cells, memory B cells, and M1 macrophages in the inflamed phenotype and enrichment of M0 macrophages, naïve B

and FOXP3 cells in the immune-excluded phenotype. Enrichment of M2 macrophages, neutrophils, and $CD68^+$ cells was observed in the immune-dessert phenotype [224].

The TME comprise a complex interplay between different regulatory mechanisms which also affect the function of the tumor cells and the TILs. Other immune cells such as natural killer cells, DCs, B cells, MDSCs, and TAMs also play important roles in tumor evolution [225]. Furthermore, cancer-associated fibroblasts, cytokines, chemokines, metabolites, hypoxia and lactate affect the function of both tumor and immune cells in the TME [226]. Recently, targeting TAM receptors has been proposed as a novel therapeutic target to overcome ICI resistance [227].

The association between gut microbiome and ICI efficacy has been investigated, and abnormal gut microbiome and use of antibiotics have been associated with primary ICI resistance [228]. This may be explained by the association between gut bacteria and peripheral immune cell dynamics [229].

The one aim to perform these biomarker studies is to improve the quality of life and survival of patients with NSCLC. However, statistical considerations according to biomarker discovery and validation remain crucial in establishing the application of biomarkers into routine cancer care [190].

CHAPTER 8. STRENGTHS AND LIMITATIONS

8.1 STUDY I-II

8.1.1 STRENGTHS

The major strength of these studies is the nationwide character and the substantial inclusion of all consecutive patients with advanced NSCLC treated with ICI in Denmark. Due to the equal and free access to health care (including ICIs within the framework of national guidelines) for all Danish patients, the risk of selection bias is minimal. Furthermore, the large sample sizes allowed for subgroup analyses of patients that are usually underrepresented in the RCTs, but widely treated in routine clinical cancer care, such as patients with PS ≥2, moderate-to-severe comorbidity, organ metastases, and age ≥75. Because of the Danish CPR system, the completeness of follow-up is very high. Data from the DLCR enabled the OS comparison before and after the implementation of ICIs, which was not previously investigated for this patient population [1, 2]. Additionally, this study strengthened the national research collaboration within DOLG, and paved the way for future similar nationwide studies.

8.1.2 LIMITATIONS

The retrospective study design implies a lack of data accuracy and completeness. This is particularly related to the CTx cohorts from the DLCR (study I) and to patients treated with ≥2L treatment (study II). The validity of data on comorbidity, smoking status, PS, toxicity, and tumor response evaluation may be reduced due to the retrospective design. Data on potential confounders including laboratory data, concomitant use of glucocorticoids or antibiotics, and BMI was not obtained [1, 2]. Study I and II were not appropriate for causal inference, partly due to the low internal validity [230]. This would require application of inference methods such as propensity score matching of the patients in our studies and participants in the ICI-RCTs, of the pre- and post-approval DLCR cohorts, and of the DLCR-ICI and DLCR-CTx cohorts. However, propensity score matching of the DLCR cohorts were considered futile due to the unequal distribution of missing data on disease stage, and lack of information on significant prognostic factors such as PS and metastatic sites. Furthermore, no direct comparison of the results in study I and II (1L vs ≥2L) should be made because of minor differences in study definitions and covariates, and primarily due to the lack of statistical comparisons. The optimal design for studying ICI effectiveness is randomization in a real-world setting, however, observational prospective or retrospective studies are less prone to selection bias, less resource-intensive and hence more feasible [231].

8.2 STUDY III

8.2.1 STRENGTHS

The patients were treated in daily clinical practice, were consecutively included, and the tissue samples used for GEP were routine diagnostic samples. Both factors increase the external validity and the possibility to clinically implement a potential new biomarker. Thus, the biomarker study complied with the clinical context; patients with unresectable, incurable advanced NSCLC treated with ICIs and availability of only scarce tissue samples. The bridge between basic science and clinical research enable to correct for potential confounding prognostic and/or predictive clinical factors in the assessment of new biomarkers. The comparison of patients with and without GEP showed the representativeness of the GEP cohort and possible differences in patients who are eligible for GEP in routine clinical practice. No patients were lost to follow-up.

8.2.2 LIMITATIONS

The final number of samples that qualified for GEP was small, which affected the probability to discover significant gene expression signatures according to DCB. Most patients treated with ≥2L ICI, had received CTx between the time of diagnostic tissue sampling and the initiation of ICI treatment, which could affect the gene expression and hence the predictive or prognostic value. Furthermore, heterogeneity according to biopsy site (primary tumor versus distant metastasis), NSCLC histopathological subtype, TNM stage, and PS could affect biomarker performance. Additionally, we did not have a validation cohort and randomization between ICI and CTx has become difficult due to the wide approval of ICI-based 1L regimens. The final analysis did not include criteria on treatment duration or a minimum duration of survival. The study specific methods were applied after routine diagnostic testing; hence the histological material was scarce and of poor quality. RNA amplification could have been performed in low input samples leading to increased Qubit concentrations prior to hybridization. The pathological evaluation was performed after study termination, and the number of included patients were not based on tissue samples suitable for GEP and NGS. The blood samples could have contained more known prognostic and potential predictive/prognostic values such as CRP, lactate dehydrogenase, and additional differential counting of blood cells.

CHAPTER 9. CONCLUSIONS

In advanced NSCLC patients without EGFR mutations or ALK rearrangements receiving 1L systemic antineoplastic treatment, the mOS and long-term survival have improved after the implementation of ICI treatment in Denmark. The mOS increased from 7.8 months before ICI-approval to 11.0 months after ICI-approval. This increase was primarily driven by the patients treated with 1L ICI, since the mOS in this cohort reached 19.0 months compared to 9.5 months in patients receiving 1L CTx [1]. The previously almost unknown phenomenon of long-term survival in patients with advanced NSCLC was observed in patients receiving 1L ICI, with a 3-year OS rate of 29% compared to only 6% in patients receiving 1L CTx before the ICI-implementation in Denmark [1].

Compared to the pivotal anti-PD-1 RCTs, the mOS was lower in patients treated with ≥2L ICI, but comparable in patients treated with 1L ICI. In fact, the mOS of patients with PS 0 was higher compared to the RCTs in both 1L and ≥2L ICI [1, 2]. PS was the only significant prognostic factor for both mTTD, mPFS, and mOS regardless of ICI treatment line. The subgroup of patients with PS ≥2 is heterogeneous and includes patients with very early progression but also long-term survivors [1, 2]. Therefore, ICI treatment of patients with PS ≥2 should be carefully considered.

The presence of bone- and liver metastases were significantly associated with impaired OS regardless of ICI treatment line whereas age ≥75 years and comorbidity according to CCI were not [1, 2]. Therefore, metastatic sites should be reported in future RCTs. ICI treatment is also an option in patients with high chronological age.

In study I, II, and III approximately half of the patients discontinued ICI due to PD [1-3]. In study I and II, PD occurred within 6 ICI cycles in half of the patients [1, 2]. Hence, 20-25% of all ICI-treated patients experienced PD within around 4 months, and those patients may rather benefit from other treatment options if they could be identified up front [1, 2].

Study III was a hypothesis-generating biomarker study that included 123 patients with advanced NSCLC treated with ICI in first- or subsequent treatment line in routine clinical cancer care. Around half of the patients had DCB [3]. Absence of liver metastases and high ALC were significantly associated with DCB, and an ALC above 1.0 10^9/l may predict DCB in patients with advanced NSCLC treated with ICI in daily cancer care [3]. GEP was performed in 25 of the patients. GEP-assessed PD-L1 was highly correlated with IHC-assessed PD-L1 and treatment line, which indicate a clinical relevance of GEP in routine diagnostics [3]. Higher JAK/STAT loss signature scores were observed in patients with DCB whereas higher DC, myeloid and TGF-β signature scores were observed in patients without DCB [3]. However, no single gene expressions or gene expression signatures were significantly associated with DCB

when adjusting for multiple testing. The low proportion of GEP-suitable tissue samples should be considered in future GEP-studies that include routine diagnostic biopsies from patients with advanced NSCLC.

CHAPTER 10. FUTURE PERSPECTIVES

RWE is increasingly used for marketing authorization applications and extensions of indications for already authorized treatments and helps to identify areas that require further investigation [232]. Based on experience from this dissertation, international guidelines should be devised in order to improve the RWE, including harmonization and standardization of real-world study designs (sample size, prospective data recording, etc.) and definitions of covariates, endpoints, and statistical methods. Furthermore, revision of the CONSORT statement could increase the transparency and transferability of RCT results, and thereby improve the applicability for clinicians using ICI treatment in daily cancer care.

As demonstrated in this dissertation, PS remains the major prognostic OS factor in patients with NSCLC, and PS misclassification by the physicians may occur. Detailed analysis of factors contributing to poor PS could be performed and a more nuanced and standardised PS evaluation should be developed and validated. Afterwards, interventions to improve the outcome for poor PS patients should be prioritized.

Improvement of the DLCR according to oncology data and indicators is warranted, and this work is prioritized and ongoing. Furthermore, the cross-regional and cross-sectorial health IT infrastructure should be harmonized, which includes effortless data sharing between the EHRs and the RKKP. This could partly be facilitated by structured real-time registration of clinical, molecular, and patient-reported outcomes and quality of life data in EHRs for both clinical and research use.

Investigation of predictive ICI biomarkers including resistance biomarkers should be ongoing. The biologically relevant gene expression signatures should be validated in future larger studies. Comparison of gene expression signatures in early and advanced stage NSCLC, metastatic and primary lesions, and pre- and post-treatment biopsies may add knew knowledge to the biological mechanisms of ICI response and resistance. Furthermore, future biomarker studies should consider the daily clinical practice according to the intention-to-treat patient populations based on national/international treatment guidelines. However, in order to obtain sufficient biological material for multiple biomarker-testing in routine cancer care and for concomitant biomarker research, the diagnostic work-up for patients with advanced NSCLC should be optimized. Additionally, comparison with control cohorts should be considered in order to distinguish between prognostic factors in NSCLC and predictive biomarkers for ICI efficacy.

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PUBLISHED PAPERS AND PAPER IN PREPARATION

PAPER I

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Article

Nationwide Survival Benefit after Implementation of First-Line Immunotherapy for Patients with Advanced NSCLC—Real World Efficacy

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Simple Summary: The expected change in overall survival (OS) in patients with advanced non-small cell lung cancer (NSCLC) after the clinical implementation of immune checkpoint inhibitor therapy (ICI) has not been substantially investigated in large real-world cohorts outside randomized controlled trials (RCTs). In this nationwide study, we compared OS before and after the implementation of ICI and found that 3-year OS tripled from 6% to 18%. Patients receiving ICI had a lower OS than demonstrated in RCTs, except for patients with performance status (PS) 0. More than a fifth of the patients progressed early within the first six ICI cycles. Adverse prognostic factors were PS $\geq$ 1 and metastases to the bone and liver.

Abstract: Background The selection of patients with non-small cell lung cancer (NSCLC) for immune checkpoint inhibitor (ICI) treatment remains challenging. This real-world study aimed to compare the overall survival (OS) before and after the implementation of ICIs, to identify OS prognostic factors, and to assess treatment data in first-line (1L) ICI-treated patients without epidermal growth factor receptor mutation or anaplastic lymphoma kinase translocation. Methods Data from the Danish NSCLC population initiated with 1L palliative antineoplastic treatment from 1 January 2013 to 1 October 2018, were extracted from the Danish Lung Cancer Registry (DLCR). Long-term survival and median OS pre- and post-approval of 1L ICI were compared. From electronic health records, additional clinical and treatment data were obtained for ICI-treated patients from 1 March 2017 to 1 October 2018. Results The OS was significantly improved in the DLCR post-approval cohort (n = 2055) compared to the pre-approval cohort (n = 1658). The 3-year OS rates were 18% (95% CI 15.6–20.0) and 6% (95% CI 5.1–7.4), respectively. On multivariable Cox regression, bone (HR = 1.63) and liver metastases (HR = 1.47), performance status (PS) 1 (HR = 1.86), and PS $\geq$ 2 (HR = 2.19) were significantly associated with poor OS in ICI-treated patients. Conclusion OS significantly improved in patients with advanced NSCLC after ICI implementation in Denmark. In ICI-treated patients, PS $\geq$ 1, and bone and liver metastases were associated with a worse prognosis.

Keywords: real-world evidence; cancer immunotherapy; immune checkpoint inhibitors; anti-PD-1; first-line treatment; non-small cell lung cancer; advanced lung cancer; clinical prognostic factors; overall survival; Danish registry

1. Introduction

Lung cancer remains the leading cause of cancer-related death worldwide; in Denmark, lung cancer is one of the most common cancer types with an annual incidence of approximately 5000 cases [1]. Non-small cell lung cancer (NSCLC) accounts for more than 80% of the cases; most Danish patients present with stage IIIB–IV disease at diagnosis and have poor 5-year survival rates of 3% [2]. During the past 5 years, treatment with immune-checkpoint inhibitors (ICIs) has transformed the advanced NSCLC treatment landscape. Improved OS was observed in patients receiving ICIs in the second or later lines of treatment [3–5]. Furthermore, in the first-line (1L) randomized controlled trials (RCTs), KEYNOTE-024 and KEYNOTE-042, the median overall survival (mOS) improved to 26.3 and 20 months with ICIs compared to 14.2 and 12.2 months with chemotherapy (CTx), respectively [6–8]. These results led to the approval of 1L ICI treatment in Denmark on 1 February 2017. Programmed Death-Ligand 1 (PD-L1) is currently used as a predictive biomarker for ICI treatment. PD-L1 $\geq$ 50% is the cut-off for 1L ICI monotherapy based on RCTs that enrolled patients with different PD-L1 cut-offs [5]. However, the efficacy of ICIs in highly selected patients included in the RCTs may not be reproducible in patients treated in a routine clinical setting because of the impact of patient-, provider-, and system-related factors [9,10]. Therefore, real-world studies (RWS) on ICIs in consecutively treated patients have focused on patient-related factors (age, Eastern Cooperative Oncology Group (ECOG) performance status (PS) $\geq$ 2, and brain metastases) [11]. These studies indicate that patients aged > 70 years have an mOS comparable to that of younger patients [12]. In addition, patients with brain metastases have an mOS comparable to that of patients without brain metastases [13,14]. By contrast, PS $\geq$ 2 has been associated with significantly reduced mOS, independent of treatment line, and a systematic review demonstrated a pooled mOS hazard ratio (HR) of 2.72 compared to PS 0–1 [15,16]. RWS indicate significantly reduced response rates and impaired mOS in patients with bone metastases (BoM) compared to those without [17,18]. This suggests a reduced ICI effect in patients with BM; however, more data from RCTs and larger RWS are warranted. The expected change in overall survival (OS) in patients with advanced NSCLC after the clinical implementation of ICIs has only been sparsely investigated [19,20].

This nationwide RWS aimed to compare the OS before and after the implementation of 1L ICI in patients with advanced NSCLC without epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) molecular alterations. Furthermore, the aim was to uncover prognostic factors for OS and report on treatment data in patients treated with 1L ICI.

2. Material and Methods

2.1. Patients

2.1.1. Cohorts from the Danish Lung Cancer Registry (DLCR)

The DLCR, a part of the Danish Clinical Quality Program (National Clinical Registries), includes data automatically transferred from other national registries [21,22]. From the DLCR, baseline demographics and clinical data were extracted for patients with NSCLC, without EGFR/ALK molecular alterations, who started 1L palliative antineoplastic treatment from 1 March 2013 to 1 October 2018 (n = 6890) (Figure 1; Figure S1). This cohort was separated into a *DLCR pre-approval cohort*, comprising patients who started treatment before the approval of ICIs in any line (1 March 2013 to 1 August 2014; n = 1658), and a *DLCR post-approval cohort*, comprising patients who started treatment after the approval of 1L ICI in Denmark (1 March 2017 to 1 October 2018; n = 2055). To minimize the impact of second-line ICI (implemented in Denmark in September 2015), patients who started 1L treatment between 2 August 2014 and 28 February 2017 (n = 3177), were excluded (Figure 1).

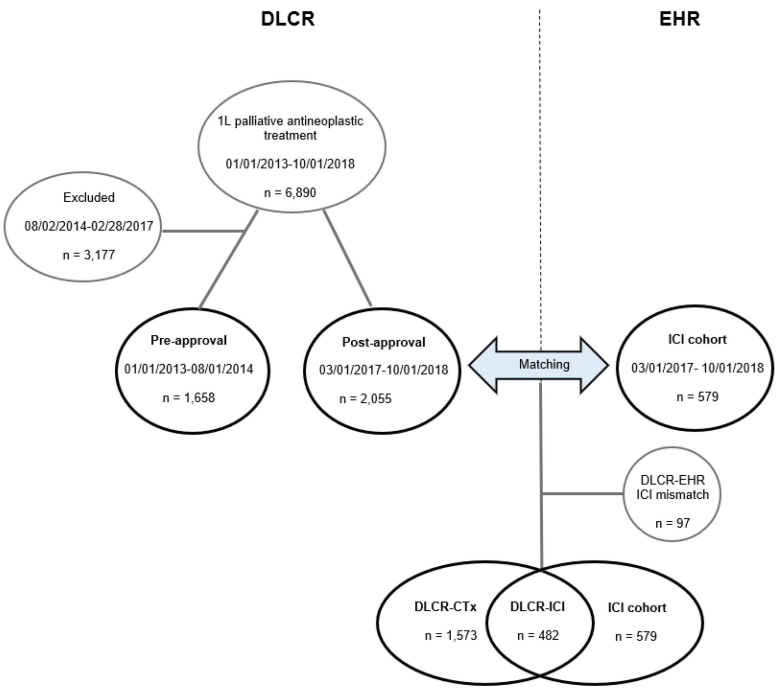

Figure 1. Flowchart showing the generation of the Danish Lung Cancer Registry (DLCR) cohorts before and after the approval of immune checkpoint inhibitors (ICIs). Treatment data from the electronic health records (EHRs) were applied on the DLCR post-approval cohort to divide patients into the DLCR-chemotherapy (CTx) and DLCR-ICI cohorts. Due to missing and inaccurate data in the DLCR, 97 ICI-treated patients identified from institutional records were not registered in the DLCR.

2.1.2. ICI Cohort Identified from Electronic Health Records (EHRs)

Data on PS and metastatic sites, and antineoplastic treatment details are lacking in the DLCR. To obtain these data on the 1L ICI-treated patients, the nationwide *ICI cohort* of consecutive patients initiating 1L ICI-treatment between 1 March 2017 and 1 October 2018 (n = 579) in all oncology departments administering ICIs in Denmark (n = 11) was identified. EHRs were reviewed in order to obtain clinical and treatment data on the ICI-treated patients.

2.1.3. Matching of the DLCR Post-Approval Cohort and the EHR-Identified ICI Cohort

Stratification according to systemic antineoplastic treatment in the *DLCR post-approval cohort* was accomplished by matching with the EHR-identified *ICI cohort*. A match of 83% was observed, and the ICI-treated patients in the *DLCR post-approval cohort* were identified (*DLCR-ICI cohort*, n = 482). Thus, 97 patients identified from institutional records were not included in the *DLCR post-approval cohort* (*mismatch*; Figure 1). According to the national treatment guidelines at that time, the standard 1L treatment of the remaining patients in the DLCR post-approval cohort was platinum-doublet CTx (*DLCR-CTx cohort*; n = 1573) (Figure 1).

Hence, two different ICI cohorts were identified. The *DLCR-ICI cohort* that was used in the analyses comparing the OS before and after the implementation of 1L ICI, and the EHR-identified *ICI cohort* that was used in the detailed analyses of ICI-related clinical outcomes and treatment data.

2.2. Data Management of the EHR-Identified ICI Cohort

Due to our study definition of 1L treatment (first palliative treatment after NSCLC diagnosis or at relapse $\geq$ 6 months after curatively intended treatment), 12 patients (2%) received nivolumab (3 mg/kg every 2 weeks). ICI doses were prescribed according to Danish guidelines at the time, with a fixed pembrolizumab dose at 200 mg or 2 mg/kg every 3 weeks for a maximum of 2 years. Individual ICI dose intensities (mg/kg/time) were not recorded [23]. The reasons for ICI discontinuation were recorded, and the types of immune-related adverse events (irAEs) leading to ICI discontinuation were recorded. Additionally, hospitalization due to irAEs was recorded as a dichotomous variable (yes/no). Radiologic assessments according to the Response Evaluation Criteria in Solid Tumors were not consistently available. Therefore, the date of disease progression was defined as the date of radiologically-verified progressive disease (PD). If no radiological PD was evident, the date of PD was defined as the first clinical evidence of PD leading to ICI discontinuation. The index date was defined as the date of the first ICI administration. For patients still alive, the censoring date was 1 March 2020, and the date of last follow-up was defined as the last EHR-documented patient contact. Time-to-event measures were OS, progression-free survival (PFS), and time to treatment discontinuation (TTD).

2.3. Statistical Methods

2.3.1. The DLCR Cohorts

The chi-square test was used to test for differences in categorical baseline characteristics between the pre- and post-approval cohorts, similarly to the DLCR-CTx and DLCR-ICI cohorts. The TNM stage was not considered due to the large proportion of missing values in the DLCR. Kaplan–Meier (KM) estimates were used to assess OS, and the log-rank test was used to compare the estimated survival curves.

2.3.2. The EHR-Identified ICI Cohort

KM estimates were used to assess OS, PFS, and TTD, and log-rank tests were used to test for differences according to baseline characteristics. In the survival analyses, the Charlson Comorbidity Index Score (CCIS) was categorized as 0–1 and $\geq$2. Smoking status was excluded from the analyses due to a limited number of "never smokers" and the heterogenous smoking patterns in the "former smoking" group. TNM stage was excluded

as a covariate from the survival analyses because of its interaction with metastatic sites. The remaining baseline characteristics were included as covariates and, for each of them, the assumption of proportional hazard function was assessed. Since the ECOG PS violated the assumption, weighted univariable and multivariable Cox regressions were used [24]. Multivariable Cox regression analysis was extended with an interaction between sex and histopathology. Survival analyses were not adjusted for age-related background mortality. The median follow-up was calculated using the reverse KM estimate.

All analyses were performed using R version 4.0.2 (R Core Team, Vienna, Austria) [25]. The survival- and ggsurvplot-packages were used to construct the KM estimates, and the coxphw package was used to perform the weighted Cox regressions.

3. Results

3.1. The DLCR Cohorts

3.1.1. Baseline Characteristics

Comparing baseline characteristics between the DLCR pre-approval (n = 1658) and post-approval (n = 2055) cohorts showed a significant increase in the median age (from 68 to 70 years, $p < 0.0001$) (Table S1). Compared to the pre-approval cohort, the post-approval cohort comprised a significantly higher proportion of female patients (50.2% vs. 46.9%, $p = 0.05$) and adenocarcinomas (58.8% vs. 53.3%, $p < 0.0001$) (Table S1). Additionally, significant differences in TNM stage was found ($p < 0.0001$) before and after the implementation of ICIs; however, large differences in missing values were also observed (the post-approval cohort n = 246, the pre-approval cohort, n = 69) (Table S1). No differences in CCIS were found (Table S1).

The DLCR-ICI cohort (n = 482) had a larger proportion of female patients than the DLCR-CTx cohort (n = 1573) (58.3% vs. 47.7%, $p < 0.0001$) (Table S2). Significant differences were found in the distribution of NSCLC histopathology, with a higher proportion of squamous cell carcinomas in the DLCR-CTx cohort, and higher proportions of adenocarcinomas and "other" in the DLCR-ICI cohort (Table S2).

3.1.2. OS before and after the Implementation of ICIs

Significant differences were seen in OS between the DLCR cohorts (p-value < 0.0001), with notable differences in mOS, and 1-, 2-, and 3-year survival rates (Figure 2 and Table 1). The greatest survival improvement was observed in patients receiving ICIs with a mOS increase from 7.8 months (95% CI 7.4–8.2) to 19.0 months (95% CI 16.0–22.0), 1-year OS rate from 31% to 64%, 2-year OS rate from 12% to 42% and 3-year OS rate from 6% to 29%.

Table 1. Survival of patients with advanced NSCLC treated with systemic antineoplastic treatment before and after the introduction of ICIs.

DLCR Cohorts	*n* (%)	mOS (Months) (95% CI)	1-Year OS (%) (95% CI)	2-Year OS (%) (95% CI)	3-Year OS (%) (95% CI)
Pre-approval cohort	1658 (100)	7.8 (7.4–8.2)	31 (29–33)	12 (10–14)	6 (5–7)
Post-approval cohort	2055 (100)	11.0 (10.2–11.9)	48 (46–50)	27 (25–29)	18 (16–20)
CTx	1573 (77)	9.5 (8.9–10.3)	43 (40–45)	22 (21–25)	14 (12–17)
ICI	482 (23)	19.0 (16.0–22.0)	64 (60–68)	42 (38–47)	29 (24–35)

Median overall survival (mOS), 1-, 2-, and 3-year overall survival (OS) rates with 95% confidence interval (CI) before and after the approval of ICI treatment (the pre-approval cohort 1 January 2013–1 August 2014 and the post-approval cohort 1 March 2017–1 October 2018). NSCLC; non-small cell lung cancer; DLCR, Danish Lung Cancer Registry; *n*, number of patients; CTx, chemotherapy; ICI, immune checkpoint inhibitor.

3.2. The EHR-Identified ICI Cohort

3.2.1. ICI Efficacy

The baseline characteristics of the EHR-identified ICI-treated patients (n = 579) are presented in Table 2.

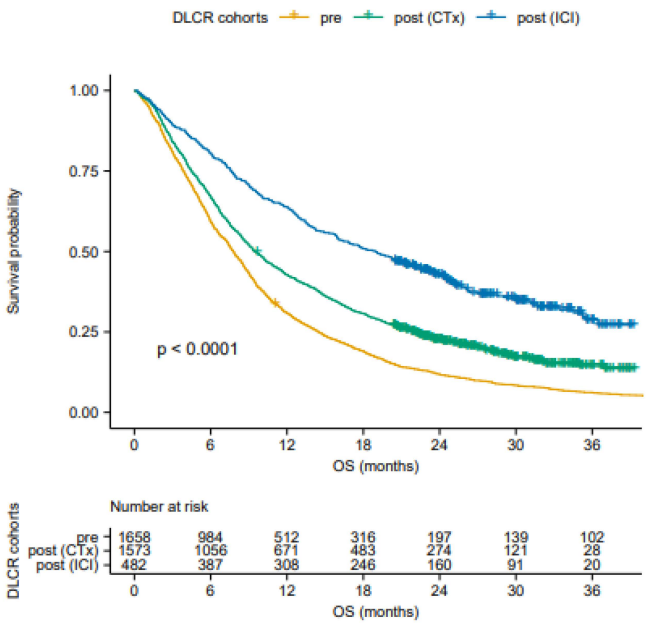

Figure 2. Overall survival (OS) of patients in Denmark before and after the approval of first-line immune checkpoint inhibitor (ICI). The survival of patients treated with chemotherapy (CTx) before the approval (pre) was compared to survival of patients treated with either CTx or ICI after the approval (post (CTx) and post (ICI)). DLCR, Danish Lung Cancer Registry.

Table 2. Baseline characteristics, ICI cohort.

Baseline Characteristics	***n*** **(%)**
All patients	579
Age, median years (range)	70 (45–88)
<75	441 (76)
≥75	138 (24)
Sex	
Male	246 (42)
Female	333 (58)
ECOG performance status	
0	194 (34)
1	295 (51)
≥2	90 (15)
CCIS	
0 (none)	217 (37)
1 (mild)	169 (29)
2 (moderate)	103 (18)
3+ (severe)	90 (16)
Smoking status	
Current	189 (33)
Former	343 (59)
Never	26 (4)
Unknown	21 (4)

Table 2. *Cont.*

Baseline Characteristics	*n* (%)
TNM stage and metastatic sites	
III	109 (19)
IV [a]	470 (81)
Brain	38 (7)
Bone	162 (28)
Liver	63 (11)
Adrenal	86 (15)
Distant lymph nodes	174 (30)
NSCLC histopathology	
Adenocarcinoma	409 (71)
Squamous cell carcinoma	135 (23)
Other [b]	35 (6)
PD-L1	
Negative	3 (0.5)
≥1% and <50%	20 (3.5)
≥50%	552 (95.3)
Unknown	4 (0.7)
Prior treatment with curative intention	
Surgery ± adj. CTx	39 (7)
CRT	46 (8)
Surgery and CRT	16 (3)
None	478 (82)
Prior palliative RT [c]	
Yes	71 (12)
No	508 (88)

[a] Patients may be registered with more than one metastatic site; [b] 'Other' includes NSCLC NOS (not otherwise specified) and adenosquamous carcinoma; [c] Prior palliative radiotherapy for NSCLC (primary lesion or metastatic site). *n*, number of patients; ECOG, Eastern Cooperative Oncology Group; CCIS, Charlson Comorbidity Index Score; TNM, tumor-node-metastasis classification of malignant tumors; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; adj. CTx, adjuvant chemotherapy; CRT, chemoradiotherapy; RT, radiotherapy.

ICI was administered following the primary diagnosis in 477 (82%) patients. The remaining patients received ICI after curatively intended surgery +/− adjuvant CTx (*n* = 39; 7%), chemoradiotherapy (CRT) (*n* = 46; 8%), or both (*n* = 16; 3%). PD-L1 was unknown or <50% in 27 patients (4.7%). The treatment data and reasons for treatment discontinuation are shown in Table 3.

At the censoring date, 38 patients (7%) were still on ICI treatment. The median follow-up period was 27.2 months (95% CI 26.7–28.2), and the median TTD was 4.8 months (95% CI 4.1–5.5) (Table S3).

PD was the most common reason for ICI discontinuation (*n* = 250, 46%), and half of the patients discontinued ICIs within six cycles (Figure S2). More reasons for ICI discontinuation were irAEs only (28%), poor PS (11%), completion of 2 years ICI (7%), and "other reasons" (9%) (Table 3). Following ICI treatment, systemic antineoplastic treatment was administered to 179 patients (33%). Of these patients, 28% received ≥ 2 treatment lines.

3.2.2. Clinical Outcomes

The mOS was 18.3 months (95% CI 16.0–21.3); 15.2 (95% CI 13.0–18.3) in male and 21.5 (95% CI 18.0–25.1) in female patients. The mOS for patients with PS 0 was 28 months (95% CI 21.5–NR) compared to the 14.6 (95% CI 12.7–19.0) and 12.8 months (95% CI 7.6–16.1) in patients with PS 1 and PS ≥ 2, respectively. In patients with BoM, the mOS was 12.0 months (95% CI 9.5–14.9) compared to the 21.5 months (95% CI 19.0–24.9) in patients without. The mPFS was 8.2 months (95% CI 7.2–9.3); 7.1 (95% CI 6.0–8.5) in male and 8.8 (95% CI 7.9–11.8) in female patients. The mPFS for patients with PS 0 was 11.0 months (95% CI 8.5–13.9) compared to the 7.7 (95% CI 6.4–8.8) and 6.0 (95% CI 3.3–8.7) in patients with PS 1 and

PS ≥ 2, respectively. In patients with BoM, the mPFS was 5.7 months (95% CI 4.4–7.8) compared to the 9.4 months (95% CI 8.1–12.0) in patients without.

Table 3. ICI treatment and irAEs.

Treatment Characteristics	*n* (%)
All patients	579
Median number of cycles (range)	7 (1–41)
Median days on treatment [a] (range)	127 (1–826)
Ongoing ICI treatment [b]	38 (7)
ICI discontinuation	541 (93)
ICI discontinuation due to [c]:	
PD	250 (46)
Poor performance status	62 (11)
Two years of ICI [d]	39 (7)
IrAEs [e]	170 (31)
Pneumonitis	41 (8)
Hepatitis	31 (6)
Skin	10 (2)
Endocrinopathy	18 (3)
Diarrhea/colitis	37 (7)
Other [f]	52 (10)
IrAE only [g]	150 (28)
Other reasons	51 (9)
Hospitalization due to irAE	135 (23)
Grade 5 toxicity (death)	12 (2)

[a] Median time of ICI treatment = time to treatment discontinuation (TTD). [b] At date of censoring. [c] Each patient could be registered with more than one cause of treatment discontinuation. [d] Patients who received at least 2 years of ICI treatment. [e] Each patient could be registered with more than one type of irAE as a cause of treatment discontinuation. Percentage (in parentheses) describes the proportion of patients who stopped ICI because of the specific irAE compared to all patients who discontinued ICI (*n* = 541) [f] "Other" are not specified irAEs. [g] Proportion of patients with irAE as the only cause of treatment discontinuation. ICI, immune checkpoint inhibitor; irAE, immune-related adverse event; *n*, number of patients; PD, progressive disease.

For information on mOS and mPFS according to all baseline characteristics see Table S5.

In patients with PS 0–1, the estimated 3-year OS rate was 33% (95% CI 28–39) compared to the 25% (95% CI 16–39) in patients with PS ≥ 2. Furthermore, the mTTD for patients with PS ≥ 2 was 2.8 months (95% CI 1.4–4.2) (Table S3).

3.2.3. Prognostic Clinical Factors

KM estimates and log-rank tests showed that the OS was significantly reduced in male patients and in patients with PS ≥ 1, BoM, and/or liver metastases, and in patients who had received prior palliative RT (Table S4 and Figure S3). Baseline metastases in the brain, adrenal glands, and/or distant lymph nodes, age ≥ 75 years, CCIS ≥ 2, or prior curative treatment for NSCLC did not significantly affect OS (Table S5 and Figure S3). In the multivariable Cox regression analysis, PS 1 (HR = 1.86; 95% CI 1.44–2.39; $p < 0.001$) and PS ≥ 2 (HR = 2.19; 95% CI 1.5–3.18; $p < 0.001$), relative to PS 0, BoM (HR = 1.75; 95% CI 1.36–2.23; $p < 0.001$), and liver metastases (HR = 1.44; 95% CI 1.0–2.07; $p = 0.05$) remained independent of poor prognostic factors (Figure 3). Compared to patients with primary metastatic disease, patients with a relapse after prior curative treatment (surgery ± adjuvant CTx, curative CRT, or surgery + CRT) did not have a significantly improved OS.

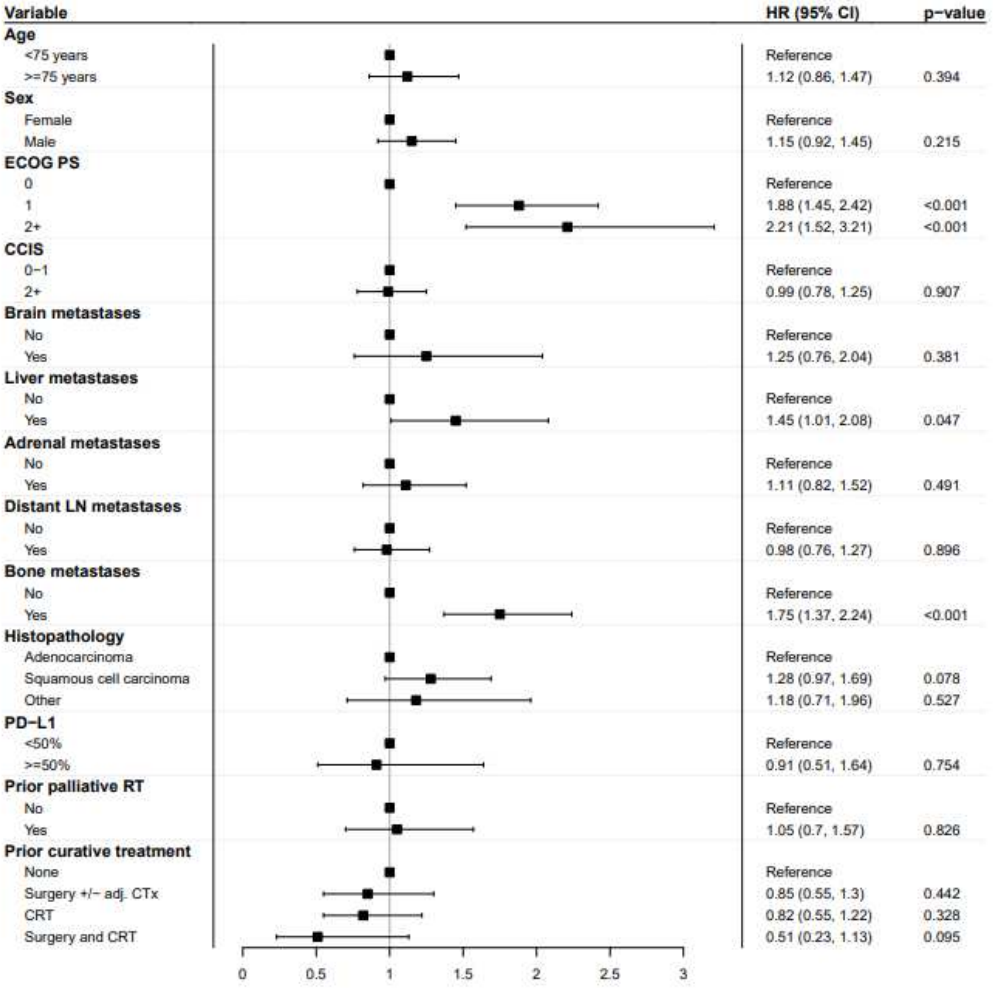

Figure 3. Weighted multivariable Cox regression analysis with forest plots showing average hazard ratios (HR) according to baseline characteristics. ECOG PS, European Cooperative Oncology Group performance status; CCIS, Charlson Comorbidity Index Score; RT, radiotherapy; CTx, chemotherapy; CRT, chemoradiotherapy.

In the interaction analysis of sex and histopathology, male patients with squamous cell carcinoma had significantly poorer survival than those with adenocarcinoma (HR = 1.70; 95% CI 1.18–2.47; $p = 0.01$). Univariable Cox regression results are given in Table S5.

4. Discussion

This nationwide Danish study was based on a consecutive cohort and demonstrated a significantly improved 3-year OS rate of 29% in 1L ICI-treated NSCLC patients compared to the 6% in those treated with 1L CTx before ICI implementation. However, more patients with PS $\geq$ 2 may have been treated with 1L CTx than 1L ICI as the Danish ICI recommendation applies to patients with PS 0–1 only. To our knowledge, this is the first RWS of patients with NSCLC without EGFR/ALK molecular alterations that included both large ICI cohorts and comparative cohorts since ICI treatment was implemented. An increase in OS in CTx-treated patients was also observed, possibly due to subsequent ICI

treatment, earlier diagnosis (including potential lead time bias), stage migration owing to improved staging diagnostics, improved palliative care, changes in histopathological subtypes, advances in molecular testing, and sex distribution over time [26,27]. Those with PS $\geq$ 2 accounted for 15% of the ICI cohort in our RWS; however, these patients were not included in previous RCTs. This may partly explain the lower 3-year OS rate and mOS compared to those obtained in the KEYNOTE-024 and KEYNOTE-042 trials [6–8]. Furthermore, the poor OS of ICI-treated PS 1 patients in our study, could reflect a possible misclassification of PS 2 patients as PS 1 patients because 1L ICI was approved only for patients with PS 0-1. This issue complicates the comparison of PS data with other studies; however, this potential bias is not addressed in other RWS. In contrast, the mOS of PS 0 patients in our study was 28 months, comparable to that of patients in the KEYNOTE-024 study [6,7]. In line with other ICI RWS, we found PS $\geq$ 2 and liver metastases to be poor prognostic factors for OS [15–18,28]. Generally, the population of patients with PS 2 is heterogeneous and has worse clinical conditions due to comorbidities, higher tumor burden, or both [28,29]. Patients with BoM accounted for 28% in our study and had significantly worse mOS compared to patients without BoM. BoM has not been reported in RCTs and is rarely reported in other RWS [17,18]. However, this information is essential because the immune and skeletal systems are closely linked; for example, the receptor activator of nuclear factor-κB ligand (RANKL) stimulation suppresses T-cell killing and enhances immunosuppression in the bone tumor microenvironment [30,31]. Unfortunately, our RWS did not include information on the administration of bone-modifying agents. Clinical studies of the RANKL-inhibitor, denosumab, combined with ICIs are ongoing [32,33]. In our study, prior curative treatment did not significantly affect OS. However, tumor burden and the site of metastases at relapse, as well as the treatment strategy for oligometastatic relapse could affect the OS in these patients.

The majority of patients in our study were female (58%), as opposed to other RCTs and RWS, which reflects the higher proportion of female smokers in Denmark compared to that in other countries [34,35]. Furthermore, the proportion of female patients with NSCLC increased during the observed period.

A significant challenge with antineoplastic treatment (including ICIs) may be primary tumor resistance to treatment. In our study, 22% of patients experienced PD within six ICI cycles (i.e., 4.2 months of treatment). Various factors such as different PD-L1 intervals, inter- and intra-tumoral PD-L1 heterogeneity, host-immune-related mechanisms, and unidentified mutations such as STK11, along with currently unknown factors are possible explanations for early PD [36–38]. Those patients could potentially derive benefit from other 1L treatment options. Furthermore, pseudoprogression could be misinterpreted as PD in some cases. To optimize response evaluation in ICI-treated patients, the use of immune (i) RECIST could be implemented as a standard in the real-world setting as well as in the RCTs [39]. Additionally, a standardization of response evaluation could improve the comparability of ICI efficacy in RWS and RCTs.

RWS provide information on effectiveness in everyday clinical practice as they include patient subgroups not reported or included in RCTs [9,11]. Furthermore, new hypotheses can be generated from the RWS results. A major strength of this study is the substantial nationwide cohort, which provides new information on large consecutive subgroups seen in daily clinical practice, such as patients with PS $\geq$ 2, moderate-to-severe comorbidity, organ metastases, and age > 75 years. Furthermore, in the Danish Healthcare System, all patients have equal and free access to therapy, including ICIs (within the framework of national guidelines), thus lowering the risk of selection bias. The limitations of our study, and particularly related to the CTx-cohorts, are similar to those of other RWS with a retrospective design, which is the lack of data completeness and data accuracy.

Based on our results, some main questions still need to be answered to optimize the future ICI treatment of patients with advanced NSCLC. Primary resistance mechanisms in patients with early PD need to be further explored. In future RCTs, a higher representation of patients from daily clinical practice, and information on known prognostic factors such as

metastatic load and location, is warranted. Prospective ICI investigations should focus on: differences between RCTs and routine care; complementary tools to assess patients' daily living activities, frailty, and reasons leading to poor PS; possible differences between male and female patients. Furthermore, the optimal registration and research use of real-time clinical, molecular, and patient-reported data need to be established.

5. Conclusions

In this comprehensive nationwide study, we demonstrated that both the mOS and the long-term survival of real-world patients with advanced EGFR- and ALK negative NSCLC, treated with systemic antineoplastic treatment, has improved since the implementation of ICIs in Denmark. The survival of ICI-treated patients was lower than demonstrated in the RCTs, except for PS 0 patients. More than every fifth patient showed early PD within six cycles of ICI, and this group of patients especially may benefit from alternative treatments, if they could be identified upfront. PS $\geq$ 1, and bone and liver metastases were found to be significantly associated with worse mOS. Sex, CCIS, and age $\geq$ 75 years did not significantly affect the mOS.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13194846/s1, Figure S1: Criteria applied to the DLCR dataset, Figure S2: ICI treatment discontinuation due to progressive disease, Figure S3: Kaplan–Meier curves for the EHR-identified ICI cohort according to age, bone metastases, performance status, and sex and histopathology, Table S1: Comparison of baseline characteristics in the DLCR pre- and post-approval cohorts, Table S2: Comparison of baseline characteristics in the post-approval DLCR-CTx and DLCR-ICI cohorts, Table S3: Time to treatment discontinuation (TTD), Table S4: Median OS and PFS according to selected baseline characteristics of ICI-treated patients, Table S5: Univariable Cox regression analysis.

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Informed Consent Statement: Patient consent was waived by the Danish Patient Safety Authority, due to the retrospective design of the study, and the use of routinely collected data.

Data Availability Statement: The study data can be available on request from the corresponding author, Mette T Mouritzen. The data are not publicly available due to the General Data Protection Regulation.

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ORIGINAL ARTICLE

Clinical features affecting efficacy of immune checkpoint inhibitors in pretreated patients with advanced NSCLC: a Danish nationwide real-world study

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ABSTRACT

Background: Immune checkpoint inhibitors (ICIs) are implemented as standard treatment for patients with advanced non-small cell lung cancer (NSCLC) in first-line and subsequent-line treatment. However, certain subgroups such as patients with older age, poor performance status (PS), and severe comorbidity are underrepresented in the randomized controlled trials (RCTs). This study aimed to assess overall survival (OS), treatment data, and clinical features affecting second- or subsequent-line ICI efficacy in an unselected, Danish, nationwide NSCLC population.

Methods: Patients with advanced NSCLC who started nivolumab or pembrolizumab as second-line or subsequent-line treatment between 1 September 2015, and 1 October 2018, were identified from institutional records of all Danish oncology departments. Clinical and treatment data were retrospectively collected. Descriptive statistics and survival analyses were performed.

Results: Data were available for 840 patients; 49% females. The median age was 68 years (19% were ≥75 years), 19% had PS ≥2, and 36% had moderate to severe comorbidity. The median OS (mOS) was 12.2 months; 15.1 months and 10.0 months in females and males, respectively. The median time-to-treatment discontinuation (mTTD) and median progression-free survival (mPFS) was 3.2 and 5.2 months, respectively. Patients with PS ≥2 had a mOS of 4.5 months, mTTD of 1.1 month, and mPFS of 2.0 months. In multivariable Cox regression analysis, male sex (HR = 1.35, 95% CI 1.11–1.62), PS >0 (PS 1, HR = 1.88, 95% CI 1.52–2.33; PS ≥2, HR = 4.15, 95% CI 3.13–5.5), liver metastases (HR = 1.72, 95% CI 1.34–2.22), and bone metastases (HR = 1.27, 95% CI 1.03–1.58) were significant poor prognostic OS factors.

Conclusions: Danish real-world patients with advanced NSCLC treated with second- or subsequent-line ICI had an OS comparable to results from RCTs. Women, frail and older patients constituted a higher proportion than in previous RCTs. Clinical features associated with poor OS were male sex, PS ≥1 (in particular PS ≥2), bone-, and liver metastases.

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Background

Lung cancer is the leading cause of cancer-related mortality and morbidity worldwide, with a five-year survival rate ranging from 6% in advanced stages to 59% in early stages [1]. In the Nordic countries, the lung cancer mortality has declined since the 1980s, due to improved diagnostics and treatment strategies [2]. The latter include the implementation of immune checkpoint inhibitors (ICIs) as standard therapy, and despite the rapidly increasing use of first-line ICI as

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Supplemental data for this article can be accessed here.

monotherapy or in combination with chemotherapy, some patients are ineligible for these regimens and may still be offered second-line ICI treatment [3–11]. The pivotal randomized controlled trials (RCTs) had strict inclusion and exclusion criteria, not comparable to a real-world setting; thus, selecting patients for ICI treatment in a daily clinical setting remains challenging due to the lack of evidence in certain subgroups. These subgroups include patients with an old age, poor Eastern Cooperative Oncology Group (ECOG) performance status (PS), and severe comorbidity. Furthermore, the sex distribution in most international RCTs and real-world studies (RWS) is unequal, and thus less representative of the Nordic population, where NSCLC incidences are equal in men and women [2,12–14]. The median age of lung cancer patients in RCTs is 61 years; however, the median age in newly diagnosed Nordic patients with NSCLC is approximately 70 years [3–5,15,16]. Thus, older patients and particularly patients aged $\geq$75 years, are greatly underrepresented in RCTs [15,17]. Lung cancer patients with PS $\geq$2 also constitute a substantial proportion of patients receiving oncologic treatment in the daily clinical setting [18]. Nevertheless, frail patients with poor PS are typically underrepresented or not included in RCTs. Organ metastases are present in more than 50% of lung cancer patients at the time of diagnosis, and metastases to the brain, liver, and bone have been associated with impaired overall survival (OS) [1,19]. Moreover, comorbidity is frequent in lung cancer patients, and may affect their treatment and clinical outcome [20–22]. However, neither level of comorbidity nor location of metastatic sites are reported in the RCTs [3–5].

The primary aim of the present study was to report on OS in a Danish, comprehensive, consecutive population with advanced NSCLC, treated with ICIs in second-line or subsequent-line treatment. This implies a special attention to, and a comparison with RCTs of, the potential predictive or prognostic clinical features characterizing the subgroups of patients who are underrepresented in RCTs. These include those with higher age, poor PS, and more comorbidity. The secondary aims were to assess reasons for ICI discontinuation (including immune-related adverse events (irAEs)), treatment duration, and progression-free survival (PFS).

Methods

Study design and patients

A retrospective, nationwide real-world study (RWS) approved by the Danish Patient Safety Authority was conducted. Consecutive patients with NSCLC who received nivolumab or pembrolizumab in second-line or subsequent-line of palliative treatment between 1 September 2015, and 1 October 2018, were identified from institutional records. Data were collected from all ($n = 11$) Danish oncology departments.

Data collection and data management

Data were manually extracted from the electronic health record (EHR) systems. Clinical data were collected and stored in local databases at every oncology department. Covariates from the local databases were aligned according to variable names, values, and labels, and data were gathered into one dataset. Furthermore, data quality control was performed for each covariate. If the PS was described as a range, such as PS 1–2, in the EHR, the highest value was captured [18]. Specific irAEs causing ICI discontinuation, and hospitalization and death due to irAEs were recorded. The disease stage and metastastic sites at ICI treatment initiation were retrospectively evaluated by reviewing baseline computed tomography (CT) scan reports.

Variables and endpoints

Baseline characteristics at ICI initiation included sex, age, PS, comorbidity according to Charlson Comorbidity Index Score (CCIS), smoking status, histopathological NSCLC subtype, TNM stage, metastatic locations, programmed death-ligand 1 (PD-L1) tumor proportion score (TPS), and epidermal growth factor receptor (EGFR) mutation status. When calculating the CCIS, the actual lung cancer diagnosis was excluded. Treatment data included the ICI drug, ICI start- and stop date, number of cycles administered (one cycle equals one administered dose), treatment line, and reasons for ICI discontinuation. These reasons were categorized as progressive disease (PD), poor PS, irAEs, and "other" reasons. Hospitalization and death due to irAEs were also recorded. The irAE types that were present at ICI discontinuation were recorded and classified as pneumonitis, hepatitis, skin toxicity, endocrinopathy, diarrhea/colitis, and 'other toxicity'. Treatment could be discontinued for more than one reason, and more than one type of irAE could be present at treatment discontinuation. Patients received either nivolumab 3 mg/kg every two weeks, pembrolizumab 2 mg/kg every 3 weeks, or pembrolizumab 200 mg every three weeks. Individual dose intensities (mg/kg/time) were not recorded [23]. The dates of progression and death were obtained from the EHRs. The progression date was defined as the date of the first clinical evidence of progressive disease (PD) (clinical examination leading to discontinuation of ICI) or radiological PD as verified by a CT and/or magnetic resonance imaging (MRI). The index date was the date of the first ICI administration, and the censoring date was 1 March 2020. The date of treatment discontinuation was the date of the last ICI administration. For living patients, the last follow-up date was defined as the date of the last patient contact in the EHRs. The primary aim was to asses OS, including investigation of predictive or prognostic clinical features. The secondary aims were to assess reasons for ICI discontinuation, treatment duration, and PFS.

Statistical methods

To compare baseline characteristics between sexes and PS groups, chi-square tests were used for the categorical variables, while the distributions of age were compared using Wilcoxon rank-sum test. No correction for multiple testing was performed. Kaplan–Meier (KM) estimates stratified by

baseline variables and log-rank tests were used to assess OS, time to treatment discontinuation (TTD) and PFS. The median follow-up time was calculated using the reverse KM estimate. To adjust for multiple covariates and potential confounders, a multivariable Cox regression analysis was performed. Initially, the assumption of proportional hazard functions was assessed for each of the baseline categorical variables by visual inspection of the log-minus-log survival curves and formally tested using the Grambsch-Therneau proportional hazard test with survival times transformed by the KM estimate. PS, bone-, liver-, adrenal- and distant lymph node metastases, histopathology, and EGFR mutation status violated the proportional hazards assumption. Therefore, average hazard ratios were estimated by weighted Cox regression [24]. Weighted univariable and multivariable Cox regression models were used for analysis of the association between OS and all the baseline categorical variables (except for TNM stage). Comorbidities that were present in >5% of the cases, were included in the weighted univariable Cox regression analysis. For the KM estimate and Cox regressions, CCIS was categorized as CCIS 0–1 and CCIS ≥2 [25].

A *p*-value of 0.05 was defined as the threshold of statistical significance. All analyses were performed using R version 4.0.2 (R Core Team, Vienna, Austria) [26]. The survival package was used to assess the assumption of proportional hazard functions, the ggsurvplot package for visualizing KM estimates, and the coxphw package for the weighted Cox regression analyses.

Results

Baseline characteristics

We identified 841 consecutive patients. No patients were lost to follow up. A single patient harboring an ALK translocation was excluded, leaving 840 patients with a median follow-up time of 34.7 months (95% confidence interval (CI) 33.2–35.9) eligible for analysis.

The median age was 68 years, with 19% ≥75 years, and 5% ≥80 years. A total of 19% of the patients ($n = 158$) had PS ≥2, 57% ($n = 479$) had PS 1, and 22% ($n = 182$) had PS 0. PS was missing in 2% of the patients ($n = 21$). Distant metastases were present in 86% of the patients. CCIS ≥2 was observed in 36% ($n = 301$) of the patients. The prevalence of specific comorbidities according to CCIS is summarized in Supplementary Table 1. The baseline characteristics of the patients are summarized in Table 1.

Male patients had a higher age ($p = 0.001$) and more comorbidities ($p < 0.0001$) than females. Squamous cell carcinomas were more frequent among male (49%) than female patients (23%) ($p < 0.0001$). Brain metastases were more prevalent in women than in men ($p < 0.0001$) (Supplementary Table 2).

Patients with baseline PS ≥2, compared to PS 0–1, consisted of more male patients (58%, $p = 0.046$), and received fewer nivolumab/pembrolizumab cycles (2/3 vs. 7/8) (Supplementary Table 2).

Table 1. Baseline characteristics.

Baseline characteristics	*n* (%)
All patients	840 (100)
Sex	
Male	432 (51)
Female	408 (49)
Age, median; range	68; 22−89
Age	
<75 years	677 (81)
≥75 years	163 (19)
ECOG PS	
0	182 (22)
1	479 (57)
≥2	158 (19)
Missing	21 (2)
Charlson Comorbidity Index Score (CCIS)	
0 (no)	332 (40)
1 (mild)	207 (25)
2 (moderate)	154 (18)
≥3 (severe)	147 (17)
Smoking status	
Current	238 (28)
Former	535 (64)
Never	46 (6)
Unknown	21 (2)
TNM stage	
III	116 (14)
IV	724 (86)
Metastatic sites[a]	
Brain	95 (11)
Bone	221 (26)
Liver	133 (16)
Adrenal	127 (15)
Distant lymph nodes	233 (28)
NSCLC histopathology	
Adenocarcinoma	485 (58)
Squamous cell carcinoma	303 (36)
Other[b]	52 (6)
EGFR mutation	
No	537 (64)
Yes	25 (3)
Unknown	278 (33)
PD-L1 status	
Negative	72 (9)
≥1% and < 50%	233 (28)
≥50%	290 (35)
Unknown	245 (29)

[a]Patients may be registered with more than one metastatic site.
[b]'Other' includes NSCLC NOS (not otherwise specified) and adenosquamous carcinoma.
n: number of patients; ECOG PS: Eastern Cooperative Oncology Group performance status; NA: not available; TNM: tumor-node-metastasis classification of malignant tumors; NSCLC: non-small cell lung cancer; EGFR: epidermal growth factor receptor; PD-L1: programmed death-ligand 1.

ICI Treatment

At the censoring date, 99% ($n = 831$) had discontinued ICI. ICI treatment characteristics are demonstrated in Table 2.

The median TTD (mTTD) was 3.2 (95% CI 2.8–3.6) months. In patients with PS ≥2, the mTTD was 1.1 (95% CI 0.7–1.4) month compared to 3.3 (95% CI 2.8–3.8) and 6.0 (95% CI 5.1–7.8) months in PS 1 and PS 0 patients, respectively.

Clinical outcomes

The mOS was 12.2 (95% CI 10.8–13.8) months, and the 1- and 2-year OS rates were 50% (95% CI 47–54) and 30% (95% CI 27–33), respectively (Table 3). The estimated three-year OS rate was 20% (95% CI 17–23). The mOS was 15.1 and 10.0 months in female and male patients, respectively. The

Table 2. ICI treatment characteristics.

Treatment characteristics	*n* (%)
All patients	840 (100)
Treatment line	
2	536 (64)
3	205 (24)
4	68 (8)
≥5	31 (4)
Treatment	
Nivolumab	444 (53)
Pembrolizumab	396 (47)
Median number of ICI cycles[a]; range	
Nivolumab	6; 1–64
Pembrolizumab	6; 1–37
ICI treatment duration[a];	
Median days; range	98; 1–961
mTTD months; 95% CI	3.2; 2.8–3.6
Ongoing ICI treatment[b]	10 (1)
ICI discontinuation due to[c]:	
PD	461 (56)
Poor PS	126 (15)
irAEs[d]	179 (22)
Pneumonitis	47 (6)
Hepatitis	19 (2)
Skin toxicity	27 (3)
Endocrinopathy	15 (2)
Diarrhea/colitis	40 (5)
Other toxicity	51 (6)
irAEs only[e]	150 (18)
Other reasons[f]	145 (17)
Hospitalization due to irAEs	135 (16)
Death due to irAEs	8 (1)

[a]Patients with ongoing ICI treatment ($n = 10$) not included.
[b]At date of censoring.
[c]Each patient could be registered with more than one cause of treatment-discontinuation.
[d]Each patient could be registered with more than one type of irAE as a cause of treatment-discontinuation.
[e]Proportion of patients with irAE as the only cause of treatment discontinuation.
[f]'Other reasons' are not specified irAEs.
n: number of patients; ICI: immune checkpoint inhibitor; mTTD: median time to treatment discontinuation; PD: progressive disease; PS: performance status; irAEs: immune-related adverse events.

mOS for patients with PS ≥2 was 4.5 months compared to 12.2 and 22.1 months in patients with PS 1 and PS 0, respectively (Table 3). The mPFS was 5.2 (95% CI 4.5–6.9) months (Table 3), and 2.0 months in patients with PS ≥2.

Prognostic clinical features

Kaplan-Meier estimates demonstrated that OS was reduced in men ($p < 0.0001$), in patients with PS >0 ($p < 0.0001$), and in patients with bone ($p = 0.003$) and/or liver metastases ($p < 0.0001$) (Figure 1).

Age ≥75 years, comorbidity according to CCIS, and the presence of brain metastases at ICI initiation were not significantly associated with impaired OS (Supplementary Table 3).

In multivariable Cox regression analysis, male sex (HR = 1.35; 95% CI 1.11–1.62), liver metastases (HR = 1.72; 95% CI 1.34–2.22), and bone metastases (HR = 1.27; 95% CI 1.03–1.58) remained statistically significant poor prognostic factors. Likewise did PS ≥2 (HR = 4.15; 95% CI 3.13–5.50) and PS 1 (HR = 1.88; 95% CI 1.52–2.33) compared to PS 0. Age ≥75 years (HR = 0.99; 95% CI 0.8–1.23), and the presence of brain metastases at ICI initiation (HR = 1.1; 95% CI 0.82–1.47) did not significantly affect OS (Figure 2). EGFR mutation status and PD-L1 TPS were unknown in 33% and 29% of cases, respectively. PD-L1 ≥ 50% was associated with an improved OS (HR = 0.69; 95% CI 0.48–0.98).

Extension of the multivariable Cox regression with interaction between sex and histopathology demonstrated a significantly poorer OS in patients with adenocarcinoma, if they were male rather than female, while no difference in OS were seen between sexes for patients with squamous cell carcinoma (Supplementary Table 5).

Kaplan-Meier estimates demonstrated that factors associated with a poor PFS were male sex ($p = 0.006$), ECOG PS >0 ($p < 0.0001$), no history of smoking ($p = 0.03$), liver metastases ($p < 0.0001$), a positive EGFR mutation status ($p = 0.004$), and PD-L1 < 1% ($p < 0.0001$) (Supplementary Table 4).

Discussion

Several subgroups have been underrepresented in RCTs, and therefore, focus is increasingly placed on the importance of gathering clinically relevant data from RWS, which typically represent a more unselected treatment population. However, different global health care systems affect the populations included in RWS. In Denmark, according to the Danish Health Care Act, all patients are offered treatment according to national treatment guidelines, irrespective of their income, education, and residential and socioeconomic status, which minimizes the risk of selection bias in Danish studies [27]. Treatment with ICIs is expensive and holds a potential risk of causing severe irAEs. Thus, characterizing a large cohort of real-life patients in detail may contribute with important knowledge helping clinicians make more evidence-based decisions on whether to offer patients ICI or not.

In this large nationwide NSCLC study of real-world ICI efficacy, the mOS and the 1-year OS rate were comparable to results from previous anti-PD-1 clinical trials of pretreated patients [3–5,28,29]. An improved mPFS compared to results from the RCTs, could be explained by differences in PFS definition [3–5].

Lung cancer incidence and mortality remain higher in males than females in some countries [30,31]. However, in agreement with the narrowing gap in the lung cancer incidence between sexes in Nordic countries, half of the patients in our study were females, as opposed to a lower proportion reported in comparable RCTs and RWS [2,13,14]. In RCTs, ICI significantly improved OS in both men and women compared to chemotherapy, however, the benefit seemed to be higher in men [7,13]. In this study, PS ≥2, higher CCIS, and squamous cell carcinomas were more frequent in males as compared to females. Despite adjusting for these factors, male patients with adenocarcinomas had a worse OS than female patients with adenocarcinomas.

In our study, the median age was 68 years, which is 5–7 years older than patients included in the anti-PD-1 RCTs, and more comparable to the age of real-world lung cancer patients [3–5,15,16]. Especially data on patients aged ≥75 years is lacking in RCTs. However, in our study they constituted 19% of patients, compared to only 7%–8% in previous RCTs [3,4]. Even with this greater proportion of older patients, the mOS was comparable to results from previous clinical trials and RWS,

Table 3. Overall and progression-free survival according to sex and performance status.

Survival	mOS months (95% CI)	mPFS months (95% CI)	one-year OS rate % (95% CI)	Two-year OS rate % (95% CI)
All patients	12.2 (10.8–13.8)	5.2 (4.5–5.9)	50 (47–54)	30 (27–33)
Male	10.0 (9.0–11.7)	4.4 (3.7–5.3)	44 (40–49)	25 (21–30)
Female	15.1 (13.4–17.2)	6.4 (5.2–8.1)	57 (53–62)	34 (30–39)
PS 0–1	15.3 (13.5–16.8)	6.3 (5.4–7.5)	57 (53–61)	35 (31–38)
PS ≥2	4.5 (3.2–5.7)	2.0 (1.7–2.6)	26 (20–34)	11 (7–17)

mOS: median overall survival; mPFS: median progression-free survival; CI: confidence interval; PS: performance status.

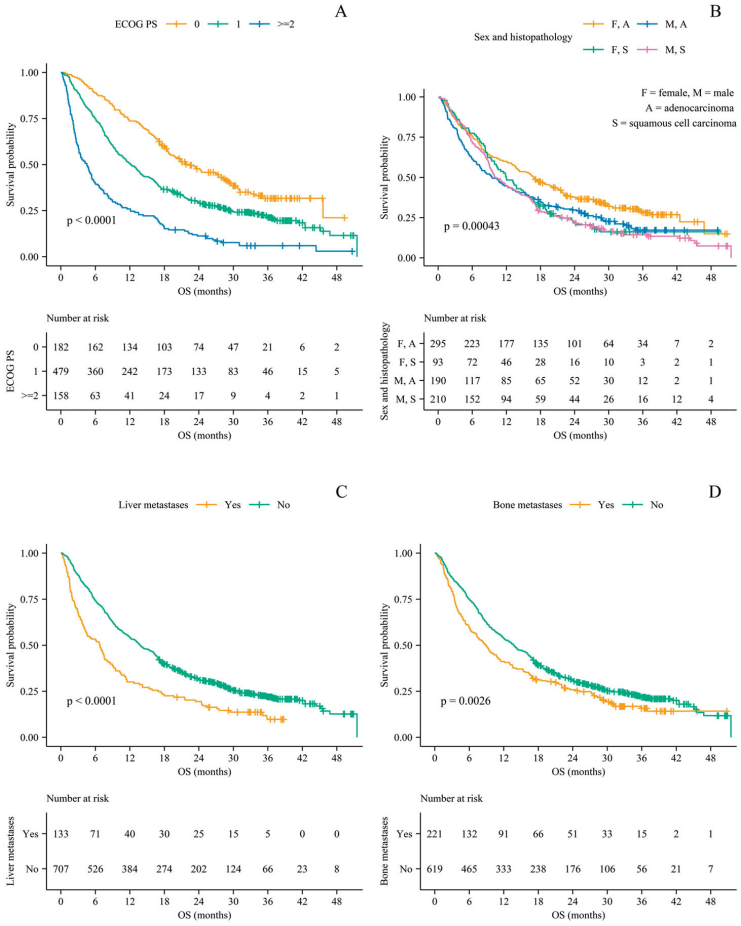

Figure 1. OS stratified by ECOG PS, sex and histopathology, liver metastases and bone metastases. OS: overall survival; ECOG PS: Eastern Cooperative Oncology Group performance status; F: female; M: male; A: adenocarcinoma; S: squamous cell carcinoma.

as age did not significantly affect OS [3,4,28,29,32,33]. Our data demonstrate that ICI should not be excluded as a treatment option because of high chronological age.

As opposed to the RCTs, the proportion of PS ≥2 patients in our study (19%) reflects the overall fraction of patients with NSCLC and PS ≥2 [18]. Thus, compared to the RCTs, our study included more frail and heavily pretreated patients, with more than one third receiving third-line or further subsequent-line ICI treatment [3–5]. Nevertheless, the mOS of patients with PS ≥2 was comparable to results from clinical trials, pooled analyses and other RWS [28,29,34]. In contrast to this, the PePS2 study assessed the efficacy of pembrolizumab in 60 patients with PS ≥2, and reported a mOS of 12.1 months in previously treated patients [35]. However, since the mPFS was only 2.0 months and the mTTD was only 1.1 month in our study, the clinical benefit of ICIs is very limited in most of these patients. On the other hand, we report a mOS of 22.1 months in patients with PS 0, which is comparable to the mOS of PS 0–1 patients treated with first-line ICI in RCTs [6,36]. This illustrates that PS 0 patients may benefit particularly from ICIs, even when

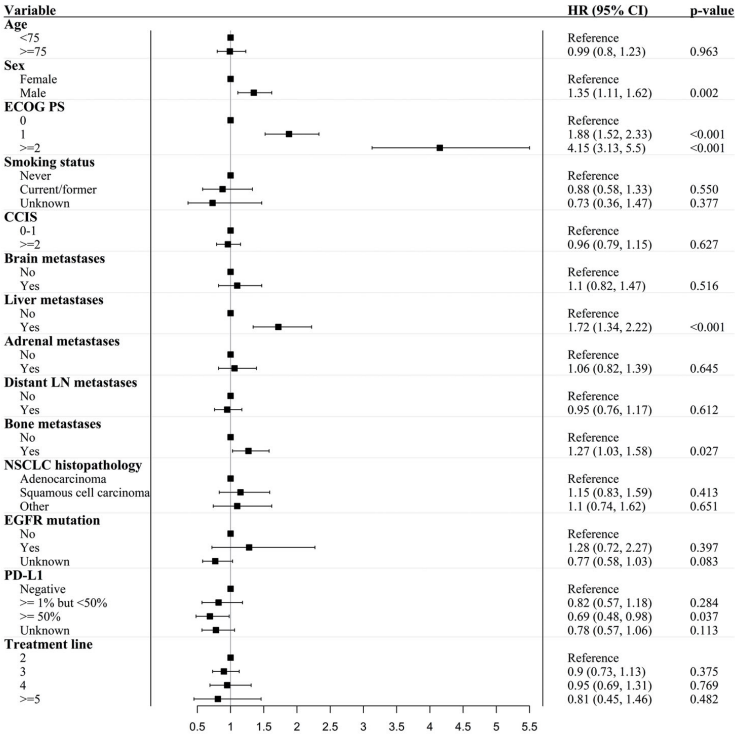

Figure 2. Weighted multivariable Cox regression analysis, with forest plots showing average hazard ratios (HR). CI: confidence interval; ECOG PS: Eastern Cooperative Oncology Group performance status; CCIS: Charlson Comorbidity Index Score; NSCLC: non-small cell lung cancer; EGFR: epidermal growth factor receptor; PD-L1: programmed death-ligand 1

administered in subsequent lines. However, the mOS of PS 0 and PS 1 patients, has not been compared in RCTs and rarely in RWS of second-line ICI [3–5,37,38].

A large proportion of the patients in our study had metastatic disease (86%), which is representative of the palliative NSCLC population. However, in most RCTs, information regarding metastatic sites is rarely available, despite the known prognostic impact [3–5,28,29]. In the present study, bone- and liver metastases were significant poor prognostic factors for OS, whereas brain metastases did not affect OS. This is comparable to results from other RWS [19,39–42]. In most patients, brain metastases are stable at ICI initiation due to previous local therapy with radiotherapy or neurosurgery. In our study, not all patients had a MRI of the brain prior to ICI initiation, thus the actual number of patients with brain metastases, as opposed to those with liver metastases, were not known at baseline. These factors may explain the lack of impact on OS of brain metastases. Poor PS, liver and bone metastases er known poor prognostic factors, and based on our results, it is difficult to assess whether these patients actually could benefit from ICI compared to best supportive care or subsequent line chemotherapy. However, our results imply that careful consideration should be made before administering ICI to particularly patients with PS $\geq$2.

In accordance with another RWS, no association between comorbidity and OS was observed [42]. However, comorbidities are rarely reported in RWS of ICI-treated patients with advanced NSCLC.

Strengths and limitations

The strengths of this study are the inclusion of a nationwide unselected population of all Danish patients with NSCLC treated with ICI in second-line or further subsequent line, the completeness of follow-up for all patients, and the large sample size, allowing for strong subgroup analyses. The study had some limitations. The retrospective nature of the study, reduced the validity of the comorbidity data, which preferably should be prospectively collected. Likewise for smoking status, ECOG PS, grade of toxicity by the Common Toxicity Criteria (CTC), and tumor response evaluation according to Response Evaluation Criteria in Solid Tumors [43,44]. Laboratory data and data regarding potential confounders such as prior or concomitant glucocorticoid and antibiotic administration and body mass index were also not obtained [44–46].

Conclusion

The OS of ICI-treated patients in our study, was comparable to the OS demonstrated in RCTs [3–5]. Women accounted for half of the patients in this Danish cohort, making the results from this cohort especially comparable to other countries (including Nordic countries) with a high proportion of female NSCLC patients eligible for ICI. Furthermore, our results showed that older age did not affect ICI efficacy, and ICIs should not be excluded as a treatment option, due to high chronological age. Patients with PS $\geq$2 had only very limited effect of ICI with a very poor prognosis, thus careful consideration should be made on an individual basis when offering ICIs to this subgroup. Data on metastatic sites should be available in future RCTs, because of the prognostic impact on OS and in order to improve the comparison between future RCTs and RWS.

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Ethics approval and consent to participate

Approved by the Danish Patient Safety Authority and reported to The Danish Data Protection Agency.

Consent for publication

Patient consent was waived by the Danish Patient Safety Authority, due to the retrospective design of the study, and the use of routinely collected data.

Disclosure statement

The funding sources were not involved in the study design, collection, analysis, and interpretation of data, in the writing of the report, or in the decision to submit the article for publication. GF Persson: Advisory board Roche, Astra Zeneca, BMS, MSD, Takeda, Pfizer. Congress travels with Roche, Astra Zeneca, BMS, MSD, Takeda, Pierre Fabre. Research grants from Varian Medical Systems. M Pøhl: Honoraria for lectures and consultancy from AstraZeneca, BMS, MSD, Pfizer, Roche. SW Langer: Advisory board MSD, Roche, Pfizer. The remaining authors declare no conflict of interest.

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Data availability statement

The study data may be available on request from the corresponding author, Mette T Mouritzen. The data are not publicly available due to the General Data Protection Regulation.

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Gene expressions, high lymphocyte count, and absence of liver metastases may predict durable clinical benefit in patients with advanced NSCLC treated with immune checkpoint inhibitors

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Abstract:

Background: Not all patients with advanced NSCLC benefit from immune checkpoint inhibitors (ICIs). Therefore, we aimed to assess the predictive potential of gene expression profiling (GEP), peripheral immune cell counts, and clinical characteristics. **Methods**: The primary endpoint of this prospective, observational study was durable clinical benefit (DCB) defined as progression-free survival >6 months. In a subgroup with histological biopsies of sufficient quality (n=25), GEP was performed using the nCounter® PanCancer IO 360 panel. **Results**: DCB was observed in 49% of 123 included patients. High absolute lymphocyte count (ALC) and absence of liver metastases were associated with DCB (OR=1.95, p=0.038 and OR=0.36, p=0.046, respectively). GEP showed clustering of differentially expressed genes according to DCB, and a strong association between PD-L1 assessed by GEP (CD274) and immunohistochemistry (IHC) was observed (p=0.00013). The TGF-β, dendritic cell, and myeloid signature scores were higher for patients without DCB whereas the JAK/STAT loss signature scores were higher for patients with DCB (unadjusted p-values <0.05). **Conclusions:** ALC above 1.0 10^9/l and absence of liver metastases were significantly associated with DCB in ICI-treated patients with NSCLC. GEP-derived signatures may be associated with clinical outcome and PD-L1 could be assessed by GEP rather than IHC.

Keywords: cancer immunotherapy; immune checkpoint inhibitors; non-small cell lung cancer; advanced lung cancer; biomarkers; gene expression analysis; lymphocyte count; liver metastases

1. Introduction

Immune checkpoint inhibitors (ICIs), anti-Programmed Death-(Ligand)-1 (PD-(L)1) antibodies, have revolutionised the treatment of patients with advanced non-small cell lung cancer (NSCLC). Randomised controlled trials (RCTs) have demonstrated improved overall response rates, progression-free survival (PFS) and overall survival (OS) compared to standard chemotherapy in patients treated with first- or subsequent-line ICI monotherapy [1-5]. Furthermore, a subgroup of patients becomes long-term responders with improved 3- and 5-year survival rates in both RCTs and daily cancer care [6-8]. In Denmark, according to national guidelines, the selection of patients with advanced NSCLC for ICI-based treatment is dependent on the PD-L1 tumor proportion score (TPS) [9]. However, PD-L1 TPS has shown limited potential as a single predictive biomarker of

response to ICIs. In patients with squamous NSCLC treated with subsequent-line (≥2L) ICI, no significant survival differences between PD-L1 negative and PD-L1 positive patients were observed, and around 40% of patients treated with first-line (1L) ICI and PD-L1 TPS ≥90% do not respond [1, 10]. Therefore, complementary biomarkers have been proposed, and may be related to both tumor cells, tumor microenvironment (TME), the immune system, and other host factors. Besides PD-L1 TPS, microsatellite instability (MSI)/ mismatch repair deficiency and tumor mutational burden (TMB) have been approved by the Food and Drug Administration (FDA) [11, 12]. Due to the continuous and dynamic nature of TMB, no gold-standard method or cut-off value exists [13]. Consensus guidelines exist for MSI due to the clinical role in cancers associated with Lynch syndrome [14]. In NSCLC though, the prevalence of MSI-high and TMB-high status is only approximately 1% and 15%, respectively, and they are not yet incorporated into clinical guidelines in NSCLC [15, 16].

Other comprehensively investigated clinical factors with prognostic value and a possible association with ICI efficacy include the immune phenotypes, the presence of tumor-infiltrating lymphocytes (TILs), and their relative abundance and location [17]. In addition, an INFγ-related 18-gene mRNA, T-cell inflamed gene expression signature (TIS) has been associated with improved ICI response across different tumor types [18]. The 18-gene TIS was also applied to The Cancer Genome Atlas (TCGA) RNA-sequencing dataset, showing high median TIS scores in NSCLC resections [19]. Gene expression profiling (GEP) holds the potential to integrate the investigation of biomarkers related to tumor cells, TME and immune cells simultaneously. Furthermore, GEP can be performed with a relatively low amount of RNA with good quality and hence should not require large tissue samples or resections [18]. However, few studies of GEP in routine clinical practice of patients with advanced NSCLC have been performed [20]. Peripheral blood biomarkers have been associated with ICI efficacy such as neutrophil-to-lymphocyte-ratio (NLR), lactate dehydrogenase, and absolute lymphocyte count (ALC) [21, 22]. A post hoc analysis of the phase III OAK trial showed predictive value of NLR in ICI-treated patients compared to patients treated with chemotherapy [23]. Furthermore, high pre- and post- ICI treatment peripheral lymphocyte count has been associated with improved survival in patients with NSCLC [24]. To increase the predictive value, and hence to improve the selection of patients for ICI treatment, different immunograms and models have included multiple of the proposed biomarkers [25-27]. Although none of the proposed biomarkers or predictive models have been implemented in the clinical treatment guidelines for patients with NSCLC, the clinical variables and gene expression signatures have shown promising predictive potential.

In this study, we aimed to assess the impact of gene expressions, clinical features, and peripheral immune cell counts on durable clinical benefit (DCB) in patients with advanced NSCLC treated with ICIs in routine clinical cancer care.

2. Materials and Methods

2.1 Study design and patients

The study was a real-world prospective, observational and explorative study. The study population consisted of consecutively included patients with advanced NSCLC, who received at least one cycle of anti-PD-1 or anti-PD-L1 monotherapy as 1L or ≥2L of treatment. Patients with EGFR-mutations, ALK-rearrangements, or curative treatment options were excluded.

At the Department of Oncology, Aalborg University Hospital, 58 patients were included regardless of treatment line. The patients were included from August 2018 to September 2019 (ClinicalTrials.gov NCT03658460). An additional cohort of 65 patients treated with 1L ICI was included at the Department of Clinical Oncology and Palliative Care, Zealand University Hospital, Naestved. These patients were included from July 2018 to

June 2020 (ClinicalTrials.gov NCT03512847). Treatment criteria, monitoring and follow-up were similar at the two recruiting departments.

2.2 Data collection and data management

Baseline characteristics were prospectively collected including age, sex, eastern cooperative oncology group (ECOG) performance status (PS), smoking status, BMI, and TNM stage (IASLC 8th edition) with additional information on metastatic sites in case of stage IV disease. Furthermore, information on biopsy modalities, NSCLC histopathological subtype, and PD-L1 TPS was recorded. From baseline peripheral blood samples, the ALC and absolute neutrophil count (ANC) were obtained and the NLR was derived (ANC/ALC).

Patients received ICI treatment according to the national treatment guidelines at that time; pembrolizumab 2mg/kg/3w or 200 mg/3w, atezolizumab 1680 mg/4w, or nivolumab 3 mg/kg/2w [28, 29]. Information describing the patient's treatment was collected, which included treatment line, ICI treatment duration, reasons for ICI discontinuation, ICI treatment beyond progression, and post-ICI systemic antineoplastic treatment.

CT scans were performed every 8-9 weeks for treatment response evaluation and were described according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1. The primary clinical endpoint was durable clinical benefit (DCB) defined as progression-free survival (PFS) >6 months. PFS was calculated from the first ICI-administration date (index date) to the date of progressive disease (PD), death, or the last follow-up or censoring date. The last follow-up date was defined as the date of the last radiological response evaluation. No patients were lost to follow up. Furthermore, OS was calculated from the index date to the date of death or the date of data cut-off. The censoring date was March 1, 2022 for patients treated at Zealand University Hospital, and May 1, 2022 for patients treated at Aalborg University Hospital.

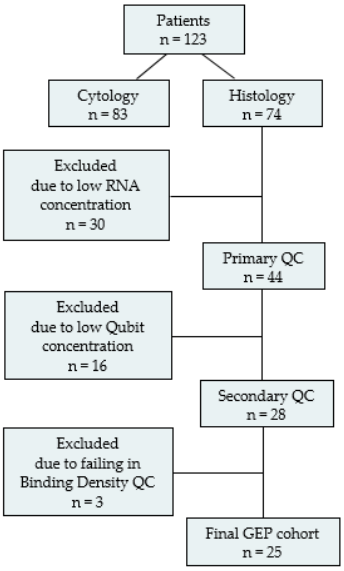
2.3 Tissue samples and routine diagnostics

The tissue samples were routinely performed histological or cytological diagnostic biopsies, formalin-fixed and paraffin-embedded (FFPE). In most of the patients receiving ≥2L ICI, systemic antineoplastic treatment was administered between the time of tissue sampling and the date of first ICI administration (n=25; 93%). The routine diagnostic framework included morphological examination and immunohistochemistry (IHC) to establish the cancer diagnosis and determine the histopathological subtype of NSCLC. Standard assessment of PD-L1 TPS was performed by IHC with the 22C3 pharmDx antibody stained on the Dako Omnis platform. PD-L1 TPS was categorized as <1%, 1-49%, and ≥50%. Next generation sequencing (NGS) was routinely performed with the TruSight® Tumor 15 assay (Illumina) (patients included at Aalborg University Hospital) or GeneRead QIAact AIT Panel (patients included at Zealand University Hospital, Naestved) to assess *EGFR, BRAF, KRAS* and *ERBB2* status. ALK rearrangements were routinely assessed by IHC, and in cases with inconclusive or positive IHC, additional fluorescence in situ hybridization (FISH) was performed to confirm the presence/absence of ALK rearrangements.

2.4 Gene expression profiling

Prior to GEP, the tumor percentage was estimated in histological samples by a pathologist. After excluding patients with cytology only, insufficient tissue, failing quality controls (QC) or failed analysis the final GEP-cohort consisted of 25 patients (Figure 1).

Figure 1. Flowchart of baseline tissue samples prior to gene expression profiling



The trajectory of tissue samples selected for gene expression profiling (GEP).
n, number of patients; QC, quality control

Histological samples were analysed using a 770-gene expression panel, the nCounter® PanCancer IO 360 panel (NanoString Technologies, Inc.). According to the recommendations of the manufacturer, extraction of total ribonucleic acid (RNA) was performed manually on 10x5 μm sections from FFPE samples using the miRNeasy FFPE kit (Qiagen). The extracted RNA was eluted in 13 μl RNAase-free water and the RNA concentrations were determined by using the Qubit 3 Flourometer (Invitrogen™). The purified RNA was stored at -80°C. Only samples with an RNA concentration ≥60ng/ul were included in the final GEP cohort. An input amount of 300 ng RNA was used for each sample during Nanostring analysis. Hybridization was performed using the nCounter® PanCancer IO360 gene expression panel (NanoString Technologies, Inc.). The technical integrity of the nCounter® profiling assay underwent further QC assessment. The sample input and reaction efficiency were assessed by the geometric mean of housekeeper genes in each sample. A minimum geometric mean count of 32 housekeeper genes was required for analysis, and geometric mean counts of 32-100 were considered borderline. Furthermore, the nCounter® profiling assay was assessed according to imaging, binding density, positive control linearity, and limit of detection. To correct for cartridge differences, background correction and data normalization were performed before the final data analysis. The final analysis included data from samples that passed all QC steps.

2.5 Next generation sequencing

TMB and MSI status was assessed by NGS. DNA was extracted from 10x5 μm sections from FFPE samples using the Maxwell® 16 FFPE Plus LEV DNA Purification Kit (AS1135). Only samples with a DNA concentration ≥3,33ng/ul were included in the final GEP cohort. The TruSight® Oncology 500 (TSO500; Illumina) gene panel was used for sequencing analysis. Library preparation was performed using the TruSight® Oncology 500 reagent kit according to the manufacturer's protocol and the samples were run on the NextSeq™ 550 instrument (Illumina®) [30]. Only samples that passed all sequencing QCs were included for further analysis. The TSO500 Local Run Manager TruSight® Oncology

500 v2.2 Analysis Module was used to generate TMB and MSI scores [31]. TMB was defined as the number of eligible variants divided by the effective panel size. The TMB-high cut-off was 10 mutations/Mb. The MSI score was defined as the number of unstable MSI sites divided by the total number of assessed MSI sites [31]. The MSI-high cut-off was 20%.

2.6 Statistical analyses

2.6.1 Descriptive statistics, logistic regression, and survival analyses

Comparisons of patients receiving ICI treatment in 1L or ≥2L were performed with ANOVA tests for the continuous variables and Fisher's exact tests for the categorical variables. Fisher's exact test was chosen to account for the expected low values. Median values of ALC, ANC, and NLR were used for the comparisons.

Logistic regression analysis was used to assess factors associated with DCB. First, univariable logistic regression analyses were conducted with DCB as the dependent variable and each of the baseline characteristics as the independent variable. Brain-, bone-, and liver metastases were included as the only metastatic sites due to the known prognostic impact on survival in NSCLC. Secondly, multivariable logistic regression analysis was conducted and included age, sex, PS, and PD-L1 and factors significantly associated with DCB in the univariable logistic regression analysis. Wald test p-values and profile likelihood confidence limits were reported.

A Cox proportional hazards model was used for the OS analysis. Analyses were restricted to patients receiving 1L ICI treatment (n=96), due to significant differences in selection criteria for ICI and prognostic clinical and pathological factors according to treatment line. Univariable Cox regression analyses were performed for baseline characteristics. Subsequently, a multivariable Cox regression analysis was performed including age, sex, PS, and factors significantly associated with OS in the univariable analyses. One patient with missing ALC was excluded from the multivariable model. Schoenfeld residuals revealed no significant nonproportionality in the multivariable model, indicating that the assumption of proportional hazards was reasonable.

A ROC curve using ALC as a predictor for DCB was drawn. A Two-sample Kolmogorov–Smirnov plot was used to find the optimal ALC cut-off for predicting DCB [32]. This optimal cut-off was determined as the cut-off value of the ALC that yielded the maximal difference between the cumulative density of ALC in the DCB negative/DCB positive group. Subsequently, this cut-off was used to dichotomize the ALC.

P-values <0.05 was considered statistically significant, and no adjustments for multiple testing were performed. Statistical analyses were performed with R version 4.2.1 [33].

2.6.2 Bioinformatics

Differential expression of genes

Gene expression analyses were performed to identify differentially expressed genes for response (DCB vs. no DCB). First, gene counts were normalised to log2 counts per million using the function Voom (Limma R package) and the trimmed mean of M-values (TMM) method from the R package edgeR [34, 35]. Next, a linear model was fit to each gene adjusting for biological factors associated with DCB using the R package limma [34]. The p-values were corrected for multiple testing using Benjamini–Hochberg false discovery rate (FDR). Significant differentially expressed genes were identified using FDR cutoff of 5%. The patterns of the gene expression of genes with a p-value below 0.05 were further explored using the ComplexHeatmap package [36]. The package was applied to cluster the patients and the genes using hierarchical clustering based on euclidean distance. ANOVA test was used to assess to the association between the categorical IHC-derived PD-L1 TPS and the continuous GEP-derived PD-L1 (CD274).

Gene expression signatures

Differences in gene expression signature scores according to DCB were also evaluated. Gene expression signature scores were calculated as a weighted linear combination of the included genes' expression values normalized to stable housekeeper gene expression as described by the manufacturer [18]. As in the gene expression analysis, a linear model was fit to each gene adjusting for biological factors associated with DCB using the R package limma [34]. The p-values were using FDR, and FDR <0.05 was considered statistically significant.

3. Results

3.1. Baseline patient characteristics

The study included 123 patients. Overall, 44% of patients were female, and the median age was 67 years (range: 46-86). ICI was administered in 78% of the patients as 1L and 22% as ≥2L. Significant differences in PS, PD-L1 TPS, NSCLC histopathological subtype, lung and peripheral lymph node metastases were observed between patients treated with 1L and ≥2L ICI. No significant differences in median ALC (p=0.33), ANC (p=0.84), and NLR (p=0.21) were observed according to treatment line (Table 1).

Table 1. Baseline characteristics and peripheral immune cell counts according to treatment line

Baseline characteristics	**1L** **n (%)**	**≥2L** **n (%)**	**Total** **n (%)**	**p-value**
Patients	96 (78)	27 (22)	123 (100)	
Age, median years (range)	66 (46-86)	70 (52-83)	67 (46-86)	0.12
Sex				
Male	52 (54)	17 (63)	69 (56)	0.51
Female	44 (46)	10 (37)	54 (44)	
Performance status				
0	38 (40)	1 (4)	39 (32)	<0.001
1	48 (50)	16 (59)	64 (52)	
≥2	10 (10)	10 (37)	20 (16)	
Smoking status				
Current	30 (31)	12 (44)	42 (34)	0.37
Former	64 (67)	15 (56)	79 (64)	
Never	2 (2)	0 (0)	2 (2)	
BMI, median (range)	25 (16-41)	23 (18-40)	24 (16-41)	0.36
TNM stage				
III	14 (15)	3 (11)	17 (14)	0.76
IV	82 (85)	24 (89)	106 (86)	
Metastatic sites[a]				
Brain	8 (8)	2 (7)	10 (8)	1.0
Bone	28 (29)	5 (19)	33 (27)	0.33
Liver	18 (19)	8 (30)	26 (21)	0.29
Adrenal glands	23 (24)	7 (26)	30 (24)	0.81

Distant lymph nodes	9 (9)	7 (26)	16 (13)	0.05
Lung	19 (20)	14 (52)	33 (27)	0.002
Pleura[b]	35 (37)	8 (30)	43 (35)	0.65
Soft tissue[c]	5 (5)	0 (0)	5 (4.1)	0.59
Other	22 (23)	2 (7)	24 (20)	0.10
NSCLC subtype				
Adenocarcinoma	69 (72)	15 (56)	84 (68)	
Squamous cell carcinoma	17 (18)	12 (44)	29 (24)	0.008
Other[d]	10 (10)	0 (0)	10 (8)	
PD-L1				
<1%	0 (0)	10 (37)	10 (8)	
≥1% and <50%	1 (1)	14 (52)	15 (12)	<0.001
≥50%	95 (99)	3 (11)	98 (80)	
Blood values, median (range)*				
ALC (10^9/l)	1.42 (0.30-3.60)	1.27 (0.43-2.99)	1.40 (0.30-3.60)	0.33
ANC (10^9/l)	6.60 (2.90-36.3)	6.78 (3.14-16.2)	6.70 (2.90-36.3)	0.85
NLR	4.40 (1.16-34.7)	4.40 (1.99-37.7)	4.40 (1.16-37.7)	0.21

a) Patients could be registered with more than one metastatic site. Each metastatic site was recorded as a categorical variable (yes or no), and the p-values reflect the distribution of the two levels for each metastatic site.
b) 'Pleura' included pleural fluid
c) 'Soft tissue' included cutis, subcutis and muscles
d) 'Other' included NSCLC NOS (not otherwise specified) and sarcomatoid carcinoma
* ALC and NLR were missing in one patient treated with 1L ICI
n, number of patients; BMI, body mass index; TNM, tumor-node-metastasis classification of malignant tumors; NSCLC, non-small cell lung cancer; PD-L1, programmed death-ligand 1; ALC, absolute lymphocyte count; ANC, absolute neutrophil count; NLR, neutrophil-to-lymphocyte ratio

3.2 Treatment characteristics

The median time to treatment discontinuation was 105 days (range 1-763), without significant differences between 1L and ≥2L (p=0.14). ICI treatment was discontinued due to PD (n=68; 55%), toxicity (n=33; 27%), completion of 2 years of ICI treatment (n=10; 8%), poor PS (n=8; 7%), death (n=4; 3%), and/or 'other' reasons (n=19; 15%). 'Other reasons' included lack of compliance, patient's choice, comorbidity, or high dose steroid. ICI treatment discontinuation could be registered with more than one reason. Systemic antineoplastic treatment after ICI-discontinuation was administered in 49% of the patients (n=60), without statistically significant difference according to treatment line. Treatment beyond PD was observed in 11 patients (9%). Swimmer plot showing the course of individual patients from the initiation of ICI treatment is shown in Supplementary Figure S1.

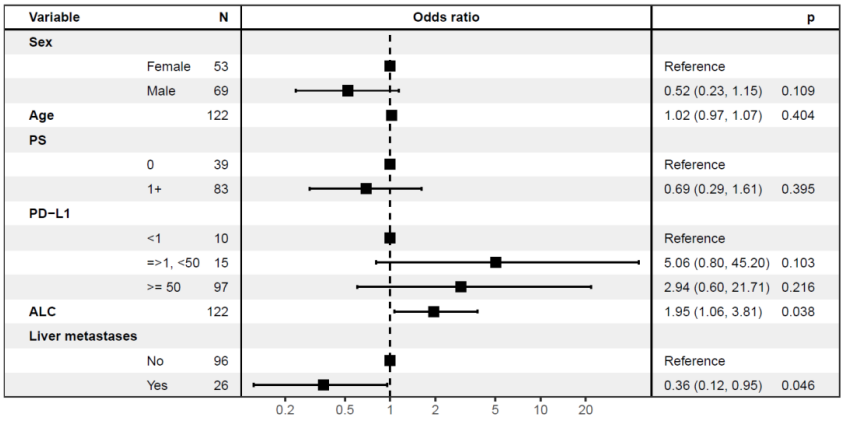
3.3 Predictive factors of durable clinical benefit

DCB was observed in 49% (n=60) of all patients and did not significantly differ in 1L compared to ≥2L (51% vs. 41%, p=0.40). A comparison of patients with and without DCB showed that the presence of liver metastases was significantly associated with not achieving DCB (30% vs. 12%, p=0.02) and ALC above median was significantly more frequent in patients with DCB (p=0.01).

Likewise, in the univariable logistic regression analysis liver metastases (OR 0.31, p=0.01) and ALC (OR 2.05, p=0.02) were significantly associated with DCB (Figure 2 and Supplementary Figure S2). In multivariable logistic regression analysis liver metastases (p=0.046) and ALC (p=0.038) remained significantly associated with DCB (Figure 2). The

increased rate of DCB in patients with PD-L1 ≥1% did not reach statistical significance (Figure 2).

Figure 2. Multivariable logistic regression analysis assessing the association between baseline characteristics and durable clinical benefit.

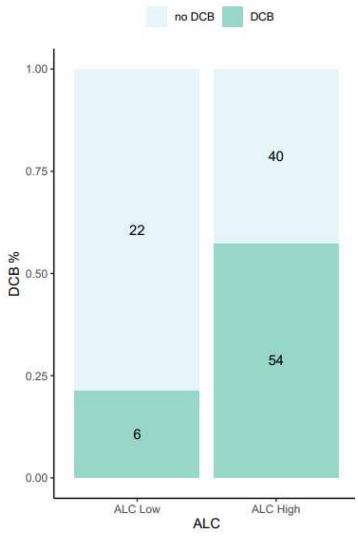


Multivariable logistic regression showing significant positive association between high median absolute lymphocyte count (ALC) and durable clinical benefit (DCB) and negative association between liver metastases and DCB.

N, number of patients; PS, performance status; PD-L1, programmed death-ligand 1

A ROC curve analysis was made to investigate the predictive potential of ALC as a single biomarker for DCB, and this yielded an AUC of 0.63 (Supplementary Figure S4). An optimal cut-point of 1.0 10^9/l was found, corresponding to the 25% quartile, and using ALC dichotomised at this cut-point as a predictive biomarker for DCB resulted in a false positive rate of 0.64 and true positive rate of 0.90. DCB was observed in 21% of all patients with an ALC below the optimal cut-point of 1.0 10^9/l, and in 57% of all patients with an ALC above the optimal cut-point (Figure 3).

Figure 3. Bar chart presenting the relationship between peripheral lymphocyte counts and durable clinical benefit



All patients (n=122) were categorized as ALC low or ALC high, separated by the optimal ALC cut-point of 1.0 10^9/l. The numbers in the bars represent the absolute number of patients in each group. ALC was missing in one patient.
DCB, durable clinical benefit; ALC, absolute lymphocyte count

The mOS was 19.2 months (95%CI 0.33-41.7) and 12.5 months (95%CI 0.16-40.8) in patients treated with 1L and ≥2L ICI, respectively (p=0.09). Increased ALC was also associated with improved OS in multivariable Cox regression analysis of patients treated with 1L ICI (Supplementary Figure S3).

3.4 The GEP subpopulation

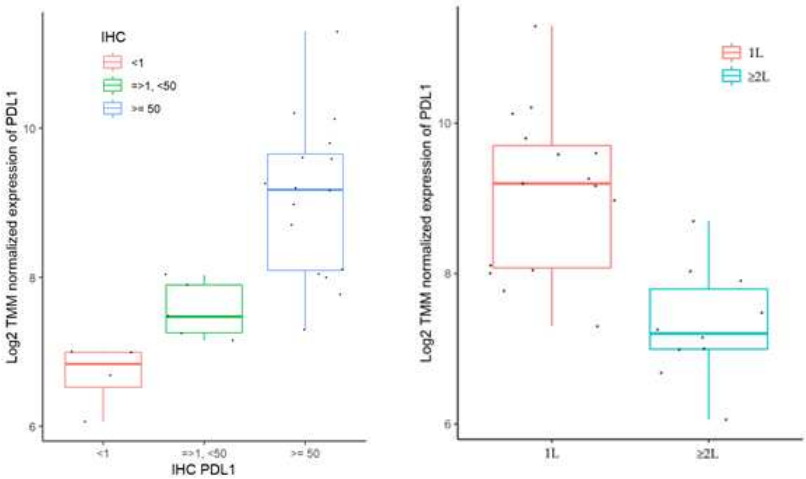
3.4.1 Baseline and treatments caracteristics and clinical outcomes

GEP was feasible in 33% (n=25) of all patients with diagnostic histological biopsies (n=74) (Figure 1). The comparison of baseline characteristics and peripheral immune cell counts in patients with and without GEP showed significantly more squamous cell carcinomas in the GEP cohort (p=0.007) (Supplementary Table S5). Significantly more patients with GEP received ICI in ≥2L (p=0.03) compared to patients without GEP. No significant differences in time to treatment discontinuation, DCB, and mOS were observed between the patients with and without GEP (Supplementary Table S6).

3.4.2 Gene expression analyses

Comparison of gene expression between patients with DCB and without DCB revealed 53 genes with a p-value below 0.05 (Supplementary Table S7). PD-L1 (CD274) was one of those genes (p=0.03), however, no genes were significant after adjustment for multiple testing (no FDR below 0.05). Pearson correlation of PD-L1 with genes differentially expressed between DCB and no DCB, showed a significant negative correlation with LTBP1 (p<0.05) and positive correlation with TAP1 and ITGAE (p<0.05). A highly significant association between the categorical PD-L1 TPS assessed by IHC and the continuous GEP-derived PD-L1 (CD274) was identified (p=0.00013). Furthermore, PD-L1 (CD274) was differentially expressed between patients receiving 1L and ≥2L ICI (p=0.0017) reflecting the treatment inclusion criteria (Figure 4).

Figure 4. The association between PD-L1 (CD274) derived by gene expression profiling and A) PD-L1 assessed by immunohistochemistry and B) treatment line

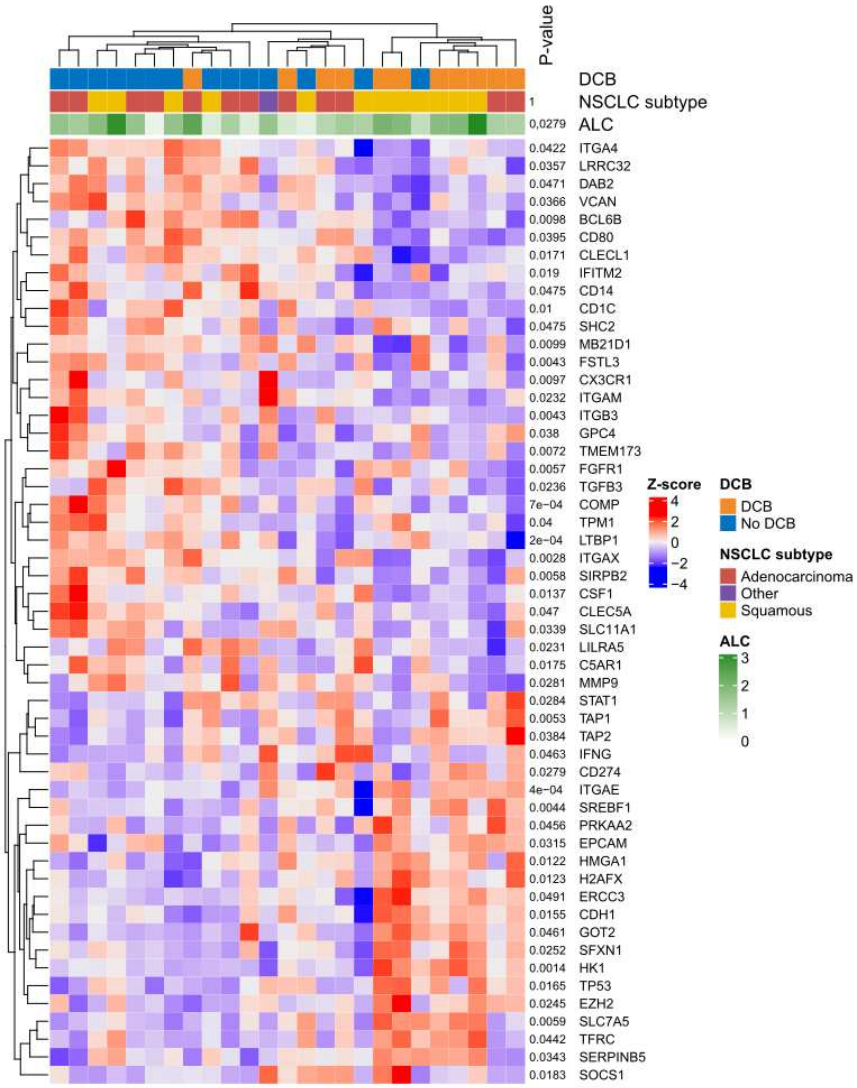


Boxplots of log2 normalized expression of PD-L1 for A) three levels of PD-L1 assessed by IHC (p=0.00013) and B) treatment line (p=0.00017).

PDL1, programmed death-ligand 1; IHC, immunohistochemistry; 1L, first-line treatment; ≥2L, treatment in second- or subsequent line

The patterns of the expression of the 53 genes with a p-value <0.05 were explored, and hierarchical clustering showed that two clusters separated the patients with and without DCB except for two patients. An intermediary heterogeneous cluster consisted of patients with or without DCB (Figure 5).

Figure 5. Hierarchical clustering of genes with a p-value below 0.05.



Heatmap of gene expression z-scores for genes with a p-value <0.05 in comparison between DCB vs. no DCB. The patients (columns) (n=25) and the genes (rows) are clustered using hierarchical clustering based on euclidean distance. The dendrogram added to the top and to the left visualize the order of the clustering. In the top three annotation rows are added to indicate each patient's DCB status, NSCLC subtype, and ALC. Finally, a p-value

is listed for each row. The p-value for NSCLC subtype and ALC compares DCB vs. no DCB using a Fisher's exact test and unpaired t-test, respectively. The p-value in front of the genes derives from the gene expression test.

The gene expression signature scores in patients with DCB and without DCB were compared. These analyses identified no signatures with FDR <0.05; however, four signatures had an unadjusted p-value <0.05. The TGF-β (p=0.047, log2FC= -0.92), dendritic cell (DC) (p=0.025, log2FC= -0.92) and myeloid (p=0.024, log2FC= -0.80) signature scores were higher for patients without DCB whereas the JAK/STAT loss signature scores (p=0.005, log2FC= 1.41) were higher for patients with DCB.

3.5 Next generation sequencing

TMB and MSI were available in only 42% (n=51) of all the patients (n=123), and 47% (n=24) of the analysed tissue samples were TMB-high. NGS was feasible in only 24% (n=6) of patients in the GEP subpopulation. No tumor samples were MSI-high, and therefore MSI status was not included in the analyses.

4. Discussion

This prospective study included 123 consecutive patients with advanced NSCLC treated with ICI in routine clinical cancer care. The association of baseline characteristics, peripheral immune cell counts, and GEP was assessed with DCB being the primary clinical endpoint. No consensus on DCB definition exists and we defined DCB as PFS>6 months to increase comparability with other GEP studies in NSCLC [37, 38]. The DCB was similar regardless of treatment line allowing for analysis of predictive factors for DCB in the combined population. Additionally, the time to treatment discontinuation, as a proxy for dose intensity, and mOS were similar regardless of treatment line.

4.1 Liver metastases and peripheral immune cell counts

As demonstrated in other real-world studies and RCTs, the presence of liver metastases was negatively associated with DCB and OS in our study [39-41]. A study of patients with malignant melanoma, showed that liver metastases had significantly lower T-cell infiltration and increased TIM-3 expression than lung and lymph node metastases [42]. A recent study in NSCLC also demonstrated that the CD8+ T-cell infiltration was lower in liver metastases compared to other metastatic lesions, and that combined PD-L1 TPS ≥1% and CD8+ T-cell infiltration in liver metastases increased PFS [43]. These biological mechanisms may contribute to the poorer ICI efficacy in patients with liver metastases.

An increase in ALC was significantly associated with DCB and improved OS in our study. We also found that an ALC of 1.0 10^9/l was the most optimal cut-point for predicting DCB, and confirmatory studies in independent, larger populations are warranted, as this variable is easily obtainable in routine clinical cancer care. High pre- and post-ICI peripheral lymphocyte counts, and specific subsets of peripheral lymphocytes have also been associated with improved outcome in ICI-treated patients with NSCLC, whereas lymphopenia has been associated with impaired survival [24, 44, 45]. Additionally, a lower percentage of peripheral lymphocytes in NSCLC has been observed in male patients and patients with bone- and liver metastases and has been associated with poor survival regardless of NSCLC histopathological subtype and disease stage [46].

In contrast, no association between ANC or NLR and DCB or OS was found in our study. A meta-analysis showed that a higher NLR was associated with poorer OS in ICI-treated patients with lung cancer; however, other factors such as NSCLC subtype may impact the predictive value of NLR [47]. A recent study demonstrated that lung adenocarcinomas had more effector and activated T cells and fewer Treg cells compared to lung squamous cell carcinomas assessed by single-cell RNA sequencing from surgical resections [48].

4.2 GEP and PD-L1 assessment

Since NSCLC histopathological subtype and lymphocyte counts may interact and impact the GEP, the analyses of gene- and gene expression signatures were adjusted for NSCLC histopathological subtype and ALC. However, no significant differentially expressed genes or signatures were found when adjusting for multiple testing (no FDRs below 0.05). Despite the lack of significant FDRs, a clustering tendency of differentially expressed genes was observed according to DCB (Figure 4) and indicates that certain gene expression phenotypes may be associated with DCB in ICI-treated patients with advanced NSCLC.

Notably, a strong association between the categorical PD-L1 TPS assessed by IHC and the continuous GEP-derived PD-L1 (CD274) was identified. Furthermore, PD-L1 (CD274) was differentially expressed between patients receiving 1L and ≥2L ICI, which correspond to the different PD-L1 cut-offs in treatment guidelines in patients with advanced NSCLC [9]. These findings indicate a clinical relevance of GEP in treatment decisions, and GEP eliminates the intra- and inter observer differences in the IHC assessment of PD-L1 [49].

4.3 GEP in ICI-treated patients with advanced NSCLC

Most NSCLC gene expression studies rely upon surgically resected early-stage tumors with large tissue resections, and thus may not be comparable to gene expressions in ICI-treated patients with advanced stage disease [19, 50]. Only few GEP studies have included patients with advanced NSCLC treated with ICI in routine clinical care. One other study used the same panel and included both histological and cytological samples [37]. However, no comparison between the two sample types was performed according to RNA quality or differences in intratumor gene expressions [37]. Two GEP studies on ICI-treated patients with NSCLC have shown that DC66b expression was associated with poor OS [51] and high immune cell scores (T cells, NK-cells and M1 macrophages) were associated with DCB, respectively [37, 38]. Another study found INF- and antigen processing/presentation signatures to be positively associated with PFS in adenocarcinomas and TME signatures to be associated with PFS in squamous cell carcinomas [52]. However, adjustment for baseline clinicopathological factors prior to the gene expression analyses have not been consistently performed, which may confound the true predictive association with ICI efficacy of the proposed gene expressions. Furthermore, the comparison of different GEP studies is very challenging due to the small sample sizes and differences in disease stage, NSCLC subtypes, tissue sample types, sample sites (primary or metastatic, and which metastatic location), RNA preparation, GEP panels, statistical analyses and endpoint definitions. Currently, no large GEP datasets of advanced stage NSCLC cancer cohorts are available for validation.

In our study, only 25 patients were included in the GEP cohort. This low number of suitable samples was primarily due to low concentration and quality of RNA, which may be explained by the thin sections (5 μm) holding a lower percentage of intact cells and more fragmented RNA compared to larger sections (10-20 μm) [53]. Therefore, the implementation of GEP in routine diagnostics requires improved RNA quality and revision of the diagnostic framework as suggested by Hirsch *et al*. [54]. In the GEP cohort, most patients treated with ≥2L ICI had received chemotherapy in between the time of the diagnostic tissue sampling and ICI initiation. A pre- and post-chemotherapy gene expression analysis of 29 paired samples has shown that the average expression of CTLA4, LAG3, TNFRSF18, CD80 and FOXP3 in an immune module were significantly decreased in post-chemotherapy samples, and dynamic changes in INF-γ expression were observed [55]. Additionally, INF-γ expression has been associated with improved outcome in ICI-treated patients [18, 55-57]. However, the direct impact of previous chemotherapy on ICI efficacy has not been assessed in clinical cohorts.

4.4 Gene expression signatures

No gene expression signatures were significantly associated with DCB when adjusting for multiple testing. However, DC, myeloid and TGF-β signature scores were higher

in patients without DCB and JAK/STAT loss signature scores were higher in patients with DCB.

DCs are important antigen presenting cells that contribute to initiation of anti-tumoral T-cell responses [58]. However, immature DCs generally induce immune tolerance, and tumors may induce immune evasion by disruption of normal DC function [58, 59]. Myeloid cells in the TME include tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) which promote tumor cell growth and invasion and suppress immune responses [60, 61]. TGFβ (TGFB1) in the TME inhibits immune activity against tumor and promotes tumor growth and survival [62]. Clinical investigation of dual inhibition of TGFβ and PD-(L)1 is ongoing in many solid tumors including NSCLC [63].

The JAK/STAT pathway plays an essential role in the differentiation of T-helper cells, and JAK/STAT inhibition in Tregs has shown downregulation of Foxp3 which attenuates the immunosuppressive function [64, 65]. Hence, the JAK/STAT function is cell specific and the impact of JAK/STAT loss on ICI-efficacy seems to be cell-dependent. However, in our study, the JAK/STAT loss signature, defined by the manufacturer, was not restricted to a specific cell type and the association with ICI efficacy remains unknown.

4.5 Strengths and limitations

The main strength of this study was the clinical relevance according to the target population (advanced/metastatic NSCLC) and treatment setting (palliative ICI treatment). Furthermore, patients were consecutively included, clinical data completeness was high, and no patients were lost to follow up. Only histological biopsies were used for GEP to increase the likelihood of the tumor, TME, and immune response biology to be represented in the samples. Additionally, RNA amplification on low quality samples was not performed, to avoid the risk of amplification bias on these samples.

The main limitation of this study was the low number of patients included in the GEP cohort (n=25) due to the poor RNA quality. Furthermore, the DCB impact of chemotherapy on GEP prior to ICI treatment was not assessed. The potential interaction between peripheral ALC and other baseline characteristics, such as NSCLC histopathological subtype, metastatic sites, sex, and age was also not assessed in this study.

5. Conclusions

In patients with advanced NSCLC treated with ICIs in routine clinical cancer care, high ALC and absence of liver metastases were significantly associated with DCB. PD-L1 assessed by GEP was highly correlated with IHC-assessed PD-L1 and treatment line, indicating a clinical relevance of GEP. DC, myeloid and TGF-β signature scores were higher in patients without DCB and JAK/STAT loss signature scores were higher in patients with DCB. However, with the current clinical diagnostic framework GEP is only feasible in one third of the patients.

Supplementary Materials: Figure S1: Swimmer plot; Figure S2: Univariable DCB; Figure S3: Multivariable OS 1L; Figure S4: ROC ALC DCB; Table S5: Baseline characteristics GEP; Table S6: Treatment characteristics and outcome GEP; Figure S7: Differentially expressed genes

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