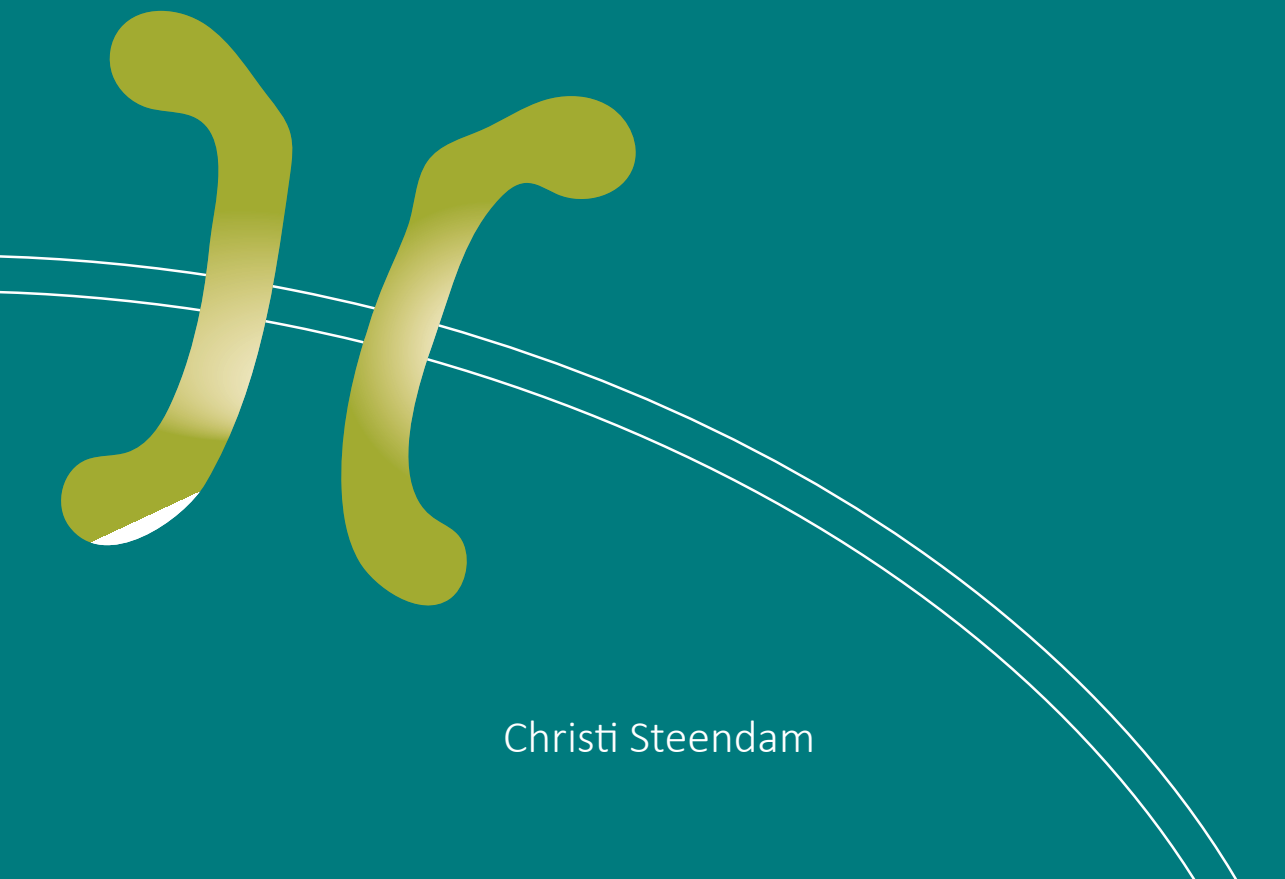
Innovations in Optimizing Treatment of Non-Small Cell Lung Cancer with **EGFR** Targeted Tyrosine Kinase Inhibitors

Exploring clinical practice and plasma



Christi Steendam

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Innovaties om behandeling van niet-kleincellige longkanker met EGFR gerichte tyrosine kinase inhibitoren te optimaliseren

Onderzoek in de klinische praktijk en in plasma

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Colofon

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ISBN: 978-94-6458-988-7

Provided by thesis specialist Ridderprint, ridderprint.nl
Printing: Ridderprint
Lay-out and cover design: Robin Weijland | persoonlijkproefschrift.nl

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Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. A.L. Bredenoord

en volgens besluit van het College voor Promoties.
De openbare verdediging zal plaatsvinden op

vrijdag 21 april 2023 om 13.00 uur

door

Christi Marja Johanna Steendam
geboren te Dordrecht.

Erasmus University Rotterdam

Promotiecommissie:

Promotoren:	prof.dr. A.C. Dingemans prof.dr. J.G.J.V. Aerts
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Chapter 1

Introduction

Introduction

Despite the advancements in diagnosis and treatment that have been made the last decades, lung cancer still has the highest mortality of solid tumors and remains a huge burden worldwide.[1] The largest histological subtype comprises non-small cell lung carcinoma (NSCLC).[2] Most patients are diagnosed in advanced or metastatic stage of disease, and the recurrence rates after definite treatment for early stage NSCLC are high.[3] Once metastasized, patients are treated in a palliative setting where systemic treatment can give opportunity to decrease symptom burden, delay the disease course and prolong life expectancy.[4] The backbone of systemic therapy has been platinum-based cytotoxic chemotherapy for a long time, which is based on inhibiting cell proliferation in the process of cell division in general. The different chemotherapy regimens proved more or less similar efficacy in improving overall survival (OS).[5, 6] In recent years the outcome of NSCLC has significantly improved by the introduction of immune checkpoint inhibitors and the identification of pathways and specific driver mutations which drive the cell to becoming a malignancy.[4, 7-9] As the focus of this thesis is on molecular altered NSCLC the developments of treatment with immunotherapy will not be discussed in depth.

During the process of DNA replication and cell division, our genetic material is susceptible to damage or errors and subsequent aberrations. In normal circumstances, the cell depends on checkpoints between the different phases of the cell cycle and on DNA repair mechanisms for protection against DNA malformations. Despite these protective mechanisms, alterations in the DNA do occur, and once present they will be preserved in future cell divisions. Although the cell DNA can contain these so-called somatic mutations, the consequences for the cell vary and depend on the location and epigenetic factors that determine the transcription of the genetic code in the DNA into RNA and subsequent translation into proteins. Changes in protein structure can enhance or diminish its function, and therefore when genes involved in regulatory circuits of cell survival and proliferation are affected, they can alter the cell cycle regulation and gather traits which drive the normal cell towards malignancy.[10]

DNA aberrations emprise multiple forms, starting from point mutations with substitution of a single nucleotide which changes the genetic code for a protein, to deletions of larger parts of DNA, and deletion-insertions when new fragments of DNA are introduced in the deleted area. Parts of the DNA code can also be duplicated and integrated in the chromosome, and whole genes can be amplified with multiple copies detectable in the cell nucleus. Another entity is formed by translocations, where chromosomes break and are rearranged with a part of another chromosome, which can lead to a new gene and protein due to the new fusion partner.

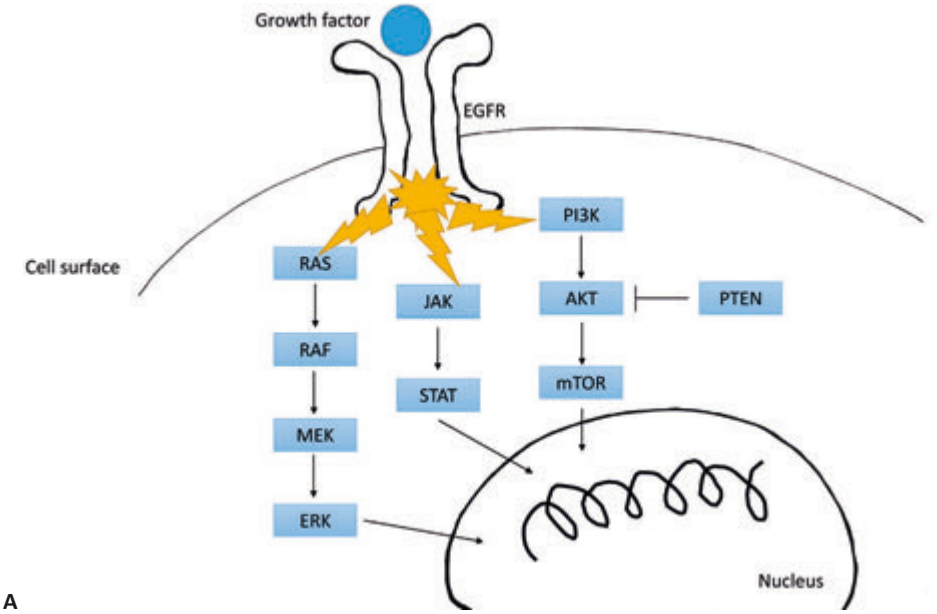
In NSCLC, and in particular adenocarcinoma, multiple driving genetic alterations have been identified over the years.[2] One of the most common affected genes is the Epithelial Growth Factor Receptor (*EGFR*), with an incidence of activating mutations of 10% in the Caucasian up to 35% in the Asian population.[11]

EGFR

EGFR (ErbB1/HER1) is a tyrosine kinase receptor on the cell surface and belongs to the ErbB family, together with ErbB2 (HER2/neu), ErbB3 (HER3) and ErbB4 (HER4). These receptors consist of an extracellular part where ligand binding takes place, a transmembrane part and an intracellular protein tyrosine kinase domain. Ligand binding results in dimerization and activation of a downstream signaling network by the activated kinase domain towards the nucleus. This cascade leads to changes in gene expression and transcription of factors associated with cell survival and proliferation.[12]

The journey of identification of EGFR as an important target in NSCLC took several years, the first challenge being distinguishing the difference between expression on one side and persistent kinase activation on the other side.[13] Increased receptor expression induces a hyperresponsiveness to limited amounts of ligand, in the absence of genetic aberrations of the *EFGR* gene (wild-type; WT). In case of oncogenic driver mutations in the *EGFR* gene in the tyrosine kinase encoding region, persistent ligand-independent signaling has a strong addictive effect on the tumor, depending mainly on this mechanism for its infinite proliferation (see **Figure 1**).[7] An important step was the development of EGFR tyrosine kinase inhibitors (TKI), small molecules designed to fit the ATP binding pocket at the tyrosine kinase domain to inhibit further downstream signaling.[13]

Most activating mutations in the cancer genome that provide a driving force of cell proliferation are found within the adenocarcinoma population of NSCLC, and their occurrence is often inversely related to smoking.[4] This explains the evolution of investigating efficacy of EGFR targeted treatment first in an unselected population, then through clinical enrichment of the treated population by selecting histology (adenocarcinoma) and smoking behavior (non- or former light smokers) eventually to biomarker selection based on detection of activating *EGFR* mutations once these were identified.[14-18]



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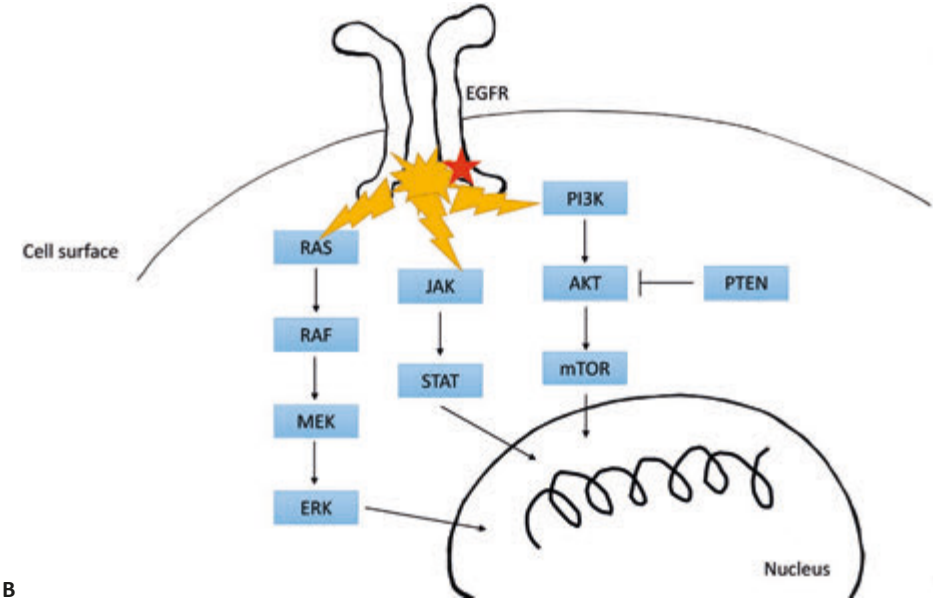

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Figure 1. *Schematic view of* ***A)*** *normal function of EGFR by stimulation by growth factor (ligand) at the extracellular domain, with subsequent dimerization and at the intracellular domain signaling by activated tyrosine kinase downstream to the nucleus through different pathways,* ***B)*** *abnormal function of EGFR with an activating mutation (red star) in the kinase domain, leading to continuous downstream signaling. RAS = rat sarcoma virus; RAF = rapidly accelerated fibrosarcoma; MEK = mitogen-activated protein kinase, ERK = extracellular signal-regulated kinase; JAK = Janus kinase; STAT = signal transducer and activator of transcription; PI3K = phosphatidylinositol-4, 5-biphosphate 3-kinase; AKT = Ak strain transforming; mTOR = mammalian target of rapamycin; PTEN = phosphatase and tensin homolog.*

EGFR in clinical practice

EGFR wild-type NSCLC

EGFR-TKI monotherapy has only modest activity in the wild-type (WT) population compared to patients with activating mutations, however the expression and stimulation of EGFR still plays a role in carcinogenesis in wild-type tumor cells.[13, 14, 16] In the normal cell evolving to a cancer cell, a trait in self-sufficiency in growth signals is acquired, which leads to sustained proliferative signaling, in which EGFR is an important player.[7, 10] The 1st generation EGFR-TKI erlotinib has been used as second line treatment after progression on systemic therapy in *EGFR*-WT NSCLC.[14, 19] This led to investigation of combination treatment with chemotherapy and EGFR-TKI in several phase I/II trials.[20-24] The multicenter randomized NVALT10 phase II study investigated the combination of chemotherapy regimens with intercalated erlotinib in the second line treatment of patients with unselected NSCLC.[25] This intercalated dosage regimen was adopted from observations in cell lines to prevent an antagonist effect.[26] A significantly prolonged survival was observed for combination therapy compared to erlotinib monotherapy (7.8 vs 5.5 months, p=0.01), restricted to the patients with non-squamous NSCLC.[25] Therefore the hypothesis was generated that WT populations also could benefit from EGFR-TKI therapy when added to regular chemotherapy in an intercalated scheme.

EGFR-mutated NSCLC

The efficacy of erlotinib and gefitinib showed superior in pretreated patients with activating mutations compared to *EGFR*-WT NSCLC, leading to phase III trials with randomization to conventional chemotherapy or EGFR-TKI as first-line treatment in these patients.[27, 28] Given the high response rates, progression free survival (PFS), favorable toxicity profile and durations of response, treatment with EGFR-TKI moved to first line for patients with *EGFR*-mutated NSCLC, despite the lack of difference in OS compared to conventional chemotherapy (see **Table 1**), which was probably due to high crossover rates.[19, 29] Although EGFR-TKI are the standard-of-care in first line therapy for patients with *EGFR*-mutated NSCLC, all patients develop resistance and subsequent progressive disease eventually. Investigation of the mechanism of resistance is able to detect acquired genetic aberrations that are responsible for treatment resistance in a subset of patients.[30] These genetic alterations can change the drug target, like *EGFR p.T790M* development blocks the binding of 1st and 2nd generation EGFR-TKI to the tyrosine kinase domain, or activate bypass tracks or downstream signaling pathways.[30] After development and implementation of 1st (erlotinib, gefitinib) and 2nd generation (afatinib) EGFR-TKI, the 3rd generation irreversible EGFR-TKI osimertinib was developed. Osimertinib proved effective in overcoming the *EGFR* exon 20 *p.T790M* gatekeeper resistance mutation, which develops in about half of the patients during treatment with 1st or 2nd generation EGFR-TKI.[31] Recently, osimertinib became the preferred treatment in first line after publication of the FLAURA trial results.[32, 33] PFS and OS were significantly longer than in the control arm treated with 1st generation reversible

EGFR-TKI's erlotinib or gefitinib, with an impressive mOS of 38.6 months (see **Table 1**) and a 3-year survival rate of 54%. [32, 33] Although toxicity is experienced as more favorable, especially concerning rash, still 32% of all subjects experienced toxicity grade 3 or higher.[32] In contrast to conventional chemotherapy, which is dosed on body surface area, EGFR-TKI are flat dosed agents. It is a known phenomenon that fixed dose TKI prescriptions generate variable drug plasma concentrations due to interpersonal pharmacokinetic differences and interactions with food and concomitant agents.[34] The concept of personalized medicine could be improved by exploring the relation of plasma concentrations and toxicity to work towards implementation of therapeutic drug monitoring (TDM).[35]

Table 1. Phase III randomized trials on EGFR-TKI monotherapy in first line treatment in patients with metastatic EGFRm+ NSCLC

Trial	Population	*n*=	Treatment	mPFS	mOS	ORR
Mok et al, NEJM 2009 (IPASS)[16]	Asia, non-/former light smokers	129 *EGFR*m+ (total 608)	Carboplatin AUC5/6 + paclitaxel 200mg/m^2	NR (1y PFS 6.7%)	21.9m [36]	47.3%
		132 *EGFR*m+ (total 609)	Gefitinib 1dd250mg	NR (1y PFS 24.9%)	21.6m [36]	71.2%
Maemondo et al, NEJM 2010 (NEJ002)[17]	Japan; *EGFR*m+	110	Carboplatin AUC6 + paclitaxel 200mg/m^2	5.4m	26.6m [37]	30.7%
		114	Gefitinib 1dd250mg	10.8m	27.7m [37]	73.7%
Mitsudomi et al, Lancet Oncol 2010 (WJTOG3405)[38]	Japan; *EGFR*m+	86	Cisplatin 80mg/m^2 + docetaxel 60mg/m^2	6.3m	37.3m [39]	32.2%
		86	Gefitinib 1dd250mg	9.2m	34.9m [39]	62.1%
Han et al, JCO 2012 (First-SIGNAL)[18]	Korea; never smokers	*EGFR*m+ 16 (total 150)	Cisplatin 80mg/m^2 + gemcitabine 1250mg/m^2	6.3m	25.6m	37.5%
		*EGFR*m+ 26 (total 159)	Gefitinib 1dd250mg	8.0m	27.7m	84.6%

Table 1. Continued

Trial	Population	*n*=	Treatment	mPFS	mOS	ORR
Patil et al, ESMO Open 2017[40]	India, *EGFR*m+	145	Carboplatin AUC5 + pemetrexed 500mg/m^2	5.6m	22.6m	45.3%
		145	Gefitinib 1dd250mg	8.4m	18.0m	63.5%
Zhou et al, Lancet Oncol 2011 (OPTIMAL)[41]	China; *EGFR*m+	82	Carboplatin AUC5 + gemcitabine 1000mg/m^2	4.6m	27.2m [42]	36.0%
		72	Erlotinib 1dd150mg	13.1m	22.8m [42]	83.0%
Rosell et al, Lancet Oncol 2012 (EURTAC)[43]	Europe, *EGFR*m+	87	Cisplatin 75mg/m^2 + docetaxel 75mg/m^2 or gemcitabine 1250mg/m^2 (or carboplatin AUC 6 or 5)	5.2m	19.5m	18.0%
		86	Erlotinib 1dd150mg	9.7m	19.3m	64%
Wu et al, Ann Oncol 2015 (ENSURE)[44]	Asia; *EGFR*m+	107	Cisplatin 75mg/m^2 + gemcitabine 1250mg/m^2	5.5m	25.5m	33.6%
		110	Erlotinib 1dd150mg	11.0m	26.3m	62.7%
Sequist et al, JCO 2013 (LUX-Lung 3)[45]	Global, *EGFR*m+	115	Cisplatin 75mg/m^2 + pemetrexed 500mg/m^2	6.9m	28.2m [46]	23.0%
		230	Afatinib 1dd40mg	11.1m	28.2m [46]	56.0%
Wu et al, Lancet Oncol 2014 (LUX-Lung 6)[47]	Asia, *EGFR*m+	122	Cisplatin 75mg/m^2 + gemcitabine 1000mg/m^2	5.6m	23.5m [46]	23.0%
		242	Afatinib 1dd40mg	11.0m	23.1m [46]	66.9%

Table 1. Continued

Trial	Population	*n*=	Treatment	mPFS	mOS	ORR
Soria et al, NEJM 2018 (FLAURA)[32]	Global, *EGFR*m+	277	Gefitinib 1dd250mg or erlotinib 1dd150mg	10.2m	31.8m [33]	76.0%
		279	Osimertinib 1dd80mg	18.9m	38.6m [33]	80.0%

Legend: n= number of patients, EGFRm+ = EGFR-mutated, m = months, y = year, NR = not reported

Chemotherapy

Unfortunately, all patients treated with EGFR-TKI develop resistance and subsequent progressive disease eventually. While investigation of the resistance mechanism gives the possibility of adjusting the targeted therapy to the new resistance mutations in some patients, for others without a targetable resistance mechanism chemotherapy is still the treatment of choice. In clinical practice there are several chemotherapy regimens to choose from, with expanding options regarding anti-angiogenic and immunotherapy agents in the spectrum of available agents in the current landscape.[19]

Detection of *EGFR* mutations, tissue and blood

Historically, tissue biopsy is the gold standard for molecular analysis and determining the driving aberrations and resistance mechanisms. However, in clinical practice this is not always feasible. In recent years the development of methods to detect tumor genetic aberrations in plasma has been an important step.[48] As the challenge of detecting the sparse molecules of cell free tumor DNA (ctDNA) between the bulk of total cell free DNA (cfDNA) is enormous, existing Polymerase Chain Reaction (PCR) and Next Generation Sequencing (NGS) techniques had to be adapted. Especially for patients with progression on 1st or 2nd generation EGFR-TKI with development of the *p.T790M* gatekeeper resistance mutation, clinical benefit of treatment started upon detection in plasma has been established.[49, 50] As more knowledge on possible resistance mechanisms and new genetic targets is becoming available, question is which technique is the test of choice in specific situations in clinical care. In case of lack of molecular analysis on tissue at all, when a patient is suspected of metastasized lung cancer, plasma analysis could possibly also be valuable for detecting targetable mutations. When a patient has a proven *EGFR* mutation in the tumor and starts treatment with EGFR-TKI, following the levels of mutations in plasma could be of added value to regular clinical and radiological assessment in clinical practice.

Aims and outline of the thesis

The one-size-fits-all approach that has been common practice in cancer treatment for a long time does not do justice to interpersonal differences, in both cancer biology as well as pharmacokinetic characteristics. Although important advances have been made in tailoring treatment guided by histology and biomarker assessment, further optimization of personalized medicine is warranted.

This thesis searches for innovations in optimizing treatment with EGFR-TKI in patients with metastatic NSCLC.

Our search begins with **treatment strategies in clinical practice**.
In **Chapter 2** we present the NVALT18 trial. As EGFR is commonly expressed in NSCLC and signs of efficacy in the phase II NVALT10 trial were favorable, we conducted this randomized phase III trial with intercalated erlotinib added to docetaxel in a non-squamous NSCLC *EGFR* WT population in second or third line.

Then, in **Chapter 3** we explore the toxic limit of osimertinib in a cohort of patients with *EGFR*-mutated NSCLC, to be able to work towards TDM and preventing severe toxicity in the future.

Nevertheless as all patients develop resistance to targeted treatment at some point in time, chemotherapy remains the option for further systemic treatment when no targetable resistance mechanism is demonstrated. In **Chapter 4** we show the efficacy of different present-day chemotherapy regimens in a real world cohort of patients with *EGFR*-mutated lung cancer and progressive disease after targeted treatment in the Erasmus MC Rotterdam and the National Cancer Institute Amsterdam.

We then continue with a **focus on plasma**.
As techniques of detecting genetic aberrations of cfDNA in plasma develop rapidly, we compare droplet digital PCR (ddPCR) to NGS and tissue results in patients with *EGFR*-mutated NSCLC and progression on current therapy in **Chapter 5**.

Thereafter **Chapter 6** shows the search for targetable mutations with NGS on cfDNA in plasma of patients with suspected metastatic lung cancer in case molecular analysis on tumor tissue was not possible.

In **Chapter 7** we describe plasma predictive features during treatment with EGFR-TKI in a *EGFR*-mutated NSCLC population.

These studies are summarized and discussed in **Chapter 8**, which ends with the conclusions and future perspectives.

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Part A

Treatment Strategies in Clinical Practice

Chapter 2

Randomized phase III study of docetaxel versus docetaxel plus intercalated erlotinib in patients with relapsed non-squamous non-small cell lung carcinoma

Lung Cancer. 2021 Oct;160:44-49

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Abstract

Background:

Earlier preclinical and phase II research showed enhanced effect of docetaxel plus intercalated erlotinib. The NVALT18 phase III study was designed to compare docetaxel with docetaxel plus intercalated erlotinib in relapsed metastasized non-squamous (NSQ) non-small cell lung cancer (NSCLC).

Methods:

Patients with relapsed Epidermal Growth Factor Receptor (EGFR) wild type (WT) NSQ-NSCLC were randomized 1:1 to docetaxel 75 mg/m^2 intravenously on day 1 every 21 days (control), or docetaxel 75 mg/m^2 intravenously on day 1 plus erlotinib 150 mg/day orally on day 2-16 every 21 days (experimental arm). Progression free survival (PFS) was the primary endpoint, secondary objectives were duration of response, overall survival (OS) and toxicity.

Results:

Between October 2016 and April 2018 a total of 45 patients were randomized and received treatment in the control (*n*=23) or experimental arm (*n*=22), the study was stopped due to slow accrual. Median PFS was 4.0 months (95% CI: 1.5-7.1) versus 1.9 months (95% CI 1.4-3.5), p = 0.01 respectively; adjusted hazard ratio (HR) 2.51 (95% CI: 1.16-5.43). Corresponding median OS was 10.6 months (95% CI: 7.0-8.6) versus 4.7 months (95% CI: 3.2-8.6), p = 0.004, with an adjusted HR of 3.67 (95% CI: 1.46-9.27). Toxicity was higher with combination therapy, with toxicity ≥ CTCAE grade 3 in *n*=6 (26%) in the control arm and *n*=17 (77%) in the experimental arm (p < 0.001), mainly consisting of gastrointestinal symptoms and leukopenia.

Conclusions:

Our study shows detrimental effects of docetaxel plus intercalated erlotinib, and strongly discourages further exploration of this combination in clinical practice.

Introduction

During the last decade the treatment paradigm for metastatic or locally advanced non-small cell lung carcinoma (NSCLC) has improved dramatically, with the introduction of immunotherapy with or without chemotherapy as first line regimen.[1] This poses a great challenge for patients progressing during or shortly after this first line of treatment. In those patients with non-squamous (NSQ) NSCLC treated with pemetrexed chemo-immunotherapy in first line, only docetaxel is left as the approved second line treatment.[1] Although erlotinib, a first generation Epidermal Growth Factor Receptor (EGFR) tyrosine kinase inhibitor (TKI), is approved for second or third line treatment, it is rarely used in the unselected population as the overall survival (OS) compared to placebo was limited and the efficacy is mainly driven by patients with activating *EGFR* driver mutations.[1, 2] EGFR is a transmembrane tyrosine kinase protein receptor binding ligands of the EGF family, which activates several intracellular signaling cascades and is commonly expressed in NSCLC.[3] Preclinical models have shown that combination therapy of erlotinib and docetaxel with schedule dependent separation, results in additive apoptosis regardless of *EGFR* and Kirsten rat sarcoma viral oncogene homolog (KRAS) mutational status.[4, 5] Several phase II studies have explored this combination hereafter.[6-9] In a previous randomized phase II study (NVALT10), we showed improved OS in advanced relapsed NSQ- NSCLC patients treated with a combination of chemotherapy plus intercalated erlotinib compared to erlotinib monotherapy.[10] Pemetrexed was used as chemotherapy backbone in the non-squamous population and docetaxel in the squamous population. However, pemetrexed has moved to treatment in first line setting. Therefore the combination of the improved outcome shown in the NVALT10 study and the pre-clinical evidence of additive effect of erlotinib and docetaxel led to the design of the NVALT18 study. The current NVALT18 study (NCT0277500) was designed to investigate the efficacy of docetaxel with intercalated erlotinib compared to standard docetaxel monotherapy in patients with relapsed (*EGFR* and Anaplastic Lymphoma Kinase (ALK) wild type (WT)) NSQ-NSCLC. The study was ended prematurely due to slow accrual.

Material and methods

Study design

The NVALT18 study is a prospective multicenter randomized open label phase III trial (NCT02775006). The protocol (see **Supplementary data**) was reviewed and approved by the Netherlands Cancer Institute (Antoni van Leeuwenhoek) medical ethical committee, written informed consent was obtained from all patients before randomization. Patients were followed until death or loss to follow up.

Study population

Patients were recruited at 12 sites in The Netherlands (Supplementary **Fig. S1**) between October 2016 and April 2018. Eligibility criteria included relapse of non-squamous cell

(*EGFR* and *ALK* WT) NSCLC after platinum-based chemotherapy and/or checkpoint inhibitor, WHO performance status 0–1, adequate organ function and measurable disease according to Response Evaluation Criteria in Solid Tumours version 1.1 (RECIST v1.1).[11] Presence of brain metastases was allowed provided cranial irradiation was completed more than 4 weeks before inclusion and steroid treatment had been stopped for at least 2 weeks before study inclusion. More details on in- and exclusion criteria are available in the Supplementary Data. Patients were stratified for WHO performance score (0 versus 1), previous immunotherapy (yes versus no) and treatment free interval after platinum-based therapy (<6 months versus greater than 6 months) and randomized by a centralized computer randomization system (TENALEA) to open-label treatment.

Study treatment

Patients were randomized 1:1 to the control arm (A): docetaxel 75 mg/m2 administered intravenously on day 1 every 21 days, or the experimental arm (B): docetaxel 75 mg/m2 on day 1 administered intravenously plus erlotinib 150 mg/day on day 2–16 orally every 21 days. Treatment was continued until progression of disease, unacceptable toxicity or patient refusal.

Assessments

Patients were assessed before each cycle of treatment. Computed tomography of the chest and upper abdomen was scheduled every 6 weeks during treatment, and response was evaluated by RECIST v1.1.[11] All adverse events (AE) equal to or exceeding Common Toxicity Criteria (CTC) version 4.03 grade 3, interstitial lung disease of any degree and all Serious Adverse Events (SAEs) were reported. The primary outcome measure was PFS, defined as the time from randomization to progression or death. Secondary endpoints were response rate, duration of response, OS (defined as time from randomization to death), and toxicity.

Statistical analysis

The intended number of inclusions was 230 with a preplanned interim analysis at 80 events. Assuming a median time-to-event of 3 months in the control group and a hazard ratio (HR) of 0.67 in favor of combination therapy, performing the final analysis after observing 198 events would yield 80% power to show combination therapy superior at either analysis at a two-sided overall confidence level of 95%. The (asymmetric) stopping boundaries for the interim analysis were based on the spending function of Hwang-Shih-DeCani with gamma = -4 for both alpha and beta spending. With a single interim at 80 events this corresponds to stopping for efficacy when the observed HR is below 0.52 and stopping for futility when the observed HR is above 1.09. Both PFS and OS were estimated by the Kaplan-Meier method and compared between arms by the log-rank test and by means of Cox proportional hazard models (R version 3.6, R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics

Between October 2016 and April 2018 a total of 45 patients were randomized and received treatment in the control arm (*n*=23) or the experimental arm (*n*=22). The study terminated prematurely due to slow accrual. As docetaxel shifted from second to third line treatment after approval of second line immune checkpoint inhibitors the study was amended on 22nd February 2016 to allow inclusion of patients who were pretreated with second line immunotherapy. Nevertheless this had negative impact on our expected inclusion rate and in practice also on the number of available patients, as less patients receive treatment in a subsequent therapy line as the disease progresses in time. Baseline characteristics are displayed in **Table 1** and **Supplementary Table S1**. Thirty patients (67%), 15 patients in each arm, were pretreated with second line immunotherapy. At time of database lock on16th May 2019 the median follow up was 16 months (95% confidence interval (CI) 11.5 – NR).

Table 1. Patient characteristics

	Control arm (A): Docetaxel monotherapy	**Experimental arm (B): Docetaxel + erlotinib**	**All**
	***n*=23 (%)**	***n*=22 (%)**	***n*=45 (%)**
Sex (%)			
Male	8 (35)	11 (50)	19 (42)
Female	15 (65)	11 (50)	26 (58)
WHO PS			
0	9 (39)	10 (45)	19 (42)
1	14 (61)	12 (55)	26 (58)
Smoking status			
Never	1 (4)	2 (9)	3 (7)
Former	18 (82)	15 (68)	33 (75)
Current	3 (14)	5 (23)	8 (18)
Histology			
Adenocarcinoma	23 (100)	19 (86)	42 (93)
Large cell carcinoma (NOS)	0	2 (9)	2 (4)
Neuro-endocrine (LCNEC)	0	1 (5)	1 (2)
Previous chemotherapy			
Yes	23 (100)	22 (100)	45 (100)
No	0 (0)	0 (0)	0 (0)

Table 1. Continued

	Control arm (A): Docetaxel monotherapy	Experimental arm (B): Docetaxel + erlotinib	All
	***n*=23 (%)**	***n*=22 (%)**	***n*=45 (%)**
Previous ICI monotherapy			
Yes	15 (65)	15 (68)	30 (67)
No	8 (35)	7 (32)	15 (33)
Total previous lines of systemic treatment chemotherapy + ICI			
0	0	0	0
1	7 (30)	8 (36)	15 (33)
2	14 (61)	13 (59)	27 (60)
3	2 (9)	1 (5)	3 (7)
4	0	0	0
Previous radiotherapy			
Yes	16 (70)	12 (55)	28 (62)
No	7 (30)	10 (45)	17 (38)
Best response on study treatment			
CR	0 (0)	0 (0)	0 (0)
PR	3 (13)	2 (9)	5 (11)
SD	11 (48)	9 (41)	20 (44)
PD	7 (30)	10 (45)	17 (38)
Unknown	2 (9)	1 (5)	3 (7)

Legend: WHO PS; World Health Organization Performance Score, NOS; not otherwise specified, LCNEC; large cell neuro-endocrine carcinoma, ICI; immune checkpoint inhibitor, CR; complete response, PR; partial response, SD; stable disease, PD; progressive disease.

Progression free survival

At final analysis all patients had developed disease progression. In the docetaxel monotherapy control arm (A) median PFS was 4 months (95% CI: 1.5–7.1 months). In the experimental docetaxel with intercalated erlotinib arm (B) median PFS was 1.9 months (95% CI 1.4–3.5 months), adjusted hazard ratio (HR) 2.51 (95% CI: 1.16–5.43), p = 0.01 (Fig. 1A).

Statistical evaluation primary endpoint

Although the data refute the Null hypothesis (in the opposite direction from what was expected at the beginning of the trial) the decision to stop the trial was made before looking at the data and hence independent of this outcome. Simulations show that had we continued the trial to the point of the first preplanned interim analysis, the study would in all probability have been stopped at that time. Under assumption of the Null

hypothesis (but given the results in the first 45 patients) the probability of crossing the stopping-for-futility boundary at the first interim analysis is 76%. Under the assumption that OS in the subsequent patients would follow the same distributions (in each arm) as seen in the first 45 patients, this probably would even be over 99%.

Tumor response

Objective response rate (best confirmed response complete or partial response) was 13% (*n*=3) in the control arm (A) and 9% (*n*=2) in the erlotinib plus docetaxel experimental arm (B), see **Table 1**. Durations of the tumor responses for these 3 patients in arm A were 14, 19 and 40 weeks, and in arm B 8 and 25 weeks, respectively.

Overall survival

Median OS from randomization was 10.6 months (95% CI: 7.0–8.6 months) in the control arm and only 4.7 months (95% CI: 3.2–8.6 months) in the experimental arm, adjusted HR 3.67 (95% CI: 1.46–9.27), p = 0.004, see Fig. 1B. The one year survival rate was 43% (95% CI: 26% − 74%) in the control monotherapy arm and 14% (95% CI: 5% − 39%) in the experimental arm.

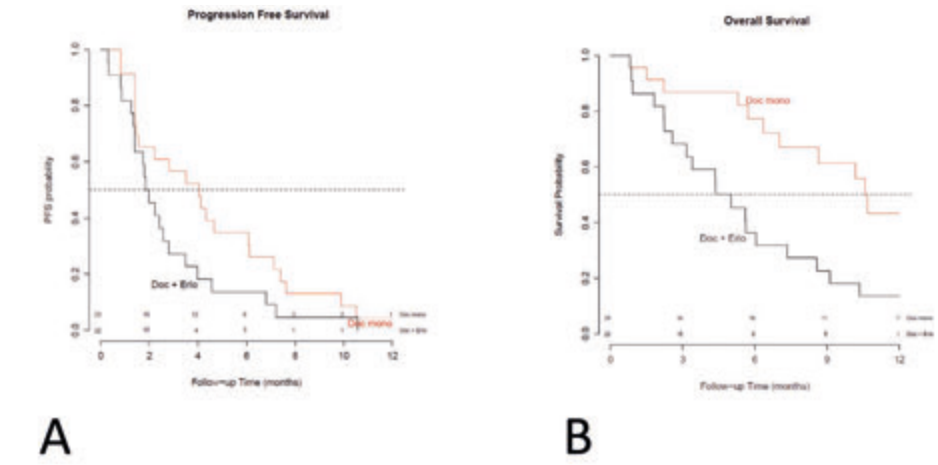

Figure 1. *Kaplan-Meier curves of PFS (figure 1A) and OS (figure 1B)*

Toxicity

In the control arm 6 patients (26%) experienced toxicity ≥ CTCAE grade 3 compared to 17 patients (77%) in the experimental arm (p = 0.0009), mainly consisting of gastrointestinal symptoms and leukopenia. There were no CTCAE grade 5 AEs reported in this study. There was one case of possible pneumonitis in a patient with pulmonary infection in the control arm (A) grade 3, treated with intravenously cefuroxime and prednisolone. The patient had a full recovery. Toxicity is summarized in **Table 2**.

Table 2. Toxicity (related to treatment)

Adverse events (grade 3 & 4)	*Control arm (A), n=23 (%)*	*Experimental arm (B), n=22 (%)*	*All n=45, (%)*
Hematological			
Leukopenia	3 (13)	5 (23)	8 (18)
Neutropenia	1 (4)	2 (9)	3 (7)
Febrile neutropenia	1 (4)	4 (18)	5 (11)
Leukocytosis	0	1 (5)	1 (2)
General			
Malaise	0	1 (5)	1 (2)
Fatigue	1 (4)	0	1 (2)
Weight loss	1 (4)	1 (5)	2 (4)
Pain	0	2 (9)	2 (4)
Syncope	0	1 (5)	1 (2)
Infection	0	1 (5)	1 (2)
Sepsis	0	2 (9)	2 (4)
Gastrointestinal disorder			
Abdominal pain	0	1 (5)	1 (2)
Diarrhea	0	2 (9)	2 (4)
Dysphagia	0	1 (5)	1 (2)
Oral mucositis	0	2 (9)	2 (4)
Nausea	0	1 (5)	1 (2)
Bilirubin increased	0	1 (5)	1 (2)
Vomiting	0	1 (5)	1 (2)
Pulmonary			
Respiratory failure	0	1 (5)	1 (2)
Dyspnea	1 (4)	0	1 (2)
Other			
Acute kidney injury	1 (4)	0	1 (2)
Palmar-plantar erytrodysesthesia syndrome	0	1 (5)	1 (2)
Pruritus	0	1 (5)	1 (2)

Treatment delivery

The median number of docetaxel courses was 2 (range 1 – 21) in the full study cohort: median 3 (range 1 – 21) in the control arm and median 2 (range 1 – 10) in the experimental arm. Patients received more than 6 cycles of therapy in 5 cases (22%) in the control arm and 2 cases (9%) in the experimental arm. In 26 courses in 16

patients administration of docetaxel was modified, i.e. reduced or delayed. A total of 16 modifications was due to adverse events; 4 events in *n*=4 in the control arm and 12 events in *n*=9 in the experimental arm. In 3 patients (control arm *n*=1, experimental arm *n*=2) an AE led to discontinuation of docetaxel treatment without progression of disease at that time point. In the experimental arm the erlotinib administration was modified in 13 out of 22 patients. In 4 patients the daily dose was reduced to 100 mg and in 1 patient further reduced to 50 mg because of non-hematological AEs. The intercalated scheme was stopped earlier or interrupted in 9 patients; twice because of a hematological AE, in 7 patients because of a non-hematological AE and once on request of the patient. In 4 patients a cycle was postponed, once on request of the patient, otherwise because of adverse events.

Discussion

Our hypothesis that a schedule dependent combination of docetaxel and intercalated erlotinib therapy is superior to docetaxel monotherapy was based on data from preclinical research and the results of the phase II NVALT10 study.[4, 5, 10] However the data reported here suggest the contrary as the primary endpoint (PFS) was significantly shorter in the experimental arm than in the control arm. In addition, the secondary endpoint OS was significantly shortened in the experimental arm. Meanwhile toxicity was worse in the combination arm. An antagonistic phenomenon could be anticipated when the two drugs are given concomitantly as cell cycle arrest in G1 due to the cytostatic effect of the EGFR-TKI might prevent the cytotoxic effect of docetaxel in the S and G2/M phase.[4, 5] However, in vitro exploration of dose scheduling showed an additional effect of cell proliferation- inhibition and apoptosis when erlotinib was administered after docetaxel.[4, 5] An intercalated scheme of chemotherapy on day 1 with EGFR-TKI on day 2–16 in a 21 day cycle was therefore proposed as an optimal trial design. In a phase I/II trial the intercalated scheme of docetaxel and erlotinib was feasible and tolerable.[6] However, reports of phase II trials show opposite results. One trial showed no additional effect of the combination therapy in 147 randomized patients.[7] On the other hand, another study reported improved PFS, OS and disease control rate in the combination arm in 68 randomized patients.[8] Another phase II study conducted in male patients with squamous NSCLC was ended prematurely and showed no improvement in PFS at 6 months.[9] The most important differences between these studies and our study are the continuation treatment (erlotinib versus docetaxel plus intercalated erlotinib) and the difference in mutational status. While patients in the NVALT18 were *EGFR*-WT, the other studies contained high levels of unknown mutational status which could explain the higher response rates and better outcomes. This is supported by the plasma analysis on a phase I/II trial where activating *EGFR* mutations detected in plasma were significantly associated with better outcomes.[12] A more recent single arm phase I/II trial included *EGFR*-WT patients and showed no improved overall response rate for the docetaxel and erlotinib combination.[13] The clinical trials on docetaxel with intercalated erlotinib are summarized in **Table 3**.

Table 3. Clinical trials on docetaxel with intercalated erlotinib

Study	Phase	Patient population	*EGFR* Status	Arms	Cycles	Maintenance	*n*=	ORR	PFS (months)	OS (months)
Sangha *et al,* 2011.[8]	I/II NSCLC, second line	Solid tumors/ NSCLC, any treatment line	unknown	IA docetaxel 70-75mg/ m2 every 21 days, erlotinib day 2,9 and 16 (600-1000mg)	6	E	17 (10 NSCLC)	NA	NA	NA
				II docetaxel 70-75mg/m2 every 21 days, erlotinib days 2-16 (150-300mg)	6	E	25 (12 NSCLC)	NA	NA	NA
					6	E	39	28.20%	4.1	18.2
Aliac *et al,* 2014.[9]	II	NSCLC, second line	WT 68%, unknown 32%	C docetaxel 75mg/m2 every 21 days	NA	D	74	6.60%	2.5	8.3
			WT 66%, unknown 34%	E docetaxel 75mg/m2 every 21 days, erlotinib 150mg days 2-16	NA	DE	73	12.30%	2.2	6.5

Table 3. Continued

Study	Phase	Patient population	*EGFR* Status	Arms	Cycles	Maintenance	*n*=	ORR	PFS (months)	OS (months)
Juan *et al,* 2015.1	II	NSCLC, second line	M 3%, WT 14%, unknown 83%	C erlotinib 150mg/d continuously	NA	E	35	9%	2.1	5.2
			WT 3%, unknown 97%	E docetaxel 75mg/ m2 every 21 days, erlotinib 150mg days 2-16	4	E	33	3%	3	7.5
Gridelli *et al,* 2016.[11]	II	Male SQ-NSCLC, second line	unknown E docetaxel 75mg/m2 every 21 days, erlotinib 150mg days 2-16	C erlotinib 150mg/d continuously	NA	E	36	2.80%	2.3	5.6
				4	E	38	8.10%	2.8	8.9	
Kimura *et al,* 2019.[13]	I/II	NSCLC, second line, *EGFR*-WT	WT II docetaxel 60mg/m2 every 21 days, erlotinib 150mg days 2-16	I docetaxel 60mg/ m2 every 21 days, erlotinib 150mg days 2-16	NA	DE	12	NA	NA	NA
				NA	DE	46	17.10%	3.5	11.3	

Table 3. Continued

Study	Phase	Patient population	*EGFR* Status	Arms	Cycles	Maintenance	*n*=	ORR	PFS (months)	OS (months)
Steendam *et al*, 2020	III	NSQ-NSCLC, second (or >) line, *EGFR/ALK WT*	WT E docetaxel 75mg/m2 every 21 days, erlotinib 150mg days 2-16	C docetaxel 75mg/m2 every 21 days	NA	D	23	13%	4	10.6
				NA	DE	22		9%	1.9	4.7

Legend: NSCLC, non-small cell lung cancer; SQ, squamous; NSQ, non-squamous; EGFR, Epidermal Growth Factor Receptor; WT, wild-type; M, mutated; N, number; E, experimental; E-I/E-II, experimental phase I/II; C, control; D, docetaxel; E, erlotinib; DE, docetaxel plus intercalated erlotinib ; ORR, objective response rate; PFS, progression free survival; OS, overall survival; NA, not applicable.

An important difference between cell line experiments and clinical trials in patients is the recurrence of drug administration in cycles. Whereas cell lines typically only receive 1 cycle of 'therapy' before measurements, patients are treated with several cycles of treatment. Possibly the remaining circulating erlotinib still has an antagonistic effect on the cytotoxic action of docetaxel after the first cycle. In the NVALT10 study, erlotinib concentrations were measured in a subgroup of patients on day 22 prior to chemotherapy administration (and after 5 days of erlotinib interruption).[10] Although the plasma levels of erlotinib did not reach therapeutic levels, the drug was still detectable in 12 out of 25 patients with a mean concentration of 79 ng/mL (SD 120 ng/mL).[10] Enduring detection of erlotinib concentrations in tissue specimens after resection in a neoadjuvant setting up to 13 days after the last administration was reported earlier.[14] The mean lung tumor tissue erlotinib levels were 149 ng/g (SD 153 ng/g) after a mean of 7 days (SD 4.9 days) between last erlotinib intake and surgery. We hypothesize that erlotinib could still have activity in the intracellular compartment diminishing the cytotoxic effect of the chemotherapy after the 5 day washout period in our study, and a longer washout period could be necessary to overcome the antagonistic effect. Unfortunately we were unable to collect adequate samples for a preplanned pharmacokinetic analysis. More adverse events equal to or exceeding CTC grade 3 were reported in the docetaxel plus erlotinib arm. In addition, an earlier study reported a clinically relevant pharmacokinetic interaction between docetaxel and the TKI pazopanib, leading to a more than 50% increased systemic exposure to docetaxel.[15] Although we did not measure docetaxel concentrations in the NVALT18 study, we cannot rule out that docetaxel levels increased due to erlotinib leading to more toxicity in the combination arm. A limitation of our study was the open label design and lack of a double-blind experiment in this setting. Furthermore we did not include our prespecified sample size (as described in the study protocol in supplementary data). To our knowledge this is the first study to report a clinically relevant inferior outcome in the experimental arm by intercalating erlotinib with docetaxel over docetaxel as standard treatment in *EGFR*-WT patients with NSQ-NSCLC. Earlier phase II trials reported no significant differences in outcome in control and experimental arms.[7-9] These trials did not reveal a detrimental effect of the combination therapy. However opposed to our study, the maintenance therapy consisted only of erlotinib monotherapy and not of a combination with docetaxel and patients were not selected on *EGFR*-WT status. Our results therefore do not support the further exploration or implementation of docetaxel plus intercalated erlotinib treatment.

Conclusion

These data strongly discourage the clinical use or the further investigation of the docetaxel plus intercalated erlotinib regimen in (*EGFR* and *ALK* WT) NSQ-NSCLC. Whether these data may be extrapolated to other EGFR-TKIs and/or other taxanes is currently unknown, but caution on adverse outcomes is strongly advised.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank all patients and their families, and all the colleagues at the participating centers for their contributions to the NVALT18 trial.

Funding

This work was supported by The Dutch Cancer Society (Grant number EMCR-2015-8059). The funding source had no involvement in the conduct of the research or the preparation of the article.

Disclosures

CS reports advisory board honorarium from Boehringer Ingelheim, grants (to institution) from AstraZeneca, other (hospitality/symposium) from Roche and Lilly, outside the submitted work. SK reports personal fees from Roche, outside the submitted work. HG reports fees (to institution) from Lilly, other from Merck, Novartis, BMS, AbbVie, outside the submitted work. AD reports personal fees from Roche, Eli Lilly, Boehringer Ingelheim, Pfizer, BMS, Novartis, Takeda, Pharmamar, non-financial support from Abbvie, grants from BMS and Amgen, outside the submitted work. RM reports grants from Astellas, Bayer, Boehringer-Ingelheim, Cristal Therapeutics, Pamgene, Pfizer, Prostakan, Roche, grants and personal fees from Novartis and Servier, outside the submitted work. JA reports personal fees and non-financial support from MSD, personal fees from BMS, Boehringer Ingelheim, Amphera, Eli-Lilly, Takeda, Bayer, Roche and AstraZeneca, outside the submitted work. In addition, JA has a patent allogenic tumor cell lysate licensed to Amphera, a patent combination immunotherapy in cancer pending, and a patent biomarker for immunotherapy pending. All remaining authors have declared no conflicts of interest.

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Supplementary Data

Study protocol

Available through DOI: https://doi.org/10.1016/j.lungcan.2021.08.002

Figure S1. Study sites
Amphia hospital, Breda, the Netherlands
Maxima Medical Centre, Veldhoven, the Netherlands
Erasmus Medical Centre, Rotterdam, the Netherlands
St. Antonius hospital, Nieuwegein, the Netherlands
Haga hospital, The Hague, the Netherlands
Martini hospital, Groningen, the Netherlands
Sint Franciscus Gasthuis, Rotterdam, the Netherlands
Medical Centre Haaglanden, The Hague, the Netherlands
Gelderse Vallei hospital, Ede, the Netherlands
VieCuri hospital, Venlo, the Netherlands
Gelre hospital, Apeldoorn, the Netherlands
Maastricht University Medical Centre, Maastricht, the Netherlands

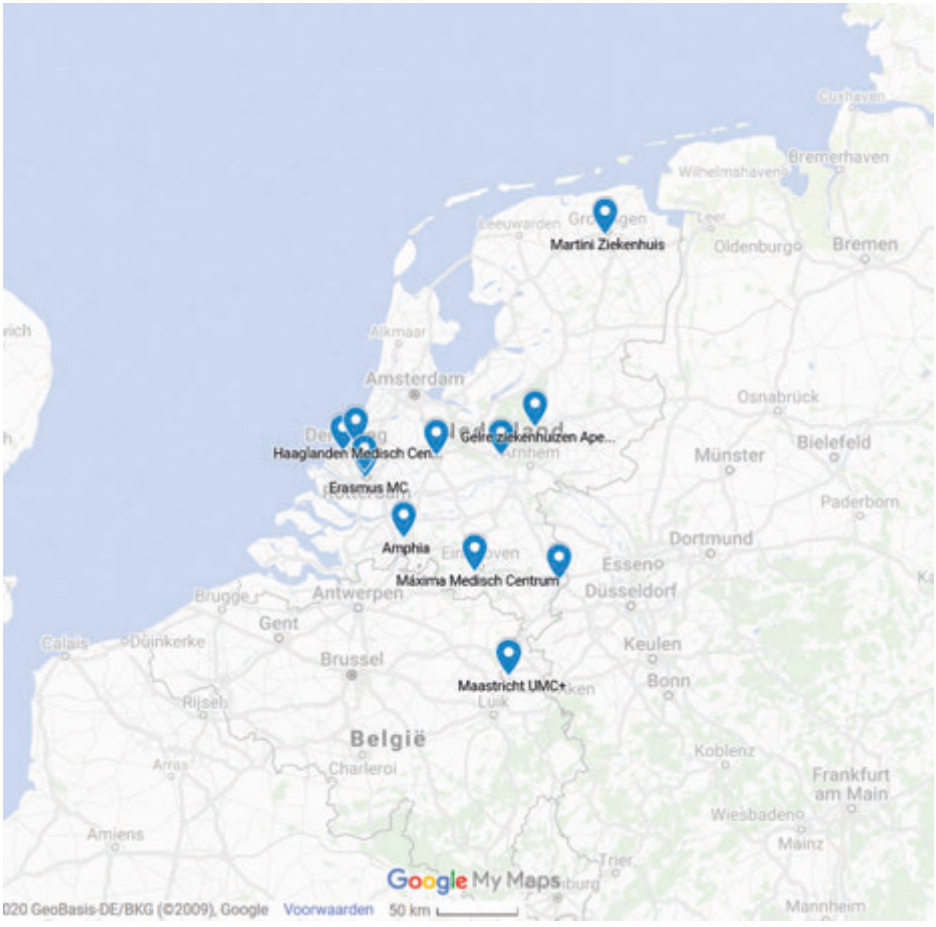

Table S1. Baseline characteristics; previous treatment

	Control arm (A) Docetaxel monotherapy	**Experimental arm (B) Docetaxel + erlotinib**	**All**
	***n*=23**	***n*=22**	***n*=45**
Previous chemotherapy (%)			
Yes	23 (100)	22 (100)	45 (100)
No	0 (0)	0 (0)	0 (0)
Previous number of lines (%)			
1	19 (83)	21 (95)	40 (89)
2	3 (13)	1 (5)	4 (9)
3	1 (4)	0 (0)	1 (2)
Previous agents			
Platinum	1	1	2
Platinum+pemetrexed	17	19	36
Pemetrexed maintenance	6	7	13
Platinum/etoposide	4	2	6
Platinum/gemcitabine	1	0	1
Carboplatin/paclitaxel/veliparib	0	1	1
Previous immune checkpoint inhibitor (ICI) monotherapy			
Yes	15 (65)	15 (68)	30 (67)
No	8 (35)	7 (32)	15 (33)
IO agent			
nivolumab	15	14	29
durvalumab	0	1	1
Total previous lines of systemic treatment chemotherapy+ICI (%)			
0	0	0	0
1	7 (30)	8 (36)	15 (33)
2	14 (61)	13 (59)	27 (60)
3	2 (9)	1 (5)	3 (7)
4	0	0	0
Previous radiotherapy (%)			
Yes	16 (70)	12 (55)	28 (62)
No	7 (30)	10 (45)	17 (38)

Chapter 3

Improving the tolerability of osimertinib by identifying its toxic limit

Therapeutic Advances in Medical Oncology. 2022 Jun 3;14.

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Abstract

Background:

Osimertinib is the cornerstone in the treatment of epidermal growth factor receptor-mutated non-small cell lung cancer (NSCLC). Nonetheless, ±25% of patients experience severe treatment-related toxicities. Currently, it is impossible to identify patients at risk of severe toxicity beforehand. Therefore, we aimed to study the relationship between osimertinib exposure and severe toxicity and to identify a safe toxic limit for a preventive dose reduction.

Methods:

In this real-life prospective cohort study, patients with NSCLC treated with osimertinib were followed for severe toxicity (grade ⩾3 toxicity, dose reduction or discontinuation, hospital admission, or treatment termination). Blood for pharmacokinetic analyses was withdrawn during every out-patient visit. Primary endpoint was the correlation between osimertinib clearance (exposure) and severe toxicity. Secondary endpoint was the exposure–efficacy relationship, defined as progression-free survival (PFS) and overall survival (OS).

Results:

In total, 819 samples from 159 patients were included in the analysis. Multivariate competing risk analysis showed osimertinib clearance (*c.q.* exposure) to be significantly correlated with severe toxicity (hazard ratio 0.93, 95% CI: 0.88–0.99). An relative operating characteristic curve showed the optimal toxic limit to be 259 ng/mL osimertinib. A 50% dose reduction in the high-exposure group, that is 25.8% of the total cohort, would reduce the risk of severe toxicity by 53%. Osimertinib exposure was not associated with PFS nor OS.

Conclusion:

Osimertinib exposure is highly correlated with the occurrence of severe toxicity. To optimize tolerability, patients above the toxic limit concentration of 259 ng/mL could benefit from a preventive dose reduction, without fear for diminished effectiveness.

Background

The most common treatable genetic aberration in patients with non-small cell lung cancer (NSCLC) is a deletion or mutation in the epidermal growth factor receptor (*EGFR*) gene. This oncogenic driver is present in almost 15% of Caucasian patients with non-squamous NSCLC, and even more frequently reported (>40%) in Asian patients.[1,2] The registration of the first- and second-generation EGFR small-molecule tyrosine kinase inhibitors (SMKIs) markedly increased survival rates compared to conventional chemotherapy in locally advanced and metastatic disease.[3–5] During treatment with EGFR-SMKIs, an *EGFR* p.T790M resistance point mutation eventually occurs in >60% of patients.[6] The third-generation EGFR-SMKI osimertinib showed significantly increased progression-free survival (PFS) and overall survival (OS) compared to the other EGFR-SMKIs and proved to be effective against T790M-mutated NSCLC.[7] These developments have hence caused the median OS of patients with *EGFR*-positive NSCLC to exceed 38 months and the 4-year survival rate to be almost 40%.[8] Additionally, recent data showed osimertinib to vastly reduce disease recurrence in the adjuvant setting.[9] As a consequence, many more patients may thus be treated with osimertinib in the future, and also for longer periods of time.

Despite its selectivity for EGFR, 20–42% of patients develop grade 3 or higher toxicity, which lead to hospital admissions, treatment discontinuations, and dose reductions.[7–9] Indirectly, severe toxicity could result in an impaired treatment effect, by interruption or even discontinuation of treatment. These undesirable consequences occurred in up to 25% and 15% of patients, respectively.[7–9] It is known from a previous population pharmacokinetic (PK) analysis that osimertinib plasma clearance (*c.q.* drug exposure) is correlated with skin rash, diarrhea, and cardiac QTc-time prolongation.[10] Nevertheless, to date, there are no indicators that can predict severe toxicity beforehand.[11]

Given the importance of osimertinib treatment continuation, in both the metastatic and adjuvant setting, a preventive dose reduction could avoid severe toxicity for patients without impairing treatment effectiveness. Therefore, we performed a prospective cohort study, using samples of patients with NSCLC treated with this agent, to study parameters that influence osimertinib exposure. Herewith, we aimed to study the relationship between drug exposure and occurrence of severe toxicity, and improve osimertinib tolerability by identifying its toxic limit.

Methods

Study design and data collection

The START-TKI study[12] is a real-life, prospective, multi-center cohort study. Patients who are treated with SMKIs at the Erasmus Medical Centre Cancer Institute in Rotterdam

and the Amphia Hospital in Breda, both in the Netherlands, between January 2017 and September 2021, were asked to participate in this study. Ethical approval was obtained from the Medical Ethics Committee of the Erasmus Medical Center (MEC 2016-643). Patients treated with osimertinib for locally advanced or metastatic NSCLC according to standard-of-care analyses, who were above the age of 18 years and able to understand and give written informed consent, were selected to be included in this analysis. Since severe toxicity was the primary endpoint of this study, patients were included regardless of disease history, treatment history, T790M- or *EGFR*-mutation, or line of treatment. Patients were only excluded if the treating physician documented possible low or absent treatment adherence. Prior to participation, patients provided written informed consent and were prospectively followed-up until end of osimertinib treatment by their treating pulmonologist. When blood was withdrawn for standard-of-care analyses, an additional blood sample for PK analyses for this study was obtained from all participants. For most patients, this meant that we obtained a PK sample every 3 months. Patients were asked to postpone the intake of osimertinib until the PK sample has been obtained to ensure trough samples. At every visit, osimertinib toxicity was assessed, and a CT scan and laboratory blood analyses (renal function, liver enzymes, and full blood count) were performed. Additionally, patients were asked at what time osimertinib was taken prior to blood withdrawal.

Severe toxicity was defined as toxicity grade $\geqslant$3 scored by the common terminology criteria for adverse events (CTCAE) criteria version 5.0,[13] or if toxicity led to dose reduction or discontinuation, hospital admission, or termination of osimertinib treatment. The date of hospital admission or dose alteration was used for time-to-event analyses. Additionally, dates of disease progression according to RECIST version 1.1[14] and death were collected for survival analyses.

Osimertinib plasma concentrations were quantified as described earlier.[15]

Population PK analysis

PK data were analyzed using nonlinear mixed-effects modeling (NONMEM) version 7.4. Model building was assisted by Perl-speaks-NONMEM version 4.2.0,[16,17] Pirana software version 2.9.5b,[18] R version 4.1.1, and Xposed version 4.4.1.[19]

The available data were transformed logarithmically and initially fitted to a one-compartmental linear model. Several model components were tested (i.e. two-compartment PK and different absorption mechanisms) to describe osimertinib PK. Residual error was estimated using an additive error model. Interindividual variability (IIV) in PK parameters was modeled using exponential models. If data below the quantification limit was present and consisted of less than 5% of the data, the M1 method was used.[20]

Continuous covariates were centered on the median and were modeled as power models to explain IIV (see **Supplemental Appendix A** for all tested covariates). Categorical covariates were modeled as proportional models. Covariate analysis was performed using stepwise forward inclusion ($p < 0.05$) and backwards elimination ($p < 0.01$). Time-varying covariates, such as laboratory parameters, were modeled using the following function:

$$\mathrm{Lab}_{\mathrm{current}} = \mathrm{Lab}_{\mathrm{previous}} + \left(\mathrm{Lab}_{\mathrm{next}} - \mathrm{Lab}_{\mathrm{previous}}\right) \times \frac{T_{\mathrm{current}} - T_{\mathrm{previous}}}{T_{\mathrm{next}} - T_{\mathrm{previous}}}$$

In this equation, Lab is the laboratory value, and *T* stands for time.

The model was evaluated numerically by changes in the objective function value (ΔOFV) and a nonparametric bootstrap procedure ($n = 30{,}000$). Changes that result in an OFV decrease greater than 3.84, correspond with $p < 0.05$ for one degree of freedom, were considered significant. Changes in the model were evaluated visually using goodness-of-fit plots and visual predictive check plots.

Exposure–toxicity relationship

After development of the population PK model, differences in median exposure were correlated with severe osimertinib toxicity. Since severe toxicity usually occurs within the first months after treatment initiation, a cut-off of 12 months was used.[7] Using Cox-regression, univariate time-to-event analyses were performed to identify confounding parameters. Variables with $p < 0.10$ were included in the subsequent multivariate Cox proportional-hazard analysis to correct for bias. Thereafter, the Fine and Gray competing risk model was performed to ensure the absence of competing risks.[21] For this analysis, a competing risk was defined as cessation of osimertinib therapy as this changed the likelihood of experiencing a toxic event for a patient (e.g. death or change of therapy because of disease progression).

In all the analyses, osimertinib clearance was used as variable for exposure. As all patients started with 80 mg/day, as is clinical practice, IIV was only modeled on clearance; thus, clearance was the best predictor for interindividual differences in exposure. Subsequently, the corresponding trough concentration was calculated to identify the toxic limit in ng/mL.

If osimertinib exposure was significantly correlated with severe toxicity, a toxic limit can be established by using a relative operating characteristic (ROC) curve. In this curve, the optimal sensitivity and specificity of different threshold are visualized. The preventive dose reduction should be effective in decreasing the exposure below the toxic limit,

which will be simulated in a large simulation cohort (n = 1,000). Thereafter, when osimertinib plasma concentrations were available in the first 2 months of treatment, the trough concentrations were associated with severe toxicity. This was especially done to test the time-to-severe toxicity relationship of the threshold and to confirm its predictive value in clinical practice. Furthermore, in order to assess the risk of toxicity after the dose reduction to 40 mg QD, patients who experienced severe toxicity, and who were dose-reduced, were screened for re-occurrence of severe toxicity.

Exposure–efficacy relationship

Median osimertinib exposure and PFS and OS were correlated using Cox proportional-hazard univariate analyses. Confounding variables with $p < 0.10$ were used in the Cox proportional-hazard multivariate analyses. If a positive exposure–efficacy relationship exists, a preventive dose reduction should not harm patients by decreasing drug concentrations below normal (*c.q.* effective) levels.

Results

Data collection

In total, 819 samples from 159 patients that were obtained between January 2017 and September 2021 were included in the population-PK analysis. A summary of patients' characteristics is shown in **Table 1**. One patient suffered from a chronic *Clostridium difficile* infection that hampered osimertinib uptake and was subsequently excluded from the analysis. Median trough level in our population was 226 ng/mL, whereas the median trough level for this patient was 62 ng/mL. Three additional samples were excluded due to non-adherence, as documented in the patient file by the treating physician.

Table 1. Patient baseline characteristics

Patient characteristics (n = 159)	***No. of patients or median***	***% or IQR***
Sex (female)	102	64%
Age (years)	66	60–75
Weight (kg)	69	60–80
Length (cm)	168	162–177
BSA	1.87	1.66–1.99
Ethnicity		
Caucasian	140	88%
Southeastern Asian	8	5%
Eastern Asian	7	4%
Western Asian	1	1%
African American	3	2%

Table 1. Continued

Patient characteristics (n = 159)	*No. of patients or median*	*% or IQR*
TKI treatment line		
First-line treatment	66	41%
Second-line treatment	79	50%
Third-line treatment	14	9%
Prior TKI treatment		
Erlotinib	56	60%
Afatinib	14	15%
Gefitinib	11	12%
Other	12	13%
WHO performance score		
0	32	20%
1	95	60%
2	27	17%
3	5	3%
Primary EGFR mutation*		
Classic exon 19 deletion	92	58%
Exon 21 L858R	43	27%
Exon 18 c.2156	5	3%
Rare or compound mutation	19	12%
Baseline TP53 mutation		
Yes	85	53%
No	67	42%
Unknown	7	4%
Follow-up		
Severe toxicity (months)	9.8	4.6–17.0
Progression free survival (months)	10.2	5.5–18.3
Overall survival (months)	16.6	10.2–25.2
Pharmacokinetic sampling (months)	11.5	5.6–19.4
No. PK samples per patient	3	2–6
No. laboratory samples per patient	9	5–15
Laboratory values		
Alkaline phosphatase (U/L)	80	65–110
ALT (U/L)	21	15–30
AST (U/L)	25	21–31
Creatine kinase (U/L)	118	73–189

Table 1. Continued

Patient characteristics (n = 159)	*No. of patients or median*	*% or IQR*
Gamma glutamyl transpeptidase (U/L)	30	19–54
eGFR (CKD-EPI) (mL/min)	71	59–84
Creatinine (µmol/L)	84	71–97
Hemoglobin (mmol/L)	7.9	7.3–8.6
Hematocrit (L/L)	0.39	0.36–0.42
Thrombocytes (10^9/L)	213	172–262
Albumin (g/L)	40	37–43
CRP (mg/L)	2.0	0.7–6.3
LDH (U/L)	206	181–241

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSA, body surface area; CKD-EPI, chronic kidney disease epidemiology collaboration; CRP, C-reactive protein; EGFR, epidermal growth factor receptor; eGFR, estimated glomerular filtration rate; IQR, interquartile range; LDH, lactate dehydrogenase; PK, pharmacokinetics; TKI, tyrosine kinase inhibitor; WHO, World Health Organization.

At data cut-off, severe toxicity occurred in 23% of patients, of which skin toxicity was the most prevalent with 6% occurrence (**Table 2**). Median time until severe toxicity was 3.7 [interquartile range (IQR) 1.8–6.6 months]. Disease progression according to RECIST occurred in 112 (70%) of patients, and 62 (39%) patients died during the study. Median follow-up is reported in **Table 1**.

Table 2. Incidence of severe osimertinib toxicity in total study cohort.

Specific severe toxicity	***n* = 36 (23%)^**	**CTCAE gr 1–2**	**CTCAE gr 3–4**	**Hospital admission**	**Dose reduction**	**Dose termination**	**Treatment stop**
Skin toxicities*	10 (6%)	4	6		9	7	1
CK elevation	7 (4%)	1	6		4	6	
Pneumonitis	5 (3%)	1	5	4	1	3	4
Creatinine increase	4 (3%)	1	3	2	4	4	
AST/ALT increase	3 (2%)	2	1		2	3	
Fatigue	3 (2%)	2	1		2	3	
QTc time prolongation	1 (1%)	.	1		1		
Heart failure	1 (1%)	.	1				1
Diarrhea	1 (1%)	1			1	1	
Thrombocytopenia	1 (1%)	1			1		
Nausea and vomitus	1 (1%)	1			1		

Table 2. Continued

Specific severe toxicity	*n* = 36 (23%)^	CTCAE gr 1–2	CTCAE gr 3–4	Hospital admission	Dose reduction	Dose termination	Treatment stop
Palpitations	1 (1%)	1			1	1	1

** Rash, paronychia, and acrodermatitis.*
^ Two patients experienced two different severe toxicities at the time of dose modification
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, creatine kinase; CTCAE, common terminology criteria for adverse events.

Population PK analysis

A one-compartment model with first-order absorption, first-order elimination, and additive error was best described osimertinib PK (**Supplemental Appendix B**). Introduction of C-reactive protein (CRP), thrombocyte count, hemoglobin, and alkaline phosphatase as covariates affecting osimertinib clearance improved the model significantly. Other tested covariates did not significantly improve the model (**Supplemental Appendix A**). The model was particularly improved when adding CRP as a covariate. A 20% increase in exposure is already seen when CRP levels are 20 mg/L. Introduction of all covariates decreased the additive error from 0.221 to 0.176 and decreased the IIV from 33.4% to 27.0%. All evaluations showed that a one-compartment model adequately described the data (**Supplemental Appendix C**).

Exposure–toxicity relationship

Osimertinib median clearance in this population was 14.7 (IQR 11.6–18.5) L/h. Osimertinib exposure and age were significantly correlated with severe toxicity in univariate Cox proportional-hazard analysis (both $p < 0.01$) (**Supplemental Appendix D**). Multivariate competing risks regression analysis showed median osimertinib exposure (HR 0.93, 95% CI 0.88–0.99), and age (HR 1.06, 95% CI 1.02–1.09), to be significantly correlated with severe toxicity. This means that for every liter per hour increase in osimertinib clearance, the risk of severe toxicity is reduced with 7%.

When the incidence of severe toxicity and osimertinib exposure was visualized in an ROC curve (**Figure 1**), the area under the curve was 62.5%. The most sensitive (true-positive) and specific (true-negative) toxic limit would be 259 ng/mL osimertinib. This target concentration divides the cohort into two groups: the risk of severe toxicity in the >259 ng/mL group – 25.8% of the cohort – is 34% *versus* 14% in the <259 ng/mL group. A log-rank test showed the groups to be significantly different (**Figure 2**). A preventive dose reduction to 40 mg osimertinib QD in the high-exposure group would reduce the risk of severe toxicity by 53%. This is underlined by the finding that from the 21 patients who were dose-reduced to 40 mg QD, only three (14%) experienced re-occurrence of severe osimertinib toxicity.

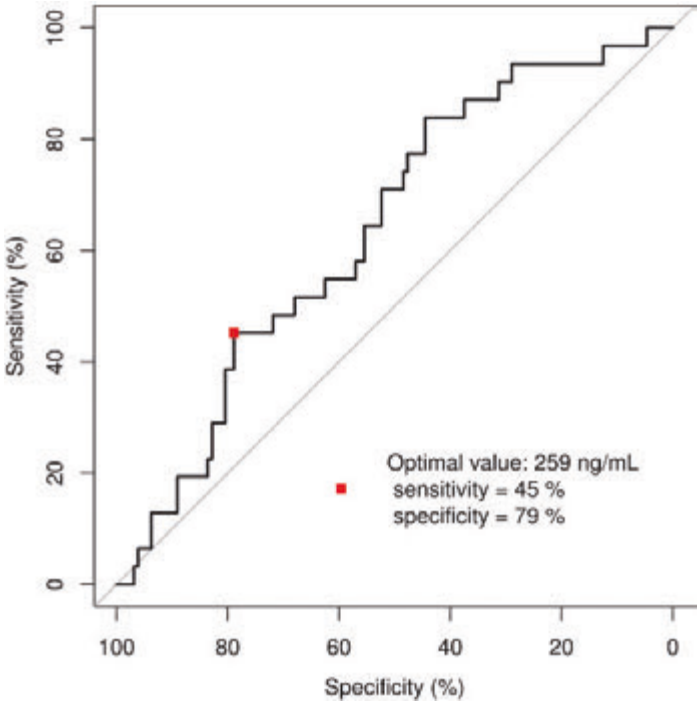

Figure 1. *Relative operating characteristic (ROC) curve to determine the optimal osimertinib trough level threshold for toxicity.*

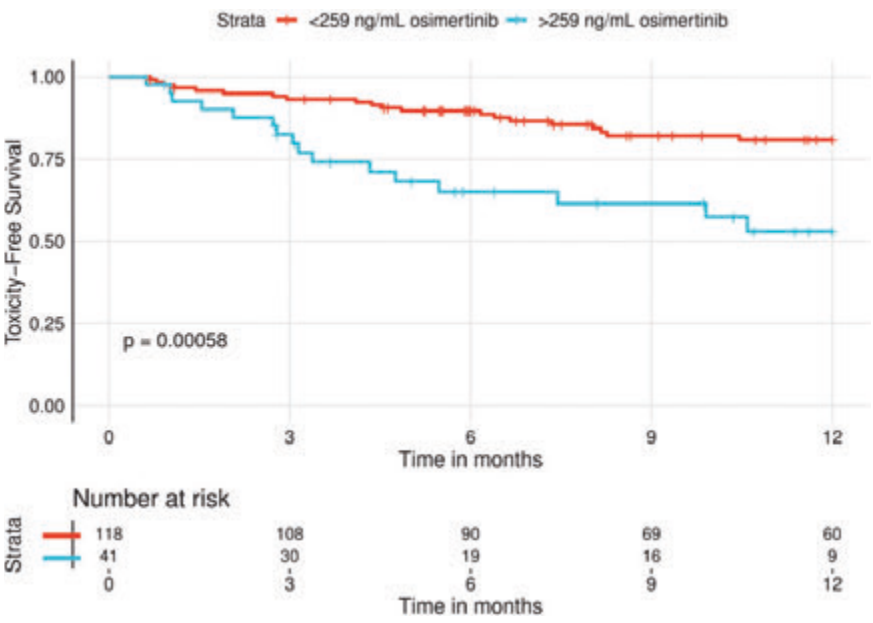

Figure 2. *Kaplan–Meier estimates of toxicity-free survival. Patients were stratified as having a higher or lower median osimertinib trough concentration compared to the toxic limit of 259 ng/mL.*

When stratifying on the occurrence of pneumonitis, which leads to permanent discontinuation of osimertinib treatment, a trend toward increased exposure for patients who experienced pneumonitis was observed (pneumonitis: median plasma concentration [MPC] = 251 ng/mL, standard deviation [SD] = 72 ng/mL; other toxicities: MPC = 241 ng/mL, SD = 85 ng/mL; no toxicities: MPC = 214 ng/mL, SD = 92 ng/mL). Due to the small number of patients who experienced a pneumonitis, this difference was nonsignificant (p = 0.25).

In the study cohort, osimertinib concentrations in the first 2 months after start of treatment were available for 90 patients. After this time period, most events of severe toxicity started to occur (**Figure 2**). Correlation of the first plasma trough concentrations in this time period revealed a similar difference in severe toxicity of almost 50% (31% *versus* 17%), when dividing the cohort into two by the toxic limit of 259 ng/mL osimertinib (**Supplemental Appendix E**).

When the osimertinib exposure was simulated after the proposed 50% dose reduction, the range in exposure was similar to the exposure in the patients without a dose reduction (median trough levels: 173.1 *versus* 180.1 ng/mL, and SDs: 45.3 *versus* 46.3 ng/mL) (**Figure 3**).

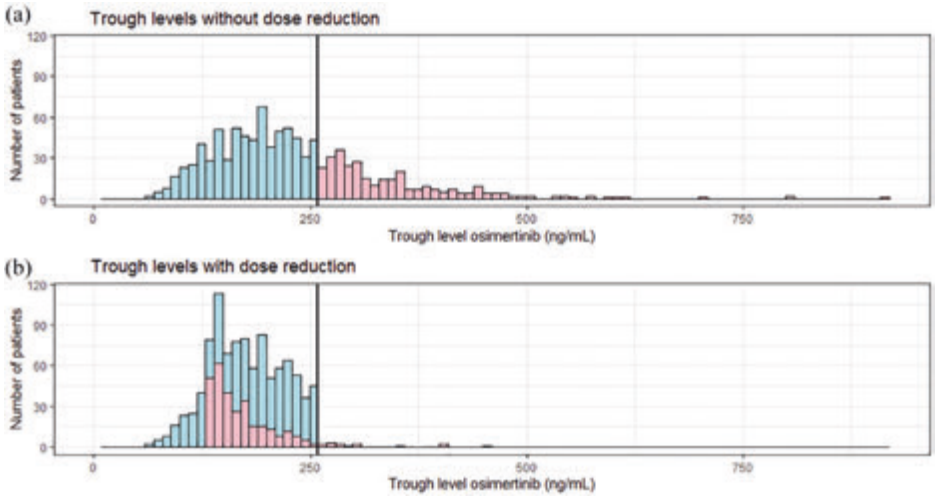

Figure 3. *Dose reduction effectively lowers osimertinib trough levels. (a) Distribution of osimertinib trough levels in a simulation cohort consisting of 1000 patients. The proposed toxic limit is visualized as a black vertical line (259 ng/mL). (b) Simulated distribution if the proposed 50% dose-reduction is applied for patients who were above the toxic limit in part (a).*

Exposure–efficacy relationship

Osimertinib exposure was significantly and negatively correlated with PFS in univariate Cox regression (p = 0.04) (**Supplemental Appendix D**). After correction for median CRP, median alkaline phosphatase, sex, age, *EGFR* mutation type, and TP53 mutations, the effect became non-significant (HR 0.95, 95% CI 0.91–1.00; p = 0.05). For OS, a similar correlation was observed in univariate Cox regression (p < 0.01). After correction for CRP, alkaline phosphatase, hemoglobin, primary *EGFR* mutation, and WHO performance status >1, only a trend toward significance remained for osimertinib exposure (HR 0.95, 95% CI 0.89–1.00; p = 0.10).

Discussion

This is the first study that describes osimertinib exposure to be significantly correlated with the occurrence of severe toxicity, and to suggest a safe, preventive dose reduction based on a toxic limit concentration of 259 ng/mL osimertinib.

Our data are supported by a prior study that also found a correlation with any grade toxicity.[10] The proposed toxic limit of 259 ng/mL osimertinib from our real-life study could result in a 53% reduction in severe toxicity for 26% of patients. This could prevent treatment discontinuation and subsequent treatment failure. Of course, in real life, other environmental factors may still influence the exposure to the drug (e.g. drug–drug and food–drug interactions),[22,23] which might therefore result in other toxicity outcomes, and the findings in this study should therefore be prospectively validated.

Importantly, we did not find a significant multivariate correlation between median osimertinib exposure and survival. The initial univariate–inverse relationship between exposure and survival was confounded by known parameters that are associated with cachexia (CRP, alkaline phosphatase, and hemoglobin) and important baseline characteristics (primary *EGFR* mutation and WHO performance status).[24-26] These results are in line with a prior osimertinib PK model study that reported an absent exposure–efficacy relationship over the 20–240 mg dose range.[10] A dose reduction of 50% would thus be safe, but should be validated prospectively.

The toxic limit is based on the median exposure during the total treatment time. When only samples are used prior to the occurrence of the majority of severe toxicity (*c.q.* before 2 months after treatment initiation), a similar effect occurred. This underlines the predictability and clinical implementability of our results. Since osimertinib reaches a steady-state concentration after 14 days of treatment, we suggest to perform osimertinib quantification after 14 days to forestall early toxicity.

The principle of a toxicity-preventing dose reduction based on therapeutic drug monitoring (TDM) is very common and frequently applied in daily clinical practice, for

example, in the field of infectious diseases and transplantation medicine. [27, 28] In the field of medical oncology, a preventing dose reduction based on TDM is less common. Most anticancer drugs, SMKIs in particular, are flat-dosed at the maximum tolerated dose and are only dose reduced after severe toxicity occurs.[29] Whereas, ideally, this should be done beforehand to avoid toxicity. For example, chemotherapeutic agents are sometimes individually dosed on expected exposure, which is predicted on individual patient characteristics (e.g. *DPYD* polymorphisms, body weight, estimated glomerular filtration rate [eGFR], and length), as is the case for capecitabine and carboplatin.[30,31] For pemetrexed and taxanes, exposure–toxicity relationships have been studied and also here, dose adjustments have been proposed to further optimize the treatment of individual patients.[32, 33]

Osimertinib drug costs of 80 and 40 mg QD in the Netherlands are exactly the same, currently both €6.150 per patient per month.[34] It would, hence, be financially interesting to consider dosing patients, eligible for a toxicity-preventing dose reduction, 80 mg every other day instead of 40 mg QD. This would potentially save 13% of total osimertinib drug costs. Since osimertinib has a long half-life of more than 40 h, this would be pharmacologically feasible.[35]

The validity of our population PK model is indirectly confirmed by the similarity with a previously published model.[10] In our model, especially CRP proved to be a strong, clinically relevant biomarker to predict osimertinib exposure. This is not surprising, since inflammation causes downregulation of CYP450 enzymes and subsequently affects the PK of various other drugs.[36] This finding could further lead to a temporary dose reduction when patients suffer from inflammation. Since quantification of osimertinib is not routine practice for most hospitals, a faster and simple CRP test would be more feasible to include in routine laboratory checks and should be validated prospectively.

A limitation of our study was an absent *a priori* power analysis, which causes the statistical analyses to be of a retrospective nature. However, the chance of a statistical type II error of these results is relatively small, because of the relatively large size of this cohort. A second limitation could be the different covariates that influence osimertinib exposure that complicate clinical interpretation. Nevertheless, despite the smaller group of 90 patients with samples during the first 2 months, the uncorrected values from these months predicted severe toxicity as well. This confirms that clinical extrapolation is definitely warranted.

To conclude, osimertinib exposure is significantly correlated with the occurrence of severe toxicity. Tolerability of osimertinib could, if prospectively validated, be optimized by implementation of a safe, preventive dose reduction in patients above the toxic limit of 259 ng/mL.

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Supplementary materials

APPENDIX A

COVARIATE EQUATIONS

Power model:

$$P_i = CL_{pop} * \left(\frac{COV}{median\ COV}\right)^{\theta COV} * exp^{(\eta_i}$$

Exponential model:

$$P_i = CL_{pop} * \left(\frac{COV}{median\ COV}\right) * \theta_{COV} * exp^{(\eta_i}$$

Categorical model

$$P_i = CL_{pop} * \theta_{Cat}{}^{FLAG} * exp^{(\eta_i}$$

Tested covariates on clearance:

- Demographics
    - Age
    - Weight
    - BMI
    - BSA
    - Sex
    - Ethnicity
    - WHO performance status

- Laboratory liver/kidney parameters
    - Alkaline phosphatase
    - ALAT
    - ASAT
    - Albumin
    - Creatine kinase
    - Gamma-glutamyl transferase
    - Estimated glomerular filtration rate
    - Creatinine

- Complete blood count
    - Haemoglobin
    - Haematocrit
    - Thrombocyte count

- Other
    - Lactate dehydrogenase
    - C-reactive protein

APPENDIX B: MODEL BUILDING AND DIAGNOSTICS

Model building

At first, a 1-compartmental model with first-order absorption was fitted to the log transformed data. A 2-compartmental model let to a numerically better fit according to OFV but to a poorer fit according to the AIC. Additionally, the 2-compartment model did not visually improve the fit and was therefore not incorporated in the model. As the estimated absorption rate deviated from prior reported values and the RSE was large, we tested multiple mechanistic absorption models, lag time, zero-order absorption, fixing it on the reported values, adding IIV, and adding transit compartments. This did not lead to improvement of the model and therefore the deviation of the absorption rate constant was accepted. A one-compartment model with first-order absorption, first order elimination and a proportional error was subsequently used for the covariate analysis.

Introduction of CRP as a covariate decreased the OFV by 254 points and reduced the error from 0.221 to 0.187. It also stabilized and decreased absorption rate constant and explained 6% of the IIV. Thereafter, the introduction of thrombocytes (dOFV=-63), haemoglobin (dOFV=-28), and alkaline phosphatase (dOFV=-25) proved to be a significant improvement and were incorporated in the final model. Initially, LDH (dOFV=-4.6) and sex (dOFV=-4.6) also improved the model significantly. However, LDH and sex were excluded from the model after the more stringent backward elimination ($p < 0.01$). Other covariates such as albumin did not significantly improve the model after addition of CRP, thrombocytes, haemoglobin and alkaline phosphatase. CRP, thrombocytes, haemoglobin and alkaline phosphatase were incorporated in the final model. The covariates decreased the proportional error from 0.221 to 0.176 and decreased the IIV from 33.4% to 27**%.** Model estimates and bootstrap results are shown in **Table A1**.

Table B1. Parameter estimations and bootstrap results of the final osimertinib model

Parameter (unit)	Parameter estimate [shrinkage]	RSE (%)	Bootstrap Median	95% CI bootstrap
Ka (h^{-1})	0.332	30.7	0.349	0.20 – 0.77
V/F (L)	1150	7.4	1154	1019 – 1372
CL/F (h^{-1})	14.50	2.4	14.59	13.9 – 15.3
Covariates on CL/F				
CRP	-0.119	11.4	-0.118	-0.15 - -0.09
Thrombocytes	-0.317	20.3	-0.319	-0.44 - -0.19
Haemoglobin	0.576	26.2	0.585	0.30 – 0.89
ALK-P	-0.155	25.0	-0.150	-0.23 - -0.07
IIV				
CL (CV%)	26.8 [6.3]	12.3	26.8	23.2 – 30.5
Residual error				
Proportional (%)	17.50 [8.9]	3.2	17.42	16.3 – 18.6
Conditional number	5.09			

Abbreviations: Ka =absorption constant; V/F = distribution volume divided by bioavailability; CL = drug clearance; CRP = c-reactive protein; ALK-P = alkaline phosphatase; IIV = inter-individual variance.

APPENDIX C MODEL DIAGNOSTICS

Figure C1. Goodness of fit plots for the final osimertinib model. Abbreviations: IWRES: individual weighted residuals

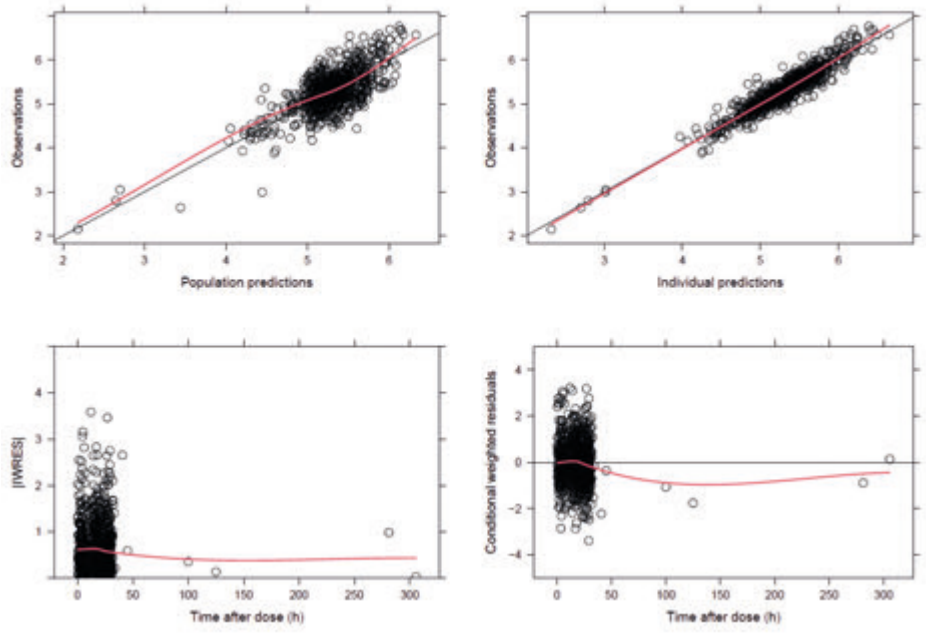
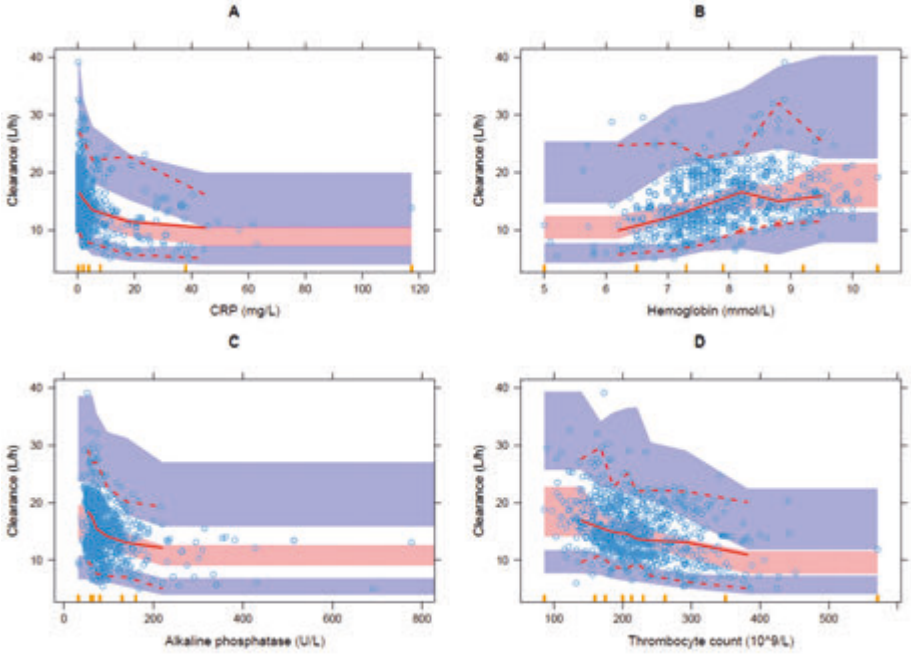


Figure C2. Visual Predictive Checks (VPC's) for covariates included in the final model.



APPENDIX D OUTCOMES OF COX PROPORTIONAL-HAZARD MODEL ANALYSES.

Table S1. Outcomes of univariate Cox proportional-hazards models for severe toxicity.

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.92 †	0.85 - 0.99	Median per person (L/h)
CRP	0.98	0.92 - 1.05	Median per person (mg/L)
ALKP	1.00	1.00 - 1.01	Median per person (U/L)
Hemoglobin	0.80	0.50 - 1.27	Median per person (mmol/L)
Thrombocytes	1.00	1.00 - 1.00	Median per person (*10^9/L)
Sex	1.16	0.55 - 2.46	Male vs. Female
Age at start	1.05 †	1.01 - 1.08	(Years)
EGFR mutation	0.65	0.34 - 1.26	Exon 19 del vs. others
	1.29	0.64 - 2.63	L858R vs. others
	1.47	0.64 - 3.35	Compound/rare vs. other
TP53 mutation	0.92	0.46 - 1.84	No mutation vs. mutation
TKI-line	0.60	0.30 - 1.22	First line vs. Second/third line
Metastases in CNS	1.02	0.37 - 2.82	No CNS metastases vs. CNS metastases
WHO PS	1.60	0.71 - 3.58	WHO 0&1 vs. WHO 2&3

* = p<0.10
† = p<0.05
‡ = p<0.01

Table S2. Outcome of multivariate Cox proportional-hazards model for severe toxicity

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.90 ‡	0.84 - 0.97	Median per person (L/h)
Age at start	1.06 ‡	1.02 - 1.10	(Years)

* = p<0.10
† = p<0.05
‡ = p<0.01

Table S3. Outcomes of univariate Cox proportional-hazards models for progression-free survival.

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.95 †	0.91 - 1.00	Median per person (L/h)
CRP	1.05 †	1.03 - 1.05	Median per person (mg/L)
ALKP	1.00 †	1.00 - 1.01	Median per person (U/L)
Hemoglobin	0.98	0.78 - 1.24	Median per person (mmol/L)
Thrombocytes	1.00	1.00 - 1.01	Median per person (*10^9/L)
Sex	0.67 †	0.46 - 0.99	Male vs. Female
Age at start	0.98 *	0.97 - 1.00	(Years)
EGFR mutation	0.66 †	0.45 - 0.96	Exon 19 del vs. others
	1.36	0.91 - 2.04	L858R vs. others
	1.41	0.82 - 2.40	Compound/rare vs. other
TP53 mutation	1.77 †	1.22 - 2.60	No mutation vs. mutation
TKI-line	1.10	0.85 - 1.44	First line vs. Second/third line
Metastases in CNS	0.80	0.47 - 1.36	No CNS metastases vs. CNS metastases
WHO PS	1.34	0.84 - 2.15	WHO 0&1 vs. WHO 2&3

* = p<0.10
† = p<0.05
‡ = p<0.01

Table S4. Outcome of multivariate Cox proportional-hazards model for progression-free survival

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.95*	0.91 - 1.00	Median per person (L/h)
CRP	1.03‡	1.01 - 1.06	Median per person (mg/L)
ALKP	1.00	1.00 - 1.00	Median per person (U/L)
Sex	0.64 †	0.41 - 0.99	Male vs. Female
Age at start	0.99	0.96 - 1.00	(Years)
EGFR mutation	0.65 †	0.44 - 0.97	Exon 19 del vs. others
TP53 mutation	1.60 †	0.63 - 1.06	No mutation vs. mutation

* = p<0.10
† = p<0.05
‡ = p<0.01

Table S5. Outcomes of univariate Cox proportional-hazards models for overall survival.

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.90‡	0.85 - 0.96	Median per person (L/h)
CRP	1.05‡	1.03 - 1.08	Median per person (mg/L)
ALKP	1.01‡	1.00 - 1.01	Median per person (U/L)
Hemoglobin	0.68†	0.50 - 0.93	Median per person (mmol/L)
Thrombocytes	1.00	1.00 - 1.01	Median per person (*10^9/L)
Sex	0.86	0.52 - 1.45	Male vs. Female
Age at start	0.99	0.97 - 1.02	(Years)
EGFR mutation	0.53†	0.32 - 0.87	Exon 19 del vs. others
	1.31	0.76 - 2.28	L858R vs. others
	2.08†	1.14 - 3.77	Compound/rare vs. other
TP53 mutation	1.01	0.66 – 1.54	No mutation vs. mutation
TKI-line	1.15	0.67 – 1.93	First line vs. Second/third line
Metastases in CNS	1.13	0.82 – 1.56	No CNS metastases vs. CNS metastases
WHO PS	1.85‡	1.24 – 2.77	WHO 0&1 vs. WHO 2&3

* = p<0.10
† = p<0.05
‡ = p<0.01

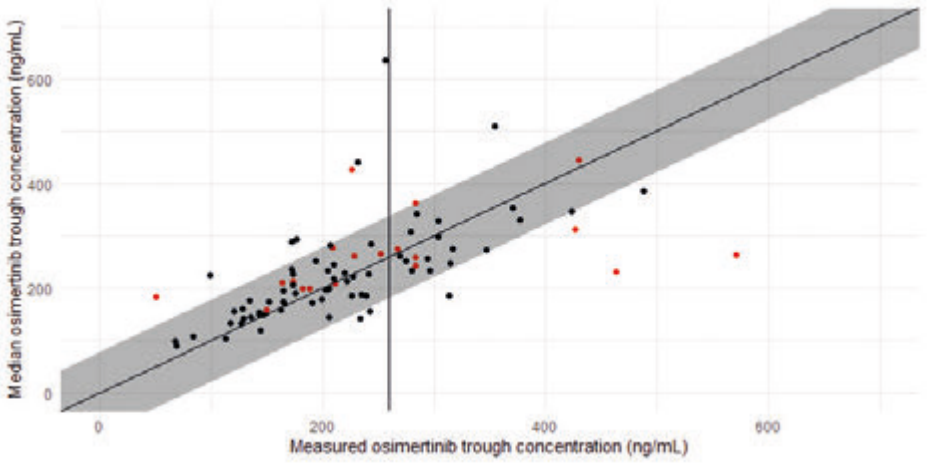
Table S6. Outcome of multivariate Cox proportional-hazards model for overall survival

Factor	*HR*	*95% CI*	*Grouping*
Clearance	0.95	0.89 - 1.01	Median per person (L/h)
CRP	1.04‡	1.01 - 1.08	Median per person (mg/L)
ALKP	1.00†	1.00 - 1.00	Median per person (U/L)
Hemoglobin	0.80	0.57 - 1.13	Median per person (mmol/L)
EGFR mutation	0.50†	0.27 - 0.91	Exon 19 del vs. others
	1.20	0.59 - 2.45	Compound/rare vs. others
WHO PS	3.06‡	1.35 - 4.40	WHO 0&1 vs. WHO 2&3

* = p<0.10
† = p<0.05
‡ = p<0.01

APPENDIX E SUBANALYSIS FIRST OSIMERTINIB PLASMA CONCENTRATION

Figure D1. First osimertinib plasma through concentration plotted median osimertinib plasma trough concentration. The vertical line is the toxic limit of 259 ng/mL, the diagonal line is the unity line, red dots are patients that experienced severe toxicity in the first 12 months of treatment, and the grey see-through pane is the 30% variability interval.



APPENDIX F FINAL MODEL CONTROL STREAM

```
$INPUT CENSOR ID DATE=DROP TIME DVLN DV DOSE=AMT ADDL II TAD CMT EVID MDV
SEX AGE LENGTH WEIGHT WHO ALKP ALKPPRE DATEALKPPRE ALKPPOST DATEALKPPOST
ALAT ALATPRE DATEALATPRE ALATPOST DATEALATPOST ALB ALBPRE DATEALBPRE
ALBPOST DATEALBPOST ASAT ASATPRE DATEASATPRE ASATPOST DATEASATPOST CK
CKPRE DATECKPRE CKPOST DATECKPOST EGFR EGFRPRE DATEEGFRPRE EGFRPOST
DATEEGFRPOST CRP CRPPRE DATECRPPRE CRPPOST DATECRPPOST
GGT GGTPRE DATEGGTPRE GGTPOST DATEGGTPOST HEMOGLOB HEMOGLOBPRE
DATEHEMOGLOBPRE HEMOGLOBPOST DATEHEMOGLOBPOST HEMATOCR
HEMATOCRPRE DATEHEMATOCRPRE HEMATOCRPOST DATEHEMATOCRPOST KREAT
KREATPRE DATEKREATPRE KREATPOST DATEKREATPOST LDH LDHPRE DATELDHPRE
LDHPOST DATELDHPOST THROMBO THROMBOPRE DATETHROMBOPRE THROMBOPOST
DATETHROMBOPOST ETHNIC BASEWEIGHT LASTDOSE CLMED ROWNUM TIMEAE
LASTTIME
$DATA Dataset.csv IGNORE=C;

$SUBROUTINES ADVAN2 TRANS2

$PK
CALLFL=-2
MTDIFF=1

MTIME(1) = DATEALKPPRE
MTIME(2) = DATEALKPPOST
ALKPDIFF = ALKPPOST-ALKPPRE
ALKPTIME = TIME-MTIME(1)
IF(ALKPTIME.LE.0) ALKPTIME=0.001
ALKPINT = MTIME(2)-MTIME(1)
IALKP = ALKPPRE+(ALKPDIFF*(ALKPTIME/ALKPINT))

MTIME(1) = DATECRPPRE
MTIME(2) = DATECRPPOST
CRPDIFF = CRPPOST-CRPPRE
CRPTIME = TIME-MTIME(1)
IF(CRPTIME.LE.0) CRPTIME=0.001
CRPINT = MTIME(2)-MTIME(1)
ICRP = CRPPRE+(CRPDIFF*(CRPTIME/CRPINT))

MTIME(1) = DATEHEMOGLOBPRE
MTIME(2) = DATEHEMOGLOBPOST
HEMOGLOBDIFF = HEMOGLOBPOST-HEMOGLOBPRE
```

```
HEMOGLOBTIME = TIME-MTIME(1)
IF(HEMOGLOBTIME.LE.0) HEMOGLOBTIME=0.001
HEMOGLOBINT = MTIME(2)-MTIME(1)
IHEMOGLOB = HEMOGLOBPRE+(HEMOGLOBDIFF*(HEMOGLOBTIME/HEMOGLOBINT))

MTIME(1) = DATETHROMBOPRE
MTIME(2) = DATETHROMBOPOST
THROMBODIFF = THROMBOPOST-THROMBOPRE
THROMBOTIME = TIME-MTIME(1)
IF(THROMBOTIME.LE.0) THROMBOTIME=0.001
THROMBOINT = MTIME(2)-MTIME(1)
ITHROMBO = THROMBOPRE+(THROMBODIFF*(THROMBOTIME/THROMBOINT))

KA = THETA(2)
TVCL = THETA(3)  *  ((ICRP/2.1)**THETA(5))  *  ((ITHROMBO/214)**THETA(6))  *
((IHEMOGLOB/7.8)**THETA(7)) * ((IALKP/81)**THETA(8))
CL = TVCL * EXP(ETA(1))
V = THETA(4)
S2 = V/1000 ;scaling from ng/mL to mg/L

$THETA
(0, 0.24) ;1 prop err
(0, 0.0084)          ;2 Ka
(0, 14.2) ;3 CL
(0, 1986)            ;4 V
(-10, 1)             ;5 CRP
(-10, 1)             ;6 THROMBO
(-10, 1)             ;7 HEMOGLOB
(-10, 1)             ;8 ALKP

$ERROR ;; Calculation based on log-transformed data
 IPRED=LOG(0.0001)
 IF(F.GT.0)IPRED=LOG(F)
 W=1
 IF(F.GT.0)W = SQRT(THETA(1)**2)
 IRES = DV-IPRED
 IWRES = IRES/W
 Y = IPRED+W*EPS(1)

$OMEGA
(0.46) ; IIV CL
```

```
$SIGMA
1 FIX ; Proportional error PK

$EST METHOD=1 INTER MAXEVAL=2000 NOABORT SIG=3 PRINT=1 POSTHOC
$COV PRINT=E
```

Chapter 4

Chemotherapy for patients with EGFR-mutated NSCLC after progression on EGFR-TKI's: exploration of efficacy of unselected treatment in a multicenter cohort study

In preparation

Christi M.J. Steendam *, Sophie M. Ernst *, Sushil K. Badrising, Merle Hattink, Joachim G.J.V. Aerts, A. Joop de Langen, Anne-Marie C. Dingemans *
These authors contributed equally

Abstract

Background:

In patients with Epidermal Growth Factor Receptor (EGFR)-mutated non-small cell lung (NSCLC) chemotherapy remains standard of care after progression on EGFR-tyrosine kinase inhibitors (TKIs). With the development of anti-angiogenic agents and immune checkpoint inhibitors the landscape of systemic regimens has changed significantly. This cohort study aims to evaluate the efficacy of chemotherapy regimens after progression on EGFR-TKI in an European population.

Materials and methods:

All consecutive patients treated with chemotherapy after progression on EGFR-TKI for EGFR-mutated NSCLC, were identified in two tertiary centers in the Netherlands. Data on best response, progression free survival (PFS) and overall survival (OS) were extracted from medical records.

Results:

In total, 171 lines of chemotherapy were identified: platinum/pemetrexed (PP, n=95), carboplatin/paclitaxel/bevacizumab/atezolizumab (CPBA, n=32), paclitaxel/bevacizumab (PB, n=36) and carboplatin/paclitaxel/bevacizumab (CPB, n=8). Of the 171 lines, 106 were given as first-line after EGFR-TKI. Median PFS did not differ significantly between the first-line regimens (p=0.50), with the highest PFS in PP (5.2 months [95% CI 4.5-5.9]) and CPBA (5.9 months [95% CI 3.8-80]). The majority of the PB group (n=32) received this regimen in a second- or later line with a median PFS of 4.9 months (95% CI 3.3-6.6). First-line regimens had a median OS of 15.3 months (95% CI 11.6-18.9) with no significant difference between regimens (p=0.85).

Conclusion:

After progression on EGFR-TKI, patients with EGFR-mutated NSCLC show substantial benefit on different chemotherapy regimens. In particular, favorable outcomes were seen in patients treated with PP and CPBA as first-line chemotherapy, and PB in further lines of chemotherapy.

Introduction

The treatment paradigm of patients with locally advanced or metastatic non-small cell lung cancer (NSCLC) with an Epidermal Growth Factor Receptor (*EGFR*) mutation has changed impressively over the past decades. EGFR tyrosine kinase inhibitors (TKI) are considered standard of care first-line systemic therapy in patients with *EGFR*-mutated NSCLC, due to the higher efficacy and favorable toxicity profile compared to conventional chemotherapy.[1, 2] Osimertinib, a third generation EGFR-TKI, showed longer progression free survival (PFS), overall survival (OS), less toxicity and better central nervous system (CNS) efficacy compared to first- and second generation EGFR-TKI.[3-5] Therefore, osimertinib is currently the preferred first-line treatment, with an impressive median OS of 38.6 months and 54% of patients still alive after 36 months.[4] However, all tumors eventually become resistant to TKI treatment.[6, 7] While several studies are investigating targeted therapies after TKI failure[8-10], for now chemotherapy remains the standard treatment when no targetable resistance mechanism is found. Currently, it is yet to be determined which systemic regimen is most appropriate after progression on EGFR-TKI.

For non-squamous non-oncogene driven NSCLC, platinum-doublet chemotherapy with or without immunotherapy, is standard of care. Additionally, weekly paclitaxel plus bevacizumab has shown to be a valid treatment option in second- or further lines.[11] Although immune checkpoint inhibitors are generally not recommended for *EGFR*-mutated NSCLC, there are ongoing efforts to evaluate the efficacy of the addition of immunotherapy to the chemotherapy backbone in *EGFR*-mutated NSCLC.[12, 13] Additionally, combination strategies with anti-angiogenic agents, with or without immunotherapy, are under investigation after the promising results of platinum-doublet chemotherapy plus bevacizumab with or without atezolizumab in the IMpower150 trial.[14, 15] While awaiting results of ongoing randomized trials, there is a need to evaluate the real-world efficacy of commonly used chemotherapy regimens to determine the current most effective treatment option. A few Asian and North-American groups have already attempted to shed light on this issue, but results were conflicting.[16-18] Additionally, due to the heterogeneity in prescribed chemotherapy regimens worldwide, extrapolation of their findings to daily clinical practice in the European *EGFR*-mutated NSCLC population is hampered. Currently, real-world data on the efficacy of approved chemotherapy regimens in a European population is lacking.

This multicenter retrospective observational study was conducted in the Netherlands to evaluate the real-world efficacy of chemotherapy regimens in patients with *EGFR*-mutated NSCLC after progression on EGFR-TKI's.

Material and Methods

This study is a retrospective, multicenter cohort study conducted in two tertiary cancer centers in the Netherlands: the Erasmus MC Cancer Institute (Rotterdam) and The Netherlands Cancer Institute (Amsterdam). In the Erasmus MC Cancer Institute, all consecutive patients between January 1st 2015 and July 20th 2021 were included in the cohort. In the Netherlands Cancer Institute, patients between January 1st 2018 and July 20th 2021 were included. The data cutoff point was 13th of February 2023.

Patients

All patients with advanced *EGFR*-mutated NSCLC who were treated with EGFR-TKIs and subsequently with chemotherapy regimens containing paclitaxel or pemetrexed were identified by checking all EGFR-TKI and chemotherapy prescriptions through the in-hospital pharmacy. All lines of chemotherapy were eligible for inclusion. The chemotherapy regimen given as first subsequent line after EGFR-TKI was included in the first-line cohort. Patients who continued osimertinib during chemotherapy remained eligible.

Data collection

Information on medical history, patient demographics, disease and pathological characteristics, TKI treatment history, chemotherapy regimens and efficacy were collected from the medical records. Treatment modifications defined as interruption, delay, dose reduction or discontinuation of compounds were scored when performed for medical reasons (e.g. toxicity, clinical deterioration). Data collection was performed according to the Declaration of Helsinki and the European Union General Data Protection Regulation statements.

Outcome measures

Tumor response and date of radiological or clinical progression as determined by treating physician were identified from the records. Durable benefit was defined as a PFS of ≥ 6 months. Primary outcome was PFS in all first-line chemotherapy regimens, which was defined as time from start of chemotherapy until radiological or clinical progression or death of any cause. Secondary outcomes were OS in the first-line chemotherapy cohorts, defined as time from start of first-line chemotherapy until death of any cause, PFS of individual regimens independent of line of therapy and objective response rate (ORR) in the first-line and entire cohort. Patients who had not progressed or died at data cutoff were censored.

Statistical analysis

Differences in PFS and OS in the first-line chemotherapy cohort were compared by log-rank test in Kaplan Meier survival analysis. The PFS in the different chemotherapy regimen cohorts (consisting of all lines of chemotherapy) was explored using Kaplan-

Meier survival analysis, but not statistically compared because of the heterogeneity of the groups. Categorical clinical characteristics and tumor response were compared with Pearson chi-square tests. 95% confidence intervals (95% CI) for ORR and DCR were estimated by Clopper-Pearson method. All data were analyzed using IBM SPSS Statistics for Windows, version 25 (IBM Corp. Released 2017). All tests were two-sided and a *p*-value of <0.05 was considered significant.

Results

Patient characteristics

In total, we identified 135 patients who were eligible for inclusion, of which 96 (71.1%) had received osimertinib prior to chemotherapy initiation. Thirty-three patients received more than one line of chemotherapy, which resulted in the identification of a total of 171 lines of chemotherapy with pemetrexed or paclitaxel after progression on EGFR-TKIs (**Figure 1**). The details of the identified regimens are summarized in **Table 1**.

Table 1. Details of the identified chemotherapy regimens.

Regimen	Details
Platinum plus pemetrexed (PP)	Either cisplatin 75 mg/m^2 or carboplatin AUC5 plus pemetrexed 500 mg/m^2 every 21 days up to 4 cycles, followed by pemetrexed maintenance every 21 days until disease progression or intolerable toxicity.
Carboplatin plus paclitaxel plus bevacizumab plus atezolizumab (CPBA)	Carboplatin AUC6, paclitaxel 200 mg/m2, bevacizumab 15 mg/kg and atezolizumab 1200 mg every 21 days for 4 cycles, followed by bevacizumab/atezolizumab maintenance every 21 days until disease progression or intolerable toxicity, according to the IMpower150 trial.[19]
Paclitaxel plus bevacizumab (PB)	Paclitaxel 90 mg/m^2 on day 1, 8 and 15 with bevacizumab 10 mg/kg on day 1 and 15 every 28 days until disease progression or intolerable toxicity. This regimen was adapted from the ULTIMATE trial.[11]
Carboplatin plus paclitaxel plus bevacizumab (CPB)	Carboplatin AUC6, paclitaxel 200 mg/m^2 and bevacizumab 15 mg/kg every 21 days up to 4 cycles, followed by bevacizumab maintenance 15 mg/kg every 21 days until disease progression or intolerable toxicity.[20]

Abbreviations: AUC = area under the curve.

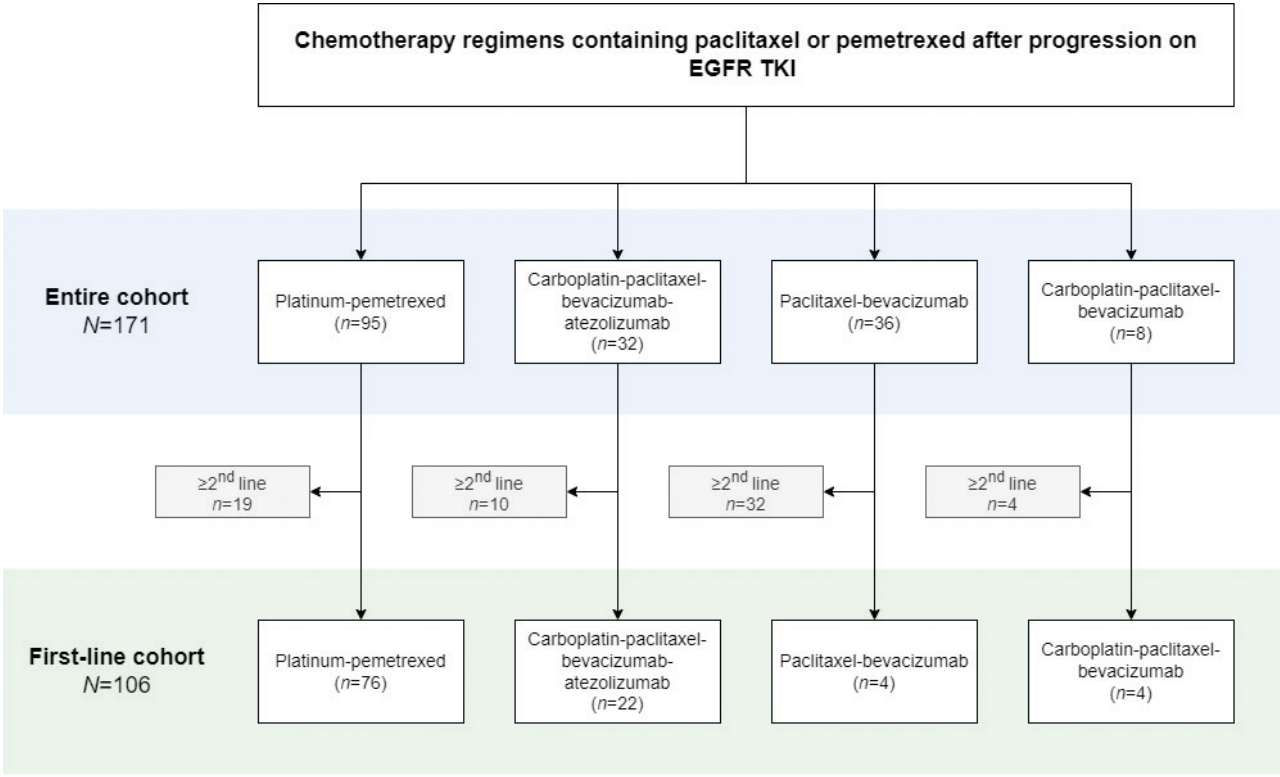


Figure 1. Overview of study cohort. *The first-line cohort consists of all chemotherapy regimens that were received as first-line of systemic treatment after progression on EGFR-TKI.*

The baseline characteristics of the entire cohort are summarized in **Supplementary Table S1**. The majority of patients (*n*=95) were treated with platinum/pemetrexed (PP), of which 42 patients (44.2%) subsequently received pemetrexed maintenance. Thirty-two patients received carboplatin/paclitaxel/bevacizumab/atezolizumab (CPBA), mainly as a first-line treatment (*n*=22, 68.8%). Thirty-six patients received paclitaxel/bevacizumab (PB), of which 20 patients (55.6%) had known CNS metastasis prior to start of the chemotherapy. In the majority of cases (*n*=32, 88.9%) PB was given as a second- or later line of chemotherapy. Eight patients were treated with carboplatin/paclitaxel/bevacizumab (CPB). Notably this cohort had a relatively poor WHO performance score (PS), 37.5% of patients had WHO PS ≥2, and treatment in a later line, with half of patients receiving CPB in a second- or later line.

Of the total 171 lines of chemotherapy, 106 lines were included in the first-line cohort, of which the majority (*n*=76, 71.7%) received PP, followed by CPBA (*n*=22, 20.8%). The baseline characteristics of the first-line cohort are summarized in **Table 2**. Median age was 63 years (range 33-80), the majority of patients were female (61.3%) and had never smoked (57.5%). The majority harbored *EGFR* exon 19 deletions (51.9%) and *EGFR* exon 21 L858R mutations (30.2%), and had a concomitant *TP53* aberration at baseline (67.0%).

Table 2. Baseline characteristics of first-line cohort.

Characteristic, *n* (%)	**PP (*n*=76)**	**CPBA (*n*=22)**	**PB (*n*=4)**	**CPB (*n*=4)**	**Total (*n*=106)**
Sex					
Male	26 (34.2%)	12 (54.5%)	0 (0%)	3 (75.0%)	41 (38.7%)
Female	50 (65.8%)	10 (45.5%)	4 (100%)	1 (25.0%)	65 (61.3%)
Age (median, range)	**64 (33 – 78)**	**56 (37 – 76)**	**69 (39 – 80)**	**58 (39 – 75)**	**63 (33-80)**
Tobacco exposure					
Current	3 (3.9%)	0 (0%)	1 (25.0%)	2 (50.0%)	4 (3.8%)
Former	29 (38.2%)	9 (40.9%)	0 (0%)	2 (50.0%)	40 (37.7%)
Never	43 (56.6%)	13 (59.1%)	3 (75.0%)	0 (0%)	61 (57.5%)
Unknown	1 (1.3%)	0 (0%)	0 (0%)	0 (0%)	1 (0.9%)
WHO PS					
0	20 (26.3%)	1 (4.5%)	0 (0%)	1 (25.0%)	22 (20.8%)
1	40 (52.6%)	20 (90.9%)	3 (75.0%)	2 (50.0%)	65 (61.3%)
2	7 (9.2%)	1 (4.5%)	1 (25.0%)	1 (25.0%)	10 (9.4%)
3	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
4	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Unknown	9 (11.8%)	0 (0%)	0 (0%)	0 (0%)	9 (8.5%)

Table 2. Continued

Characteristic, *n* (%)	PP (*n*=76)	CPBA (*n*=22)	PB (*n*=4)	CPB (*n*=4)	Total (*n*=106)
CNS metastasis					
Yes	21 (27.6%)	10 (43.5%)	2 (50.0%)	3 (75.0%)	36 (33.6%)
No	55 (72.4%)	13 (56.5%)	2 (50.0%)	1 (25.0%)	71 (66.4%)
Type *EGFR* aberration					
Exon 19 deletion	38 (50%)	12 (54.5%)	2 (50.0%)	3 (75.0%)	55 (51.9%)
Exon 21 p.L858R	25 (32.9%)	5 (22.7%)	2 (50.0%)	0 (0%)	32 (30.2%)
Exon 20 insertion	4 (5.3%)	1 (4.5%)	0 (0%)	1 (25.0%)	6 (5.7%)
Other*	9 (11.8%)	4 (18.2%)	0 (0%)	0 (0%)	13 (12.2%)
Concomitant *TP53* mutation					
Yes	56 (73.7%)	16 (72.7%)	3 (75%)	4 (100%)	71 (67.0%)
No	16 (21.1%)	6 (27.3%)	1 (25%)	0 (0%)	31 (30.4%)
Unknown	0 (0%)	0 (0%)	0 (0%)	0 (0%)	4 (3.8%)
Previous osimertinib					
Yes	50 (65.8%)	16 (72.7%)	4 (100.0%)	2 (50.0%)	72 (67.9%)
No	26 (34.2%)	6 (27.3%)	0 (0%)	2 (50.0%)	34 (32.1%)

*The first-line cohort consists of all chemotherapy regimens that were received as first-line of systemic treatment after progression on EGFR-TKI. Abbreviations: PS = performance score. CNS = central nervous system. * = other aberrations are specified in* ***Supplementary Data 1****.*

Treatment outcomes

At time of data cutoff, median follow-up in the first-line cohort was 48.4 months (95% CI 31.5-65.4), and 45.9 months (95% CI 37.3-54.5) in the entire cohort. In the first-line cohort 105 events of progression occurred and 93 patients had died. In the entire cohort 170 events of progression occurred. Treatment discontinuation due to toxicity was highest is the PB cohort (41.6%), followed by PP (21.1%), and CPBA (12.5%). In the CPB cohort there were no patients who discontinued treatment due to toxicity. Details on treatment delivery, treatment modifications and delivery of subsequent therapy after the chemotherapy regimens are summarized in **Table 3**. The majority of the cohort (69.0%) received subsequent systemic therapy after the included chemotherapy regimen.

Progression free survival

The median PFS (mPFS) in the first-line cohort was 5.4 months (95% CI 4.7-6.1), and did not differ between the different types of *EGFR* mutation (p=0.59), or between those with or without concomitant *TP53* mutations (p=0.11). No significant difference in PFS between the different first-line chemotherapy regimens was observed (p=0.50). The

different first-line chemotherapy regimens showed a mPFS of 5.2 months (95% CI 4.5-5.9) for PP, 5.9 months for CPBA (95% CI 3.8-8.0), 3.3 months (95% CI 0.0-6.9) for PB, and 2.8 months (95% CI N/E – N/E) for CPB (**Figure 2A**). Subsequently performed survival analysis for every regimen cohort separately independent of the line of therapy, showed a mPFS of 4.9 months for PP (95% CI 4.4-5.4), 5.8 months for CPBA (95% CI 5.1-6.5) for CPBA, 4.9 months for PB (95% CI 3.2-6.7) and 2.8 months for CPB (95% CI 0.0-7.1) (**Supplementary Figure S1**).

A total of 44 patients (41.5%) of the first-line cohort experienced durable benefit. The chemotherapy treatment, types of *EGFR* mutations and presence of concomitant *TP53* mutations did not differ significantly between those with or without durable benefit (p=0.72, p=0.79 and p=0.33, respectively). Patients with durable benefit had less known CNS metastasis at baseline than those without durable benefit (20.5% versus 43.5%, p=0.01).

Overall survival

In the first-line chemotherapy cohort median OS (mOS) was 15.3 months (95% CI 11.6-18.9), with the longest mOS in the PP and CPBA treatment groups of 15.4 (95% CI 11.8-19.0) and 11.1 (95% CI 3.4-18.8) months, respectively. mOS was 5.3 months (95% CI 0.0-20.2) in the PB group, and 8.4 months (95% CI 0.0-18.4) in the CPB group (**Figure 2B**). mOS did not differ significantly between the chemotherapy regimens (p=0.85).

Tumor response

Tumor response in the first-line and entire cohort are summarized in **Figure 3**. ORR and DCR did not differ significantly between the different first-line regimens (p=0.27 and p=0.59, respectively). In the entire cohort ORR differed significantly between the treatment regimens (p=0.015) with PP having the worse ORR (40.0% [95% CI 30.1-50.6]), but DCR did not differ (p=0.26).

Clinical benefit of PB in a chemotherapy pretreated population

Most patients (88.9%) in the PB cohort received this regimen in a second- or later line of treatment, with a median PFS of 4.9 months (95% CI 3.3-6.6). Nine patients who experienced disease progression as best overall response on PP, subsequently received PB. In seven of these cases (77.8%) disease control was achieved with the PB regimen: one patient showed stable disease with a PFS of 8.2 months, and 6 patients had a partial response with a PFS ranging from 3.2 to 8.3 months.

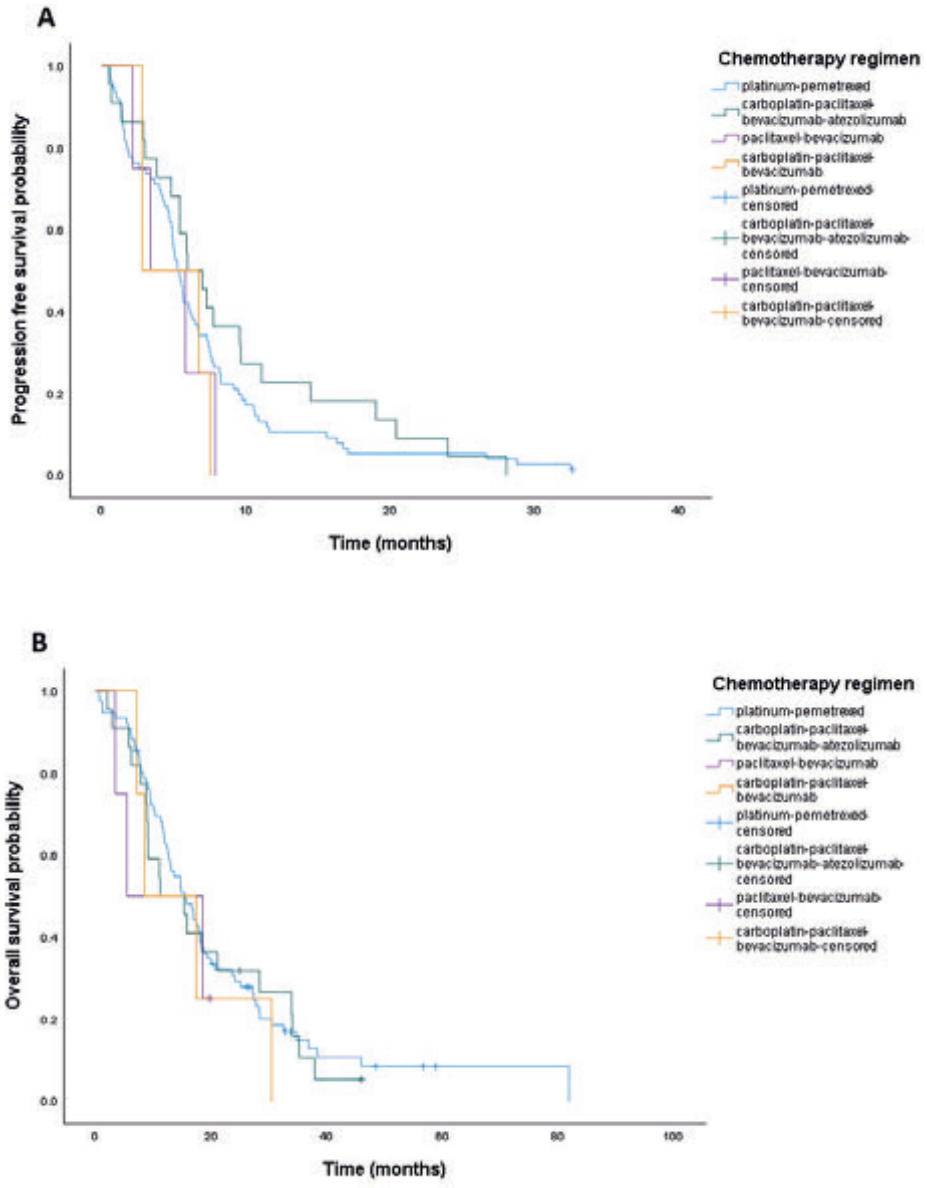
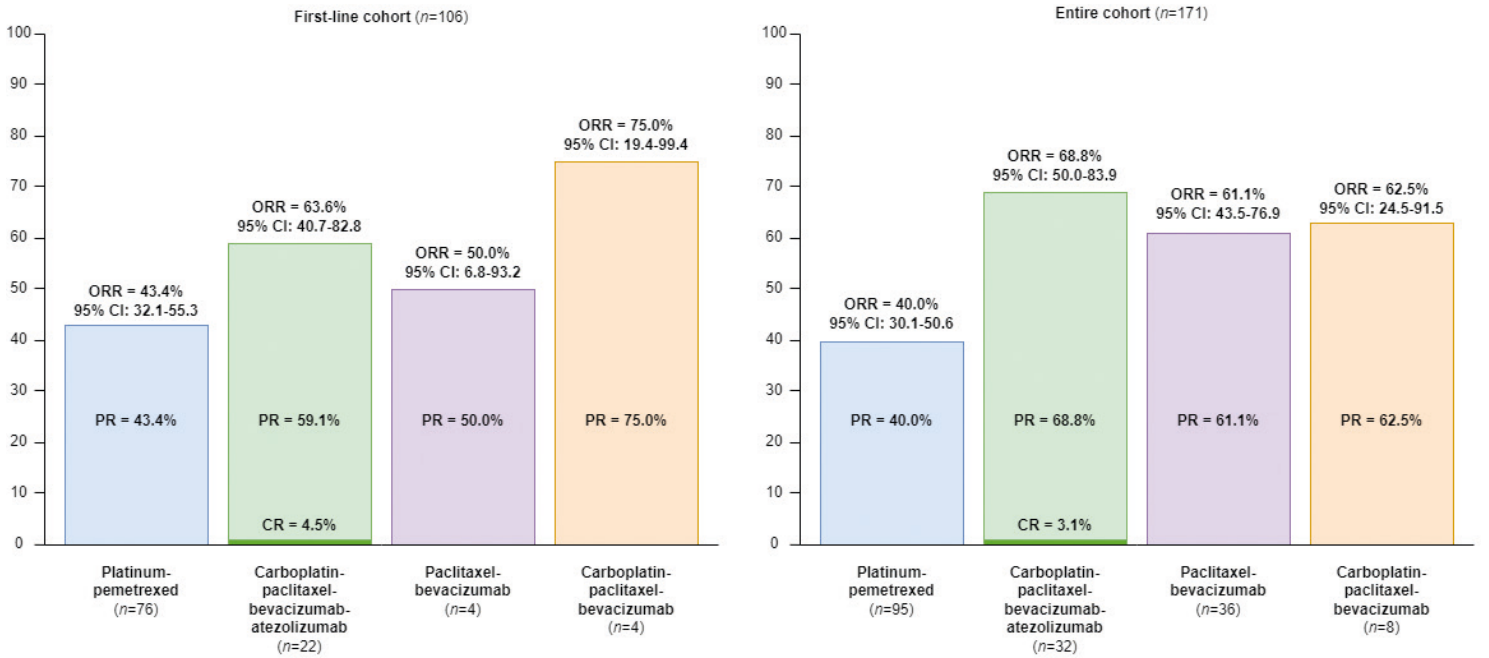


Figure 2. Kaplan-Meier estimates of (A) progression free survival and (B) overall survival for the first-line chemotherapy regimens.



	77.6% (66.6-86.4)	86.4% (65.1-97.1)	75.0% (19.4-99.4)	100% (39.8-100)	73.7% (63.6-82.2)	90.6% (75.0-98.0)	77.8% (60.8-89.9)	75.0% (34.9-96.8)
CI)	5.2 (4.5-5.9)	5.9 (3.8-8.0)	3.3 (0.0-6.9)	2.8 (N/E-N/E)	4.9 (4.4-5.4)	5.8 (5.1-6.5)	4.9 (3.2-6.7)	2.8 (0.0-7.1)
	15.4 (11.8-19.0)	11.1 (3.4-18.8)	5.3 (0.0-20.2)	8.4 (0.0-18.4)				
	30 (39.5%)	11 (50.0%)	1 (25.0%)	2 (50.0%)				

Figure 3. Treatment outcomes of first-line and entire cohort. *Abbreviations: ORR = Objective response rate. PR = Partial response. CR = complete response. N/E = not evaluable.*

Table 3. Treatment characteristics of entire cohort.

Characteristic, *n* (%)	**PP** *n*=95	**CPBA** *n*=32	**PB** *n*=36	**CPB** *n*=8	**Total** *n*=171
Reason discontinuation					
Progressive disease	52 (54.7%)	25 (78.2%)	17 (47.2%)	6 (75.0%)	100 (58.5%)
Death	5 (5.3%)	0 (0%)	2 (5.6%)	0 (0%)	7 (4.1%)
Toxicity	20 (21.1%)	4 (12.5%)	15 (41.6%)	0 (0%)	39 (22.8%)
Patient wish	2 (2.1%)	1 (3.1%)	2 (5.6%)	0 (0%)	5 (2.9%)
Planned cycles completed	13 (13.7%)	0 (0%)	0 (0%)	2 (25.0%)	15 (8.8%)
Ongoing treatment	0 (0%)	1 (3.1%)	0 (0%)	0 (0%)	1 (0.6%)
Other	3 (3.2%)	1 (3.1%)	0 (0%)	0 (0%)	4 (2.3%)
Location of progression					
Extracerebral	68 (71.6%)	15 (46.9%)	18 (50.0%)	3 (37.5%)	104 (60.8%)
Intracerebral	9 (9.5%)	10 (31.3%)	5 (13.9%)	2 (25.0%)	26 (15.2%)
Intra- & extracerebral	8 (8.4%)	5 (15.6%)	3 (8.3%)	2 (25.0%)	18 (10.5%)
Clinical progression	9 (9.5%)	2 (6.2%)	10 (27.8%)	1 (12.5%)	22 (12.9%)
N/A	1 (1.1%)	0 (0%)	0 (0%)	0 (0%)	1 (0.6%)
Treatment cycles, median (range)	4.0 (1-31)	8.0 (1-33)	4.5 (1-14)	4.5 (1-10)	5.0 (1-33)
Any modification during treatment[#]					
Yes	40 (42.1%)	21 (65.6%)	24 (66.7%)	3 (37.5%)	88 (51.5%)
No	55 (57.9%)	11 (34.4%)	12 (33.3%)	5 (62.5%)	83 (48.5%)
Subsequent therapy					
Yes	72 (75.8%)	18 (56.3%)	21 (58.3%)	7 (87.5%)	118 (69.0%)
No	22 (23.2%)	13 (40.6%)	15 (41.7%)	1 (12.5%)	51 (29.8%)
N/A	1 (1.1%)	1 (3.1%)	0 (0%)	0 (0%)	2 (1.2%)

[#] = due to medical reasons. Abbreviations: N/A = not available.

Discussion

This study shows that patients with *EGFR*-mutated NSCLC treated with chemotherapy after progression on EGFR-TKI have substantial benefit in terms of PFS and OS in a real world setting. We found a mPFS of 5.4 months for first-line chemotherapy after EGFR-TKI resistance, ranging from 2.8 to 5.9 months in the different chemotherapy cohorts. We observed no evidence of superiority of one particular first-line regimen in terms of survival or tumor response. However, the number of patients that were treated in the first-line with CPB or PB were too small to draw definite conclusions regarding the efficacy of those regimens in the first-line after EGFR-TKI failure. For subsequent lines

of chemotherapy, the PB regimen does seem to be a valid treatment option as the majority of patients receiving PB in our cohort, received this as a second- or later line of chemotherapy with a mPFS of 4.9 months. This is comparable with the reported mPFS of 5.4 months in the unselected NSCLC population of the ULTIMATE trial.[11] Next, we found a median OS of 15.3 months in our first-line cohort. Compared to the historical OS of 7-8 months for second-line docetaxel in unselected NSCLC [21, 22], our findings highlight that unselected treatment in the form of chemotherapy remains an important treatment option after progression on EGFR-TKI as it provides substantial survival benefit. The fact that the majority of our cohort received subsequent systemic treatment after the included line of chemotherapy, further illustrates this point. This also allows for potential re-treatment with EGFR-TKI after chemotherapy in selected cases.

Three other groups have also retrospectively compared the efficacy of different systemic cytotoxic regimens in patients with *EGFR*-mutated NSCLC after progression on EGFR-TKI (**Table 3**). Yu *et al.* compared chemo-anti-angiogenesis to chemo-immunotherapy in a two center cohort study in China and found comparable mPFS (p=0.552), but did not report OS.[16] However, the exact compounds of regimens were not specified, and the authors state that there was a lack of consistency in the prescribed drugs. In addition, the authors only included patients with at least one assessment of response, thereby excluding the patients with rapid clinical deterioration who were included in our study. White *et al.* reported a similar duration on treatment in those treated with chemotherapy alone, and chemotherapy in combination with pembrolizumab or bevacizumab. However, patients receiving chemo-pembrolizumab had significantly worse OS compared to the other regimens when adjusting for baseline ECOG PS and brain metastases.[17] In contrast, Chen *et al.* compared chemotherapy alone to chemotherapy plus pembrolizumab, and found that the addition of pembrolizumab was associated with improved PFS (hazard ratio (HR) 0.64 [95% CI 0.46-0.89], p=0.0076) and OS (HR 0.49 [95% CI 0.31-0.75], p=0.0052).[18] None of these studies evaluated the CPBA regimen.

Although chemotherapy in combination with immune checkpoint inhibitors or angiogenesis inhibitors, or both, have proven to be effective in the unselected NSCLC population[19, 23], clear evidence to prove their superiority over chemotherapy alone in *EGFR*-mutated NSCLC has not yet been provided. Therefore, the results of the CheckMate 722 trial (NCT02864251), in which chemo-nivolumab was compared with chemotherapy alone and with nivolumab-ipilimumab in patients with *EGFR*-mutated NSCLC after progression on EGFR-TKI, were eagerly awaited. However, the trial recently failed to meet its primary endpoint of improved PFS in the chemo-nivolumab group, although a trend for improved PFS was seen in the subgroup with sensitizing mutations and one prior line of EGFR-TKI.[12] Next, the wait is for the results of the KEYNOTE-789 trial (NCT03515837), that is evaluating chemo-pembrolizumab versus chemo-placebo in *EGFR*-mutated NSCLC after progression on EGFR-TKI.[13]

Table 3. Retrospective cohort studies investigating first-line chemotherapy regimens after EGFR-TKI failure.

Study	Cohorts	*N*=	ORR	PFS (m)	DOT (m)	OS (m)
Yu *et al.*[15]	Chemo-IO	44	29.5%*	7.59 NS	NR	NR
China	Chemo-AA	100	13.0%	6.9	NR	NR
White *et al.*[16]	Chemo	57	NR	NR	5.03 NS	12
USA	Chemo-IO	12	NR	NR	5.22 NS	10.9*
	Chemo-AA	35	NR	NR	6.01 NS	15.2
Chen *et al.*[17]	Chemo	82	20.7% NS	4.2*	NR	13.4#
China	Chemo-IO	82	34.1%	6.7	NR	26.7
Steendam *et al.*	PP	76	43.4%	5.2	NR	15.4
First-line cohort	CPBA	22	63.6%	5.9	NR	11.1
Europe	PB	4	50.0%	3.3	NR	5.3
	CPB	4	75.0%	2.8	NR	8.4

* = significant difference, # = data was immature at time of analysis. *Abbreviations: ORR = overall respons rate, m = months, PFS = progression free survival, DOT = duration on treatment, OS = overall survival, IO = immune-oncology, AA = anti-angiogenesis, NS = not significant, NR = not reported, bev = bevacizumab, PP = platinum/pemetrexed, CPB = carboplatin/paclitaxel/bevacizumab, CPBA = carboplatin/paclitaxel/bevacizumab/atezolizumab, PB = paclitaxel/bevacizumab*

Evidence of the effectiveness of adding checkpoint inhibitors in combination with anti-angiogenic agents to the chemotherapy backbone after TKI failure also remains limited. The IMpower150 investigated the addition of bevacizumab with or without atezolizumab to carboplatin-paclitaxel in chemotherapy-naïve non-squamous NSCLC, including those with *EGFR* mutations.[19] Their final exploratory analysis showed an improved mOS for CPBA compared to CPB in patients with sensitizing *EGFR*-mutations who received prior TKI treatment before inclusion (27.8 months [95% CI 18.6 – 41.4] versus 18.1 [95% CI 12.3 – 27.8], HR 0.74 [95% CI: 0.38 – 1.46]), however this did not meet statistical significance.[14] Based on these results the EMA granted this regimen approval for metastatic non-squamous NSCLC, including those with *EGFR* and *ALK* alterations after targeted treatment failure.[19] However, the FDA has only approved CPBA for metastatic non-squamous NSCLC without *EGFR* or *ALK* alterations, which explains the limited availability of real-world data of this regimen in the *EGFR*–mutated population. After EMA approval, CPBA could be prescribed in the Netherlands from 2019 onwards. In our cohort 32 patients were treated with CPBA, of which 22 patients received this regimen in the first-line after TKI failure. The results of our first-line CPBA cohort were not able to confirm the impressive OS benefit of the IMpower150 trial. However, there are several limitations of the IMpower150 exploratory analysis that could explain the large difference in the OS in their trial, and our real-world OS. First, the exploratory analysis of the *EGFR*-mutated subgroup was not adequately powered

for statistical testing as only 78 patients with *EGFR*-mutated NSCLC had received TKI treatment prior to inclusion (22 in the CPBA group, 28 in the CPA and 28 in CPB). Of note, the majority had received first- or second-generation EGFR-TKIs, and only one patient in the ABCP group received osimertinib, whereas the majority of our cohort had received prior osimertinib. Additionally, the IMpower150 trial excluded patients with untreated CNS metastasis, and the number of included patients with CNS metastases were not presented, whereas 43.5% of our first-line CPBA cohort had known CNS metastases. Itchins *et al*. also retrospectively investigated the IMpower150 regimen in an Australian pre-treated population. Within their cohort, 64 patients with sensitizing *EGFR* mutations were included, of which 57 had received previous TKI treatment. However, 42% of their cohort received a carboplatin/pemetrexed backbone instead of carboplatin/paclitaxel, and in half of the cases a lower chemotherapy dosage was used than in the IMpower150 trial. They found a median time to treatment failure of 5.2 months, and mOS of 10.5 months, which is in line with our findings of a mPFS of 5.9 months (95% CI 3.8-8.0) and mOS of 11.1 months (95% CI 3.4-18.8) in our first-line CPBA cohort.

So although at first analysis of the IMpower150 trial this regimen seemed to be potentially practice changing for *EGFR*-mutated NSCLC, the real-world data is less impressive. Nevertheless, there remains a substantial period of disease control even in the real-world setting of our study. Additionally, the IMpower150 trial generated a clinical rationale for further exploration of VEGF inhibition and immunotherapy in *EGFR*-mutated NSCLC after TKI failure. For instance, the combination of sintilimab, a PD-1 inhibitor, plus IBI305, a bevacizumab biosimilar, and chemotherapy after EGFR-TKI failure is currently under investigation in the randomized double-blind phase 3 ORIENT-31 trial (NCT03802240). Their first planned interim analysis showed that PFS was significantly longer in those receiving sintilimab-IBI305-chemotherapy versus chemotherapy alone (6.9 months [95% CI 6.0-9.3] versus 4.3 months [4.1-5.4], HR 0.46 [95% CI 0.34-0.64], $p<0.0001$).[15] Given the pre-clinical evidence of common downstream signaling shared by VEGF and EGFR pathways, there is also a biological rationale for the addition of anti-angiogenic agents to the chemotherapy backbone in this patient population.[24] VEGF has also been suggested as an immunomodulator, and thus VEGF inhibition could potentially reverse its immunosuppressive function on the tumor micro-environment. In turn, this makes the tumor more susceptible to immunotherapy.[25] However, the question remains how to properly select *EGFR*-mutated tumors that will respond to immunotherapy, and thus have the potential benefit of the addition of anti-angiogenic agents. This is of special importance in cases in which osimertinib re-treatment after chemotherapy is considered, as immunotherapy followed by osimertinib treatment has been associated with severe immune-related adverse events, such as pneumonitis.[26 27] Additionally, in some cases concomitant osimertinib treatment during chemotherapy is considered to provide greater CNS control. In those cases chemotherapy regimens combined with immunotherapy are less suitable as there is a higher chance of serious toxicities.

Our study has its limitations, the most important are the limited number of patients, the retrospective setting and the heterogeneous composition of the chemotherapy regimen cohorts. The lack of randomization increases the risk of selection bias in deciding which chemotherapy regimen was chosen. Nevertheless, as evidence on the most effective first-line chemotherapy regimen after progression on EGFR-TKI remains inconclusive, ongoing efforts to shed light on this question remain important while awaiting more informative prospective trials. Additionally, the large variety in treatment regimens worldwide hampers translatability of previous retrospective studies to daily clinical practice in several countries, which highlights the importance of adding our European cohort to this body of evidence.

Conclusion

Patients with locally advanced or metastatic *EGFR*-mutated NSCLC with progressive disease after treatment with EGFR-TKI have benefit in PFS and a substantial survival when treated with chemotherapy, especially in first-line treatment with platinum/pemetrexed or carboplatin/paclitaxel/bevacizumab/atezolizumab. The paclitaxel/bevacizumab regimen also showed a substantial PFS in further lines of therapy, providing a rationale for this regimen in patients who have already received chemotherapy, and especially in cases with previous lack of efficacy of platinum/pemetrexed.

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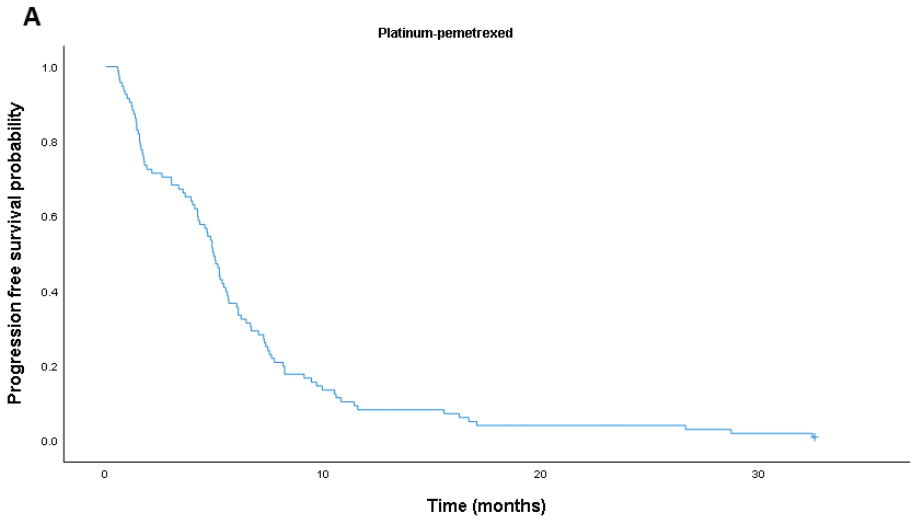
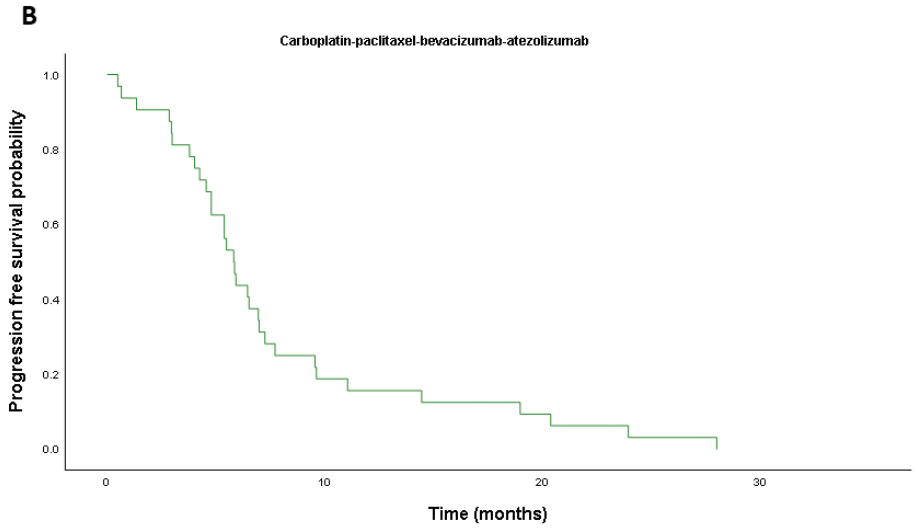
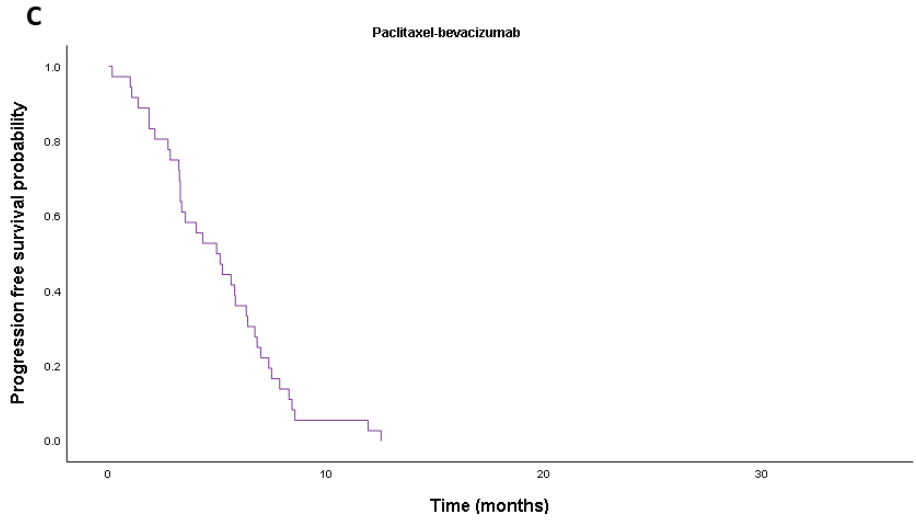
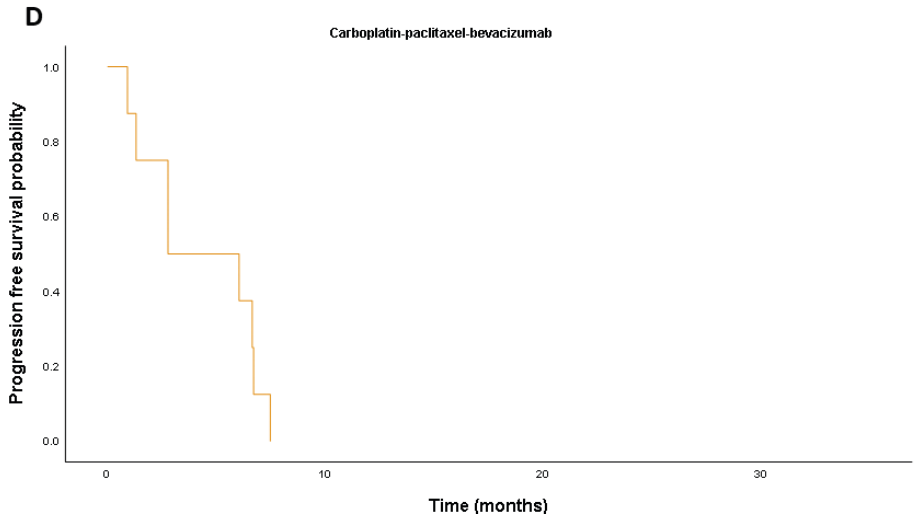
Supplementary Figure S1. Kaplan-Meier estimates of progression free survival for (A) platinum-pemetrexed, (B) carboplatin-paclitaxel-bevacizumab-atezolizumab, (C) paclitaxel-bevacizumab and (D) carboplatin-paclitaxel-bevacizumab, irrespective of treatment line.






C
Paclitaxel-bevacizumab
Progression free survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (months)


D
Carboplatin-paclitaxel-bevacizumab
Progression free survival probability
1.0
0.8
0.6
0.4
0.2
0.0
0
10
20
30
Time (months)

Supplementary Data 1. Specification of 'other' *EGFR* aberrations.

First-line cohort

Ex18 p.G719A
Ex18 p.G719A
Ex18 p.L707F + ex19 p.S752_I759del
Ex18 p.G719S + ex20 p.S768I
Ex18 p.G719A + ex21 p.L861Q
Ex18 p.G719A + ex21 p.L861Q
Ex19 p.Ile740_Lys745inframe_insertion
Ex20 p.R776H + ex21 p.L858R
Ex20 p.S768I + ex21 p.L861Q
Ex21 p.L861Q
Ex21 p.L861Q
Ex21 p.L861Q
Ex20 p.S768I

Platinum-pemetrexed

Ex18 p.G719A
Ex18 p.G719S + ex20 p.S768I
Ex18 p.G719A + ex21 p.L861Q
Ex18 p.G719A + ex21 p.L861Q
Ex20 p.S768I
Ex20 p.R776H + ex21 p.L858R
Ex20 p.S768I + ex21 p.L861Q
Ex21 p.L861Q
Ex21 p.L861Q
Ex21 p.L861Q

Carboplatin-paclitaxel-bevacizumab-atezolizumab

Ex18 p.G719A
Ex18 p.L707F + ex19 p.S752_I759del
Ex19 p.Ile740_Lys745inframe_insertion
Ex21 p.L861Q
Ex21 p.L861Q
Ex23 p.R932H

Paclitaxel-bevacizumab

Ex18 p.G719A + ex21 p.L861Q
Ex19 p.L747P
Ex21 p.L861Q

Carboplatin-paclitaxel-bevacizumab

N/A

Supplementary Table S1. Baseline characteristics of entire cohort.

Characteristic, *n* (%)	**PP** *n*=95	**CPBA** *n*=32	**PB** *n*=36	**CPB** *n*=8
Sex				
Male	33 (34.7%)	12 (37.5%)	8 (22.2%)	3 (37.5%)
Female	62 (65.3%)	20 (62.5%)	28 (77.8%)	5 (62.5%)
Age (median, range)	**63 (33-82)**	**62 (37-76)**	**64 (39-80)**	**53 (39-75)**
Tobacco exposure				
Current	3 (3.2%)	0 (0%)	3 (8.3%)	0 (0%)
Former	40 (42.1%)	14 (43.7%)	15 (41.7%)	3 (37.5%)
Never	51 (53.6%)	18 (56.3%)	18 (50.0%)	5 (62.5%)
Unknown	1 (1.1%)	0 (0%)	0 (0%)	0 (0%)
WHO PS				
0	23 (24.2%)	3 (9.4%)	0 (0%)	2 (25.0%)
1	52 (54.7%)	27 (84.4%)	28 (77.8%)	3 (37.5%)
2	10 (10.5%)	1 (3.1%)	4 (11.1%)	2 (25.0%)
3	1 (1.1%)	0 (0%)	3 (8.3%)	0 (0%)
4	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)
Unknown	9 (9.5%)	1 (3.1%)	1 (2.8%)	0 (0%)
CNS metastasis				
Yes	30 (31.6%)	12 (37.5%)	20 (55.6%)	4 (50.0%)
No	65 (68.4%)	20 (62.5%)	16 (44.4%)	4 (50.0%)
Type *EGFR* aberration				
Exon 19 deletion	48 (50.5%)	18 (56.2%)	22 (61.1%)	6 (75.0%)
Exon 21 p.L858R	32 (33.7%)	6 (18.8%)	8 (22.3%)	0 (0%)
Exon 20 insertion	5 (5.3%)	2 (6.2%)	3 (8.3%)	2 (25.0%)
Other*	10 (10.5%)	6 (18.8%)	3 (8.3%)	0 (0%)
Concomitant *TP53* mutation				
Yes	63 (66.3%)	21 (65.6%)	29 (80.5%)	5 (62.5%)
No	27 (28.4%)	11 (34.4%)	6 (16.7%)	3 (37.5%)
Unknown	5 (5.3%)	0 (0%)	1 (2.8%)	0 (0%)
Line of chemotherapy				
1	76 (80.0%)	23 (71.9%)	4 (11.1%)	4 (50.0%)
2	15 (15.7%)	9 (28.1%)	27 (75.0%)	3 (37.5%)
3	3 (3.2%)	0 (0%)	3 (8.3%)	0 (0%)
≥4	1 (1.1%)	0 (0%)	2 (5.6%)	1 (12.5%)

*Abbreviations: PS = Performance Score. CNS = Central Nervous System. * = other aberrations are specified in **Supplementary Data 1**.*

Part B

Focus on Plasma

Chapter 5

Plasma cell-free DNA testing of patients with EGFR mutant non–small-cell lung cancer: droplet digital PCR versus next-generation sequencing compared with tissue-based results

JCO Precis Oncol. 2019 Dec;3:1-9

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Abstract

Purpose:

To compare the results of plasma cell-free DNA (cfDNA) droplet digital PCR (ddPCR) and next-generation sequencing (NGS) on detection of epidermal growth factor receptor (*EGFR*) primary activating mutations and p.T790M with results of tissue analysis in patients with *EGFR* mutated non–small-cell lung cancer.

Methods:

All patients with *EGFR* mutated non–small cell lung cancer for which a pathology and a plasma specimen were available upon progression between November 2016 and July 2018 were selected. Concordance, Cohen's κ, and intraclass correlation coefficients were calculated.

Results:

Plasma cfDNA and pathology specimens of 36 patients were analyzed. Agreement between ddPCR and NGS was 86% (κ = 0.63) for the primary activating mutation and 94% (κ = 0.89) for the p.T790M detection. Allele ratios were comparable, with an intraclass correlation coefficient of 0.992 and 0.997, respectively. Discrepancies of some degree were found in 15 patients (41.7%). In six patients (16.7%), no mutations were detected in cfDNA. In three patients (8.3%), p.T790M was detected in plasma but not in the pathology specimen, whereas in three other patients (8.3%), p.T790M was demonstrated in the pathology specimen but not in plasma. Concordance of cfDNA and pathology for the primary activating mutation was 69% for ddPCR and 83% for NGS. For the detection of p.T790M, this was 75% (κ = 0.49) for ddPCR as well as for NGS.

Conclusion:

Mutual agreement is high between NGS and ddPCR in cfDNA on the level of a specific mutation, with comparable ratio results. Plasma testing of *EGFR* primary activating mutations and p.T790M shows high concordance with pathology results, for NGS as well as for ddPCR, depending on the extent of the panel used. In NGS, more genetic aberrations can be investigated at once.

Introduction

Among cancer deaths worldwide, non–small-cell lung cancer (NSCLC) is the leading cause.[1] The survival of metastasized disease is poor as illustrated by a 1-year survival rate of 23% in the Netherlands between 2010 and 2015.[2] The choice of palliative systemic treatment currently depends on histologic subtype, programmed cell death ligand 1 (PD-L1) expression, and the presence of specific genetic aberrations (also known as driver mutations) for which specific targeted therapies are available.[3] Today, for nonsquamous NSCLC, it is common practice to perform molecular analysis on a tissue biopsy specimen at the time of diagnosis.[3]

Targeted therapies have been developed and registered for treating NSCLC on the basis of the presence of genetic alterations in an expanding number of genes. The most common examples are activating mutations in the genes for epidermal growth factor receptor (*EGFR*) and B-raf proto-oncogene (*BRAF*) and translocations of the anaplastic lymphoma kinase and ROS proto-oncogene 1 genes.[4]

The population with *EGFR* mutated NSCLC is the most comprehensive of these patient groups, with an incidence of at least 10% in the white and up to 35% in the Asian population.[5] Clinical trials have shown high response rates (approximately 70%) and prolonged progression-free survival (PFS) rates up to 1 year on average to first-generation tyrosine kinase inhibitors (TKIs).[6] All patients, however, ultimately develop resistance to treatment with TKIs and show progression of disease at some point. There are two main mechanisms of acquired resistance: Pharmacologic (eg, problems with compliance, dose reductions, reduced absorption or increased metabolism, inadequate CNS penetration) and biologic (eg, altered drug target, bypass tracks, phenotypic change, downstream signaling pathways).[7]

The gatekeeper mutation p.T790M in *EGFR* exon 20 is the most common resistance mechanism to first- and second-generation TKIs (erlotinib, gefitinib, and afatinib) in NSCLC with an activating *EGFR* mutation and occurs in more than 50% of patients.[7] Because the availability of osimertinib, a drug that overcomes the p.T790M resistance mechanism that shows high response rates and a substantial median PFS of 8 months, the detection of this gatekeeper mutation has been of utmost importance.[8,9] In addition, there are other known resistance mechanisms for which targeted therapies are available in research or off-label settings. Therefore, it is strongly advised to obtain a new molecular analysis at the time of progression on first-line EGFR TKIs.[3]

Although a tissue biopsy is still considered the gold standard for diagnosis of NSCLC, the potential to detect genetic aberrations in the blood, which is often referred to as liquid biopsy, has specific advantages over a tissue biopsy in that it is easier to obtain and has a lower patient burden.[10] Currently, the use of plasma detection of p.T790M

at the time of progression on first-line EGFR TKIs is widely accepted, and prescription of osimertinib is established on the basis of *EGFR* p.T790M detection in plasma.[11,12]

Several mutation detection techniques are under investigation for application in clinical practice in which a very low detection limit is essential because the amount of circulating cell-free tumor DNA (ctDNA) in the total of cell-free DNA (cfDNA) can be very low. Traditional polymerase chain reaction (PCR) is not sensitive enough to detect these low amounts of tumor DNA. Real-time PCR slightly improves the detection limit (eg, Cobas [Roche, Basel, Switzerland], Therascreen [QIAGEN, Valencia, CA]), but a major improvement in sensitivity was achieved by the development of digital platforms that target specific mutations like droplet digital PCR (ddPCR) and beads, emulsion, amplification, and magnetics digital PCR.[13] This requires a modest amount of time and cfDNA to obtain reliable results. A more broad (untargeted) approach is represented by next-generation sequencing (NGS). A lot of effort was invested in optimizing NGS for use on cfDNA, with adjusted amplicon sizes for amplification of smaller DNA fragments and application of molecular barcodes to recognize the needle in the haystack in low concentrations of ctDNA in the total amount of cfDNA.[14] For optimal results, it is advised to use as much cfDNA in the panel as possible. Depending on the platform used, the lead time requires several working days, which is comparable to NGS on tissue specimens.

This study compares the results of ddPCR (Bio-Rad Laboratories, Hercules, CA) and NGS (Ion Torrent, Thermo Fisher Scientific, Waltham, MA) for detection of primary activating and resistance p.T790M *EGFR* mutations in plasma-derived cfDNA. Outcomes are compared with NGS results of conventional tissue biopsy or cytology.

Methods

We included all patients with *EGFR* mutated NSCLC with progression on current therapy for which a tissue specimen (histology/cytology) was available in the same time frame and line of treatment as plasma analysis. The study was conducted at Erasmus MC Cancer Institute between November 2016 and July 2018. The maximum time frame between plasma and tissue collection was limited to 3 months.

Plasma samples were collected and cfDNA analyses performed upon progression on current therapy for detection of primary activating and p.T790M *EGFR* mutations. We prospectively collected all data on requested plasma analyses.

cfDNA Isolation

Blood was collected in 10-mL CellSave Preservative Tubes (CellSearch, Menarini Silicon Biosystems, Castel Maggiore, Italy) and centrifuged for 20 minutes at 1,600 × *g*. Plasma samples were stored at −80°C until cfDNA isolation. Before extraction, samples were centrifuged for 10 minutes at 10,000 × *g*. The cfDNA was extracted using

the QIAmp Circulating Nucleic Acid Kit (QIAGEN) from 3 mL of plasma according to the manufacturer's protocol. The DNA was eluted in 50 µL of buffer.

ddPCR Analysis

The actual analysis of *EGFR* activating (exon 19 deletions and p.L858R) and resistance (p.T790M) mutations was performed using ddPCR mutation assays (Bio-Rad Laboratories) as previously described.[15]

NGS on Pathology Specimens and cfDNA

DNA was isolated from formalin-fixed paraffin-embedded tissues enriched for neoplastic cells by manual microdissection as previously described.[16] NGS analysis was performed by semiconductor sequencing with the Ion S5 System (Thermo Fisher Scientific) with the supplier's materials and protocols. Library preparation was performed with 1 to 10 ng of tissue DNA and 4 to 50 ng of cfDNA, depending on the amount of tissue or cfDNA available. Libraries of tissue DNA were prepared with a custom-made primer panel that encompassed, among others, *EGFR* exons 18 to 21, *KRAS* exons 2 to 4, *ERBB2* exons 19 to 21, *BRAF* exons 11 and 15, and the entire coding region of *TP53* using the AmpliSeq Library Kit 2.0-384 LV (Thermo Fisher Scientific); cfDNA library preparation was performed using the Oncomine Lung cfDNA Assay (Thermo Fisher Scientific). Templates were prepared using the Ion 520 & Ion 530 Kit-Chef and sequenced with the Ion S5 Sequencing Kit on an Ion 530 Chip (Thermo Fisher Scientific). Sequence data were analyzed with Variant Caller version 5.6.0.4 (Thermo Fisher Scientific). Variants detected in tissue samples were annotated by SeqNext version 4.2.2 build 503 software (JSI Medical Systems, Kippenheim, Germany). Results are reported as allele ratios (mutated alleles / [mutated + wild-type alleles] × 100%) in the case of at least three positive droplets (ddPCR) or three unique molecules (NGS).

Statistical Analysis

Concordance of ddPCR and NGS with tissue-based results was calculated for the primary activating mutation. Cohen's κ was calculated to evaluate the agreement between ddPCR or NGS and tissue-based results for p.T790M detection and between ddPCR and NGS on cfDNA for the primary activating mutation as well as for p.T790M. Intraclass correlation coefficients were calculated for the ratios of ddPCR and NGS for p.T790M as well as for the primary activating mutation when applicable.

Results

Between November 2016 and July 2018, 162 patients underwent cfDNA analysis on plasma collected in 10-mL CellSave Preservative Tubes. We selected all 36 patients with *EGFR* mutated NSCLC with progression on current treatment of which a histology or cytology specimen was available in the same time frame and line of treatment. Baseline characteristics of the population are listed in **Table 1**.

Table 1. Baseline characteristics

Characteristic	Patients, No. (%) N=36
No. of patients	**36**
Age (mean)	66 years (range 45-85)
Sex	
Male	12 (33.3%)
Female	24 (66.7%)
Smoking status	
Never	15 (41.7%)
Former	9 (25%)
Current	3 (8.3%)
Unknown	9 (25%)
Packyears	
0	15 (41.7%)
1-15	4 (11.1%)
15-30	2 (5.6%)
>30	2 (5.6%)
Unknown	13 (36%)
Activating EGFR mutation	
Exon 18	2 (5.6%)
Exon 19	24 (66.7%)
Exon 21 p.L858R	9 (25%)
Exon 21 other	1 (2.8%)
Lines of therapy	
Mean before PA specimen (range)	1,6 (1-5)
1	25 (69.4%)
2	5 (13.9%)
3	3 (8.3%)
4	1 (2.8%)
5	2 (5.6%)
Previous chemotherapy	
Yes	8 (22.2%)
No	28 (77.8)
Current therapy at time PA specimen	
Erlotinib	24 (66.7%)
Gefitinib	4 (11.1%)
Osimertinib	6 (16.7%)

Table 1. Continued

Characteristic	Patients, No. (%) N=36
Chemotherapy	1 (2.8%)
PD-1 inhibitor	1 (2.8%)
Type of PA specimen	
Histology	29 (80.6%)
Cytology	7 (19.4%)

Abbreviations: EGFR, epidermal growth factor receptor; PA, primary activating; PD-1, programmed cell death 1.

Results of Plasma Analyses

Agreement between ddPCR and NGS was 94% (κ = 0.89) for *EGFR* p.T790M detection in plasma and 86% (κ = 0.63) for detection of the primary activating *EGFR* mutation. The quantification in allele ratio (mutant / [mutant + wild type]) proved highly similar for both techniques (Fig 1), with an intraclass correlation coefficient of 0.997 and 0.992, respectively. Discrepant results were found in 15 patients (41.7%). **Table 2** lists results of plasma and tissue analyses for all patients.

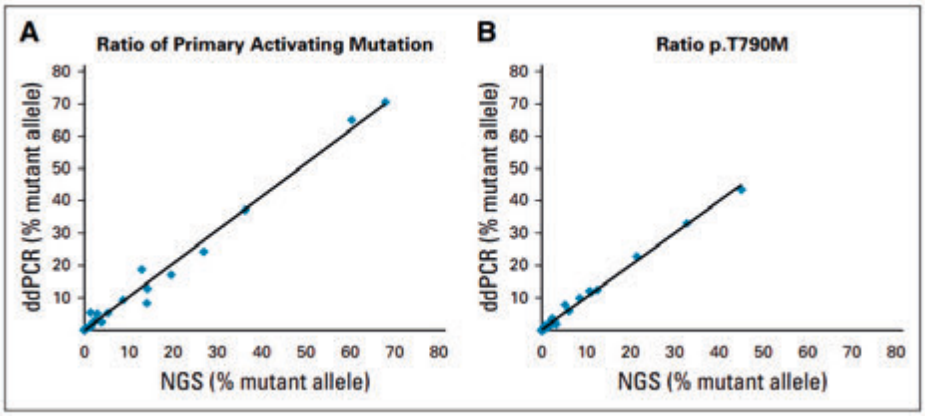

Figure 1. *Correlation between the ratio of mutant versus total cell-free DNA of next-generation sequencing (NGS) and droplet digital polymerase chain reaction (ddPCR) results. (A) The primary activation mutation (n=26 because of missing mutations in the ddPCR panel and nonquantifiable NGS results). (B) p.T790M (n=36).*

In six patients (16.7%), the primary activating mutation was a less common variant that was not present in the current ddPCR panel. Therefore, these mutations could not be detected in the plasma by ddPCR. In two of these patients, the primary activating mutation also was not detected by NGS, one of them was not present in the NGS panel. In one other patient, the primary activating mutation was not detected by ddPCR

(although present in the panel) but was shown in plasma by NGS detection. At the level of p.T790M detection, better agreement was shown, with only one patient in whom p.T790M was detected by NGS but not by ddPCR.

Comparison With Tissue Results

For NGS, concordance for the primary activating *EGFR* mutation with tissue specimens was 83% (30 of 36 confirmed results). For the mutations that could be detected by the ddPCR panel (shared mutations with the cfDNA NGS panel), 83% were confirmed. However, because of the limitation of checking only p.L858R and exon 19 deletion, the concordance of detection of all *EGFR* activating mutations (also including nonshared mutations) was 69% (25 of 36) compared with NGS-obtained tissue-based results. For *EGFR* p.T790M detection, both ddPCR and NGS showed a concordance of 75% ($\kappa = 0.49$) with the tissue-based results.

In six patients (16.7%), all with intrathoracic progression, no mutations were detected in cfDNA (mutation-negative plasma), whereas the tissue analysis showed the presence of *EGFR* p.T790M in three (50%) of these patients. The best response on initiated osimertinib treatment in these three patients was partial response in one and stable disease in two.

In three patients (8.3%), cfDNA analysis detected p.T790M, which was not demonstrated in the tissue specimen (ddPCR positive in two patients, NGS positive in three patients). In two of these patients, there was an evident extrathoracic progression site. Upon treatment with osimertinib, there was progressive disease as best response in two of these patients (one of whom had an additional *PIK3CA* mutation that was detected in the corresponding tumor tissue); the other patient had already received osimertinib treatment, and the additional clinical course was unknown because treatment was coordinated in another hospital.

In three other patients (8.3%), p.T790M was detected in tumor tissue but not in cfDNA, whereas the primary activating *EGFR* mutation was detected in the plasma. These patients all had intrathoracic progression and/or a new CNS localization. One patient showed stable disease as best response to osimertinib, another died before subsequent therapy could be initiated, and the clinical course of the third patient remained unknown because he was treated elsewhere.

Table 2. Patients and Results

Case	Age	Smoking status	Activating mutation EGFR	Drug during biopsy	Activating mutation EGFR			p.T790M EGFR exon 21			Other findings		
					PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS
1	78	Unknown	Ex19del	Erlotinib	51%	9,3%	8,7%	-	-	-	TP53 p.R273C 51%, MET amplification	-	TP53 p.R273C 3,59%
2	64	Never	Ex21 p.L858R	Gefitinib	38%	5,3%	1,36%	13%	2%	2,07%	SMAD4 p.R361H 12%	-	-
3	61	Never	Ex19del	Osimertinib	82%	13,3%	+(nq)	75%	12,1%	10,77%	EGFR p.C797S 76%, TP53 p.R213X 42%	EGFR p.C797S 9%	EGFR p.C797S 10,57%
4	53	Former	Ex19del	Osimertinib	52%	8,3%	14,05%	11%	1,9%	3,25%	EGFR p.848L 41%, TP53 p.278R 39%	EGFR p.C797S 1,9%	EGFR p.C797S 0,41%
5	52	Never	Ex18 p.E709A + Ex18 p.G719S	Erlotinib	36% 36%	† †	1,87% 1,87%	‡	1,3%	0,46%	TP53 p.R175H 43%, PIK3CA p.E542K 28%	-	TP53 p.R175H 1,22%
6	64	Former	Ex19del	Erlotinib	80%	3,8%	+(nq)	61%	2,7%	2,78%	-	-	-
7	64	Unknown	Ex19del	Osimertinib	77%	2,5%	3,80%	-	-	-	TP53 p.Q165X 29%, FGFR1 p.N546K 48%	-	-
8	53	Former	Ex19del	Erlotinib	57%	2,6%	2,10%	19%	1,2%	0,69%	-	-	-
9	76	Former	Ex19del	Osimertinib	35%	0,21%	0,06%	‡	‡	0,69%	-	-	-

Table 2. Continued

Case	Age	Smoking status	Activating mutation EGFR	Drug during biopsy	Activating mutation EGFR			p.T790M EGFR exon 21			Other findings		
					PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS
10	66	Unknown	Ex18 p.G719S + Ex20 p.S768I	Osimertinib	58% 59%	† †	51,69 52,39%	14%	33%	32,73%	TP53 p.R249M 76%	-	TP53 p.R249M 51,60%
11	82	Never	Ex19del	Erlotinib	37%	48,9%	+(nq)	15%	12,4%	12,48%	TP53 c.673-1G>A 60%	-	-
12	52	Current	Ex21 p.L858R	Erlotinib	97%	18,7%	12,91%	0,10%	7,8%	5,21%	-	-	-
13	66	Former	Ex21 p.L858R	Erlotinib	53%	5,2%	5,26%	5%	1,8%	1,49%	-	-	-
14	59	Former	Ex19del	Erlotinib	38%	§	§	-	-	-	TP53 p.K132M 60%	-	-
15	61	Former	Ex19del	Erlotinib	46%	§	§	13%	‡	‡	TP53 c.980_981dup 60%	-	-
16	64	Never	Ex19del	Erlotinib	40%	§	§	-	-	-	HER2 amplification	-	-
17	65	Never	Ex19del	Erlotinib	96%	18%	+(nq)	8%	‡	‡	EGFR amplification	-	-
18	62	Never	Ex19del	Erlotinib	80%	§	§	26%	‡	‡	-	-	-
19	73	Former	Ex19delins	Erlotinib	58%	†	§	28%	‡	‡	-	-	-
20	79	Never	Ex19del	Erlotinib	25%	22,1%	+(nq)	9%	9,9%	8,52%	-	-	-

Table 2. Continued

Case	Age	Smoking status	Activating mutation EGFR	Drug during biopsy	Activating mutation EGFR			p.T790M EGFR exon 21			Other findings		
					PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS
21	73	Never	Ex19del	Erlotinib	36%	1%	1,39%	-	-	-	TP53 p.Q317* 33%	-	-
22	45	Unknown	Ex19del	Erlotinib	86%	5%	2,88%	41%	3,5%	2,33%	TP53 p.P177H 68%	-	-
23	76	Unknown	Ex21 p.L861Q	Erlotinib	68%	†	0,89%	-	-	-	TP53 c.96+1G>C 90%	-	-
24	77	Unknown	Ex19del	Gefitinib	45%	‡	0,23%	24%	‡	‡	-	-	-
25	76	Never	Ex19del	PD-1 inhibitor	89%	37,1%	36,25%	-	-	-	TP53 p.R273H 65%	-	TP53 p.R273H 2,40%
26	66	Never	Ex21 p.L858R	Chemotherapy	34%	1,05%	0,97%	18%	0,29%	0,48%	TP53 p.C135G 88%	-	-
27	73	Unknown	Ex19delins	Gefitinib	49%	†	0,20%	-	-	-	-	-	KRAS p.G13C 0,23%
28	62	Former	Ex19del	Erlotinib	77%	12,8%	14,20%	51%	11,7%	11,13%	TP53 c.560delG 79%	-	-
29	75	Unknown	Ex19del	Osimertinib	79%	64,9%	60,33%	65%	43,4%	45,07%	-	-	-
30	73	Current	Ex21 p.L858R	Gefitinib	44%	0,6%	0,16%	20%	‡	‡	-	-	-

Table 2. Continued

Case	Age	Smoking status	Activating mutation EGFR	Drug during biopsy	Activating mutation EGFR			p.T790M EGFR exon 21			Other findings		
					PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS	PA NGS	cfDNA ddPCR	cfDNA NGS
31	59	Never	Ex21 p.L858R	Erlotinib	65%	24,3%	26,91%	16%	5,8%	6,10%	TP53 c.673-1G>T 71%	-	-
32	85	Never	Ex21 p.L858R	Erlotinib	40%	2,70%	2,24%	‡	0,80%	1,43%	TP53 p.H214D 60%	-	-
33	50	Current	Ex21 p.L858R	Erlotinib	29%	1,20%	1,13%	-	-	-	EGFR ex20 p.S768I 38%, CTNNB1 p.G34V 26%	-	EGFR ex20 p.S768I 2,72%%
34	71	Never	Ex19delins	Erlotinib	26%	†	-	-	-	-	-	-	-
35	63	Unknown	Ex21 p.L858R	Erlotinib	93%	17%	19,55%	-	-	-	TP53 p.R158P 61%, MET amplification	-	TP53 p.R158P 0,68%,
36	58	Never	Ex19del	Erlotinib	+(nq)	70,60%	67,89%	+(nq)	22,8%	21,41%	-	-	-

Abbreviations: cfDNA, cell-free DNA; Chemo, chemotherapy; ddPCR, droplet digital polymerase chain reaction; EGFR, epidermal growth factor receptor; HER2, human epidermal growth factor receptor 2; MET, mesenchymal-epithelial transition factor; +(nq), not quantifiable; NGS, next-generation sequencing; PA, pathology specimen; PD-1, programmed cell death 1; Unk, unknown

†Mutation not in panel.

‡Discrepancy in results.

§No mutations detected in liquid biopsy

Discussion

This study compared two accurate and promising techniques for detection of targetable genetic aberrations in NSCLC in the same plasma samples and confirms the high concordance of cfDNA with tumor tissue analysis as published earlier.[13,17] We did not calculate negative predictive value or positive predictive value because we did not consider the tissue analysis as the gold standard; it is a known phenomenon that tumor heterogeneity might result in a mutation-negative biopsy specimen while a mutation can be present in another (metastasized) region. Our findings support this because three patients had p.T790M detected in plasma but not in tissue. We do not believe that they had false-positive findings but, rather, that the biopsy specimen did not represent the whole spectrum of genetic aberrations of the disease.

ddPCR is considered a highly sensitive PCR platform that, next to high concordance with tissue-based results, also offers high sensitivity and specificity compared with earlier PCR assays (Taqman PCR with peptide nucleic acid; Therascreen; Cobas; and beads, emulsion, amplification, and magnetics digital PCR).[12,18] NGS has proven its qualities in molecular analysis of tissue and has promising evolving capabilities for mutation analysis of cfDNA.[19-21] Both techniques yield results as a percent of the total (ratio), which is considered a benefit over methods that merely indicate positive or negative.

The moderate agreement between cfDNA and tissue-based results on p.T790M detection is partly the result of mutation-negative plasma samples. The six patients (16.7%) in whom no mutations were detected in plasma could reflect the limited sensitivity of cfDNA analysis in NSCLC, which was earlier reported to be approximately 60% to 80%.[22,23] This is defined not only by the platform limitations but also by the lack of shedding of tumor DNA in the circulation in some patients (eg, the patients in our study were all found to have intrathoracic progression) and the limitation of the volume taken for plasma analysis.[24,25] If the primary activating *EGFR* mutation is not detectable in the plasma and p.T790M is also not detected, the result is not conclusive and of no clinical use for determining additional treatment options. Therefore, tissue biopsy specimens are desirable to detect the resistance mechanism in such patients. The mutation-negative plasma subset has a substantial negative effect on the concordance between cfDNA and tissue biopsy results.

On the other hand, the three patients (8.3%) in whom p.T790M was detected in cfDNA but not in tumor tissue also contribute to the limitation of agreement. These three patients might represent the concept of tumor heterogeneity, where the location of the biopsy does not represent the full spectrum of genetic aberrations of the disease.[24,26] The p.T790M-positive cells might represent a subclone of limited extent, or another (not yet detected) alternative resistance mechanism might be of greater influence (illustrated by the patient with a *PIK3CA* mutation in the pathology specimen). The patients who

showed p.T790M in tissue analysis but not in plasma all had intrathoracic and/or CNS progression, which again supports the theory that those sites are associated with a lesser rate of shedding of tumor DNA into blood.[25]

The concordance between NGS and ddPCR in cfDNA of a specific genetic aberration that is targetable for ddPCR, like p.T790M, is higher than for the broader and heterogeneous group of activating *EGFR* mutations because not all activating *EGFR* mutations were present in the current ddPCR panel and, thus, will be missed. In such cases, it is unclear whether plasma is false negative for p.T790M because of limited sensitivity of ddPCR or true negative because of absence of ctDNA, as the primary activating *EGFR* mutation also could not be detected.

Because osimertinib showed improved PFS when used in first-line treatment in the AZD9291 Versus Gefitinib or Erlotinib in Patients With Locally Advanced or Metastatic NSCLC (FLAURA) trial[27] and registration for this indication by the Food and Drug Administration and European Medicines Agency is a fact, the expectation is that in the near future, most patients will be treated with osimertinib upfront, and p.T790M detection will be of lesser importance. However, mechanisms of acquired resistance on first-line osimertinib presented at the European Society for Medical Oncology 2018 Congress showed a shift toward more mesenchymal-epithelial transition factor amplifications (14%); some secondary *EGFR* mutations, like p.C797S (7%); and human epidermal growth factor receptor 2 amplifications (2%).[28] In this light, a broader approach to investigate the resistance mechanism upon progression seems desirable.

In practice, frequently, only a limited amount of material for DNA investigation is available, and this is also the case for blood samples. Because every primer combination in the ddPCR panel needs a new input of specimen, this is a disadvantage when looking for a resistance mechanism with a wide view. An advantage is the fast lead time because ddPCR can generate a quick answer for the clinician (ie, within 1 working day when needed).

NGS can explore a broad spectrum of genetic aberrations in a single run, and the possibilities to detect translocations and amplifications are expanding quickly. Thus, with the expanding knowledge of resistance mechanisms and possible targeted treatments (in development) for these, the detection of a broad set of genetic aberrations seems desirable. For example, a *BRAF* V600E mutation can appear next to the primary activating *EGFR* mutation for which a dabrafenib and trametinib combination can be added to the current treatment. On the other hand, NGS is more time consuming and still much more expensive than ddPCR. Both plasma-based approaches are limited by the fact that some resistance mechanisms need a tissue-based diagnosis (eg, transformation to small-cell lung cancer).

In conclusion, our study demonstrates that results of *EGFR* mutation detection in cfDNA by NGS and ddPCR are comparable, with a high agreement when the ratio of *EGFR* mutant alleles to wild-type alleles is compared. NGS was comparable with ddPCR in sensitivity for p.T790M detection. NGS performed better in detecting specific, sometimes previously unknown, genetic alterations because of the broader panel but at a higher cost. Our results confirm the ability to detect targetable aberrations in blood, which provides possibilities for new lines of targeted treatments in daily practice without the necessity of tissue procurement in many patients.

Support:

Supported in part by a grant from AstraZeneca.

Prior presentation:

Presented at the International Association for the Study of Lung Cancer 19th World Conference on Lung Cancer, Toronto, Ontario, Canada, September 23-26, 2018.

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Chapter 6

The clinical value of cell free DNA next generation sequencing in suspected metastatic lung cancer

Submitted

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Abstract

Objectives:

Although molecular analysis of tumor material is standard of care in patients with metastatic non-squamous non-small cell lung cancer, this is not always feasible. This leaves targeted-treatment options unidentified in a subset of patients. We investigated the clinical value of cell free DNA Next Generation Sequencing in suspected metastatic lung cancer.

Materials and Methods:

Erasmus MC launched project Lung Cancer Diagnosis – cell free DNA. Thoracic oncologists in the Comprehensive Cancer Network Southwest (The Netherlands) submitted plasma samples from patients with clinical evidence for metastasized lung cancer when molecular analysis of tumor tissue was not feasible. Plasma cfDNA Next Generation Sequencing was conducted. Results were discussed in the Thoracic Oncology Molecular Tumor Board.

Results:

Between January 1st 2019 and January 1st 2021, 55 plasma samples were submitted and analyzed. A potential target for treatment was identified in 7 patients (12.7%): 2 *EGFR* aberrations, 4 *KRAS* p.G12C and 1 activating *BRAF* mutation (p.G466V). In 21 samples other aberrations were detected, which currently did not affect the choice of systemic therapy (other *KRAS, TP53* and *PIK3CA* mutations).

Conclusion:

In the case that molecular analysis of tumor tissue is not possible at the time of diagnosis of metastatic lung cancer, NGS analysis of cfDNA in patient plasma samples provides an opportunity to detect genetic aberrations for subsequent targeted therapy.

Introduction

Patients diagnosed with locally advanced or metastatic non-small cell lung cancer (NSCLC) benefit in terms of improved overall survival when an oncogenic driver aberration is present that can be treated with a targeted agent.[1] For instance, in patients with activating Epidermal Growth Factor Receptor (*EGFR)* mutations, treated with osimertinib in first line, median overall survival improved to 38.6 months, and the 5-year OS for patients with Anaplastic Lymphoma Kinase (*ALK)* rearrangement treated with alectinib in first line was 62.5%.[2, 3] As expanding options for targeted therapy become available based on the molecular profile of the tumor, the genetic characterization of disease has gained considerable importance in recent years. In the diagnostic process of locally advanced or metastatic non-squamous NSCLC, molecular analysis is therefore standard-of-care.[4] Unfortunately, this is not always feasible. In daily practice it can be difficult to obtain tumor tissue or there may be insufficient tissue available for molecular investigation at the time of diagnosis. This leaves possible targeted-treatment options unidentified in a subset of patients, as has been shown for the Netherlands, but also for other countries in Europe and abroad.[5-7]

In recent years, it has been shown that plasma cell-free DNA (cfDNA) analysis can identify tumor mutations with high concordance with mutation analyses derived from tumor tissue.[8] Although shedding of cell-free tumor DNA (ctDNA) can be limited in cases with low tumor load, or solely intrathoracic or cerebral disease location, the majority of patients with an *EGFR* mutation and metastatic disease have the driver mutation detectable in plasma.[8, 9]

To improve the use of molecular testing in patients for whom adequate molecular analysis of tumor tissue is not possible, the Comprehensive Cancer Network Southwest in the Netherlands launched a project for plasma cfDNA analysis in order to identify additional patients who might benefit from targeted treatment.[10]

Materials and Methods

In 2019, Erasmus Medical Centre Cancer Institute launched the project Lung Cancer Diagnosis – cfDNA (LCD-cfDNA). Thoracic oncologists from hospitals in the Comprehensive Cancer Network Southwest (The Netherlands) were given the opportunity to submit plasma samples from patients in case adequate molecular analysis of tumor tissue was not possible, when there was confirmed or suspected metastatic lung cancer based on radiological and clinical findings. Our procedures regarding blood collection, cfDNA isolation and plasma Next Generation Sequencing (NGS) with Oncomine Lung cfDNA Assay v1 have been previously described.[8]

Results of plasma analysis were discussed in the Thoracic Oncology - Molecular Tumor Board (TO-MTB) and reported to the referring physician.

Medical ethical committee approval and informed consent was not required as molecular testing of plasma is considered standard-of-care in absence of possibility of molecular analysis of tissue.[11] Only descriptive and anonymized data were used in line with the EU General Data Protection Regulation.

We checked the availability of molecular analysis of tumor tissue for the same patient to correlate with plasma results. This was achieved by consulting the Laboratory Management System (LMS) which is connected to the Pathological National Automated Archive (PALGA), and by retrospectively checking the available general pathology results in the patient records in July 2021.

Results

Between January 1st 2019 and January 1st 2021, plasma samples from 55 patients were submitted and analyzed. Patient characteristics are shown in **Table 1**. Compared to the characteristics of the patients diagnosed with stage IV lung cancer in 2019 in the Netherlands, our population included slightly more women and more patients with poorer performance scores.[12]

Table 1. Baseline characteristics

	***n*=55**	**NL, *n*=14671[12]**
SEX		
male	23 (42%)	56%
female	32 (58%)	44%
AGE		
WHO PS	**72 (50-87)**	**69% ≥65 year**
0	6 (11%)	20%
1	15 (27%)	29%
≥2	25 (45%)	24%
unknown	9 (16%)	26%
SMOKING STATUS		
never	6 (11%)	NR
former	25 (46%)	
current	7 (13%)	
unknown	17 (30%)	

Table 1. Continued

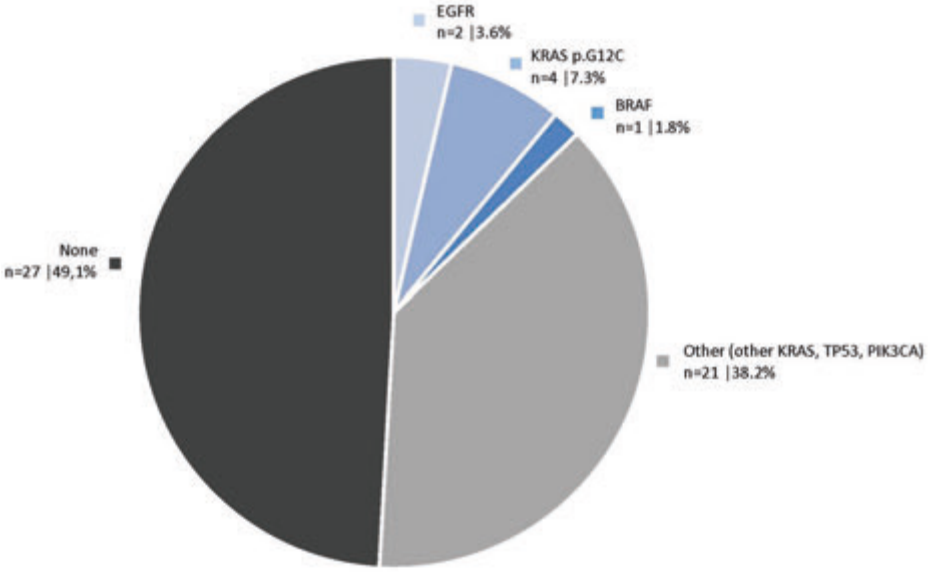
	***n*=55**	**NL, *n*=14671[12]**
TISSUE CONFIRMED NSCLC		
yes	13 (24%)	
no	42 (76%)	
REASON PLASMA ANALYSIS		
lesions unreachable for biopsy	6 (11%)	
obtained material unsuitable/insufficient for molecular analysis	32 (58%)	
patient and/or physician refused biopsy for other (medical) reasons	17 (31%)	

Legend: NL = the Netherlands; WHO PS = World Health Organization Performance Score; NR = not reported

Referring physicians reported the reason for plasma analysis as shown in **Table 1**. In 42 patients (76%) there was no lung cancer tissue diagnosis available at the time of plasma analysis. More detailed information is available in the **Supplementary Data**.

Detected aberrations in plasma

In total, in 28 of the 55 samples (50.9%) a genetic aberration was detected, as shown in **Figure 1**. Two samples (3.6%) showed an activating *EGFR* aberration. One patient, with an *EGFR* exon 19 deletion, was started on EGFR-TKI based on the plasma cfDNA analysis alone. In the plasma of the other patient an exon 20 insertion was detected. However, this patient was hesitant of participation in a clinical trial, as exon 20 insertions are not sensitive to regular EGFR-TKI. In 4 cases (7.3%), a *KRAS* p.G12C was detected. An activating *BRAF* mutation (p.G466V) was identified in plasma in 1 patient (1.8%). In 21 samples (38.2%) other aberrations were detected in *KRAS* (other than p.G12C), *TP53* and *PIK3CA*. In 10 patients (18.2%), multiple mutations were detected in plasma. In 27 cases (49.1%), no mutations were identified.



A

B

Pt	Mutation
1	**EGFR p.E746_A750del (0.63%)**
2	**EGFR p.P770_V771insG (0.12%);** TP53 p.R209Qfs*7 (0.21%)
3	**BRAF p.G466V (9.01%);** TP53 p.E339* (10.31%)
4	**KRAS p.G12C (0.86%)**
5	**KRAS p.G12C (1.39%);** TP53 p.R267W (0.91%)
6	**KRAS p.G12C (0.6%);** KRAS p.Q61H (0.76%); TP53 p.R202C (0.1%)
7	**KRAS p.G12C (1.92%)**
8	KRAS p.G12D (0.06%)
9	KRAS p.G12D (0.54%); PIK3CA p.E545K (0.23%)
10	KRAS p.G12V (5.44%); TP53 p.C275F (0.09%)
11	KRAS p.G12V (0.60%)
12	TP53 p.G244V (0.13%)
13	TP53 p.R283C (0.08%)

Pt	Mutation
14	TP53 p.Y205* (27.7%); PIK3CA p.E545K (0.06%)
15	TP53 p.G266V (0.67%)
16	TP53 p.Y234C (0.38%); TP53 c.672+1G>T; p.? (10.46%)
17	TP53 p.R249S (0.05%)
18	TP53 p.R158H (4.44%)
19	TP53 p.S215R (0.24%)
20	TP53 p.P278L (7.48%)
21	TP53 p.R283H (0.10%)
22	TP53 p.P278S (1.73%); TP53 p.G279E (0.37%); TP53 c.375+3_375+4insG; p.? (0.16%)
23	TP53 p.R267Q (0.24%)
24	TP53 p.C238F (0.86%); TP53 p.C275S (0.13%)
25	TP53 p.Y163C (54.77%)
26	TP53 p.R248W (0.34%)
27	TP53 p.S241F (3.49%)
28	TP53 p.V272G (0.17%)

Figure 1. *Detected aberrations in plasma.*
Figure 1A: Display of the ratio of detected possible targets for treatment in blue versus no target for treatment (at this moment) in grey tones. Figure 1B: Detected aberrations in plasma per case, possible targets for treatment in bold.

Correlation of plasma and tumor tissue results

The PALGA and record search revealed that in 12 out of 55 patients, tumor tissue was obtained at a later time point during the course of disease, and 1 patient had tissue results available from another entity in the past (bladder cancer). The aberration detected in plasma was confirmed in tissue in 2 cases.

In 4 patients, tissue pathology resulted in a different diagnosis than NSCLC (namely SCLC in case 37, metastasized ovarian cancer in case 12, metastasized pancreatic cancer in case 11 and Aspergillosis in case 42, **Supplementary Data**). The patient with metastasized ovarian cancer had a *TP53* p.G244V mutation in plasma, while in tissue a different *TP53* mutation (p.R181C) and a *KRAS* p.G12D mutation were detected. The case with pancreatic cancer showed a *KRAS* p.G12V mutation in plasma (molecular

analysis in tissue was not possible). In the plasma of the remaining two patients no mutations were detected.

Aberrations detected in plasma are shown in **Figure 1** and results of tissue in **Supplementary Table S1 and S2**.

Discussion

In a real-world clinical practice setting, our study is the first to show the ability to detect targets for treatment in plasma in a cohort of unselected patients with suspected metastatic lung cancer that would otherwise have no access to molecular analysis. This is important for a substantial number of patients, as in The Netherlands still in only 80.9% of newly diagnosed advanced NSCLC cases *EGFR* testing is performed, while *EGFR* mutations are detected in 11.6% of patients.[5]

The availability of targeted therapy in current practice is evident for the patient with a common *EGFR* mutation, and to a lesser extent for the patients with *EGFR* exon 20 insertion (treatment in trial possible), activating non-V600E *BRAF* mutation (BRAF-TKI), and *KRAS* p.G12C (agents in trial or patient access program). At the time of detection of the possible targets for treatment there was no available therapy in first line for *KRAS* p.G12C or non-V600E *BRAF* mutations in our center. With fast development and registration of new targeted agents, possible treatment options are expected to expand quickly in the near future. We show that NGS is an adequate instrument to detect targetable mutations in cfDNA. However, there are some pitfalls to take into account.

First, we would like to emphasize that tissue analysis remains the gold standard in diagnosing thoracic malignancies, as illustrated by the 4 cases in our study, which show other histology than NSCLC in subsequent biopsies. Nevertheless, plasma can be of added value in a subset of patients, as demonstrated by the detection of a possible target for treatment in 7 of the 55 patients in our study. This was also observed in another study, which analyzed results of 323 patients with tissue confirmed metastatic NSCLC who had received routine plasma testing in the work up.[13] In the 94 (29.1%) patients with only plasma investigation at diagnosis, a therapeutically targetable mutation was detected in 31 (33%) patients. Another more recent study also showed a high response rate of 88% in patients with initiated targeted therapy based on cfDNA only at diagnosis, in a population of histologically confirmed stage IV NSCLC.[14]

Also, the question remains how to interpret the presence of aberrations in non-targetable genes, or unexpected findings. *TP53* and *KRAS* mutations in plasma can sometimes, though rare, be explained by clonal hematopoiesis or could reflect another primary malignancy.[15] This is illustrated by the case in our study where the diagnosis turned out to be pulmonary metastasized ovarian carcinoma (case 12 **Table S1**), and

not a primary pulmonary malignancy, after comparison of the molecular profile of the earlier resected ovarian cancer. Therefore, the discussion of plasma cfDNA results in an MTB in a referral center remains of utmost importance and should take into account the medical history in order to advise the clinical physician in allied centers. Parallel assessment of radiological imaging could possibly further optimize the generated advice in the future.

Furthermore, a limitation of our study is the limited extent of the NGS panel of 11 relevant genes. The Oncomine V1 panel is able to detect mutations at the relevant hotspots but does not cover the whole range of targetable aberrations in lung cancer at this moment.[4] This is evident from the case of the patient with the ALK rearrangement in tissue analysis (case 20 **Table S1**), which cannot be detected in plasma with this panel. Another study where plasma was analyzed with the broader Guardant360 assay showed frequencies and distributions of molecular aberrations as predicted for the study population.[16] In this light, further development and introduction into clinical practice of a broader cfDNA panel with ability to detect rearrangements, fusions, and amplifications, has high urgency. However, as not all parts of the world have access to the most advanced panels of genetic investigation in plasma, it is important to realize that even a panel with a limited extent is able to identify patients with possible benefit from targeted therapies in a real life setting.

Conclusions

This study shows in a prospective unselected clinical setting of patients in whom molecular analysis on tissue cannot be performed in case of (suspected) metastatic lung cancer, that despite the limited extent of the NGS panel, analysis of cfDNA in plasma provides an opportunity to detect driver mutations for subsequent targeted therapy.

Acknowledgements: Part of these data were presented at WCLC 2021 (abstract #286). Steendam C, Atmodimedjo P, Van Der Leest C, Van Loenhout K, Van'T Westeinde S, Dumoulin D, et al. P24.08 Lung Cancer Diagnosis in Absence of Adequate Tissue Molecular Analysis in Metastatic Disease by NGS Analysis of Plasma cfDNA. Journal of Thoracic Oncology. 2021;16(10):S1034.

Funding information: This work was partly funded by AstraZeneca (grant number 2604937054, unrestricted grant). The funding source had no role in study design; in the collection, analysis and interpretation of data; in the writing of the report; or in the decision to submit the article for publication.

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Supplementary material

Table S1. Patients with detectable aberrations in plasma

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Plasma mutations (VAF)	DNA conc.	Mol. Cov.	Reason plasma analysis
1	f	60	1	never	0	**EGFR p.E746_A750del (1.63%)**	3,94	73	Material unsuitable/ insufficient for analysis
2	f	65	-	current	30	**EGFR p.P770_V771insG (0.12%);** TP53: p.R209Qfs*7 (0.21%)	1,9	4; 6	Patient and/ or physician declined biopsy
3	m	66	3	former	50	**BRAF p.G466V (9.01%);** TP53 p.E339* (10.31%)	9,08	869; 1238	Patient and/ or physician declined biopsy
4	f	61	1	-	-	**KRAS p.G12C (0.86%)**	1,12	17	Material unsuitable/ insufficient for analysis
5	f	66	2	current	-	**KRAS p.G12C (1.39%);** TP53 p.R267W (0.91%)	1,33	27; 17	Material unsuitable/ insufficient for analysis
6	m	83	-	former	35	KRAS p.Q61H (0.76%); **KRAS p.G12C (0.60%);** TP53 p.R202C (0.10%)	1,54	28; 19; 3	Lesions unreachable

Reason explanation	Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
Insufficient material for molecular analysis in EBUS and 4 pleural fluid samples	no	Start EGFR-TKI	**EGFR p.E746_A750del**	6th biopsy of lung lesion (only possible after 4x pleural fluid drainage)	patient was treated with EGFR-TKI with partial response
Poor pulmonary function, biopsy risk considered too high	no	Target for therapy in clinical trial detected, referral for information and screening is advised	NA	NA	Patient was referred and informed on clinical trials with poziotinib or afatinib+cetuximab
Poor performance score and interstitial lung disease	no	No target for TKI in first line detected	NA	NA	patient deceased week after blood withdrawal
No molecular analysis possible on material lymph node (histology)	yes	No target for TKI in first line detected	NA	NA	NA
No molecular diagnosis possible on bronchoscopy material	yes	No target for TKI in first line detected	NA	NA	NA
Anticoagulant therapy and unfavorable location of lesions	no	No target for TKI in first line detected, reconsider biopsy	NSCLC in lymph node biopsied some time later, PD-L1 80% but low cell number	4 months later pleural fluid in which adenocarcinoma	patient was treated with immunotherapy and deceased shortly after pleural drainage (in which adenocarcinoma)

Table S1. Continued

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Plasma mutations (VAF)	DNA conc.	Mol. Cov.	Reason plasma analysis
7	f	62	-	-	-	**KRAS p.G12C (1.92%)**	7,09	112	Patient and/ or physician declined biopsy
8	m	63	1	former	35	KRAS p.G12D (0.06%)	2,98	4	Material unsuitable/ insufficient for analysis
9	m	84	1	-	-	KRAS p.G12D (0.54%); PIK3CA p.E545K (0.23%)	1,29	11; 4	Material unsuitable/ insufficient for analysis
10	f	64	2	-	-	KRAS p.G12V (5.44%); TP53 p.C275F (0.09%)	4,57	326; 5	Patient and/ or physician declined biopsy
11	f	62	1	-	-	KRAS p.G12V (0.60%)	1,25	11	Lesions unreachable
12	f	62	2	-	-	TP53 p.G244V (0.13%)	3,59	10	Lesions unreachable
13	m	72	0	-	-	TP53 p.R283C (0.08%)	7,57	6	Material unsuitable/ insufficient for analysis

Reason explanation	Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
Mental disorder	no	No target for TKI in first line detected, reconsider biopsy	NA	NA	NA
No molecular diagnosis possible on bronchoscopy material	yes	No target for TKI detected	**KRAS p.G12D 6%,** *FOXL2 p.G120V 7%, PTEN p.R41K 23%*	NGS revision on earlier bronchoscopy material in expert center	NA
No diagnosis on bronchoscopy material	no	No target for TKI detected	NA	NA	NA
NA	no	No target for TKI detected	NA	NA	patient deceased two weeks after plasma withdrawal
No molecular analysis possible on lytic bone lesions	no	No target for TKI detected, reconsider biopsy	*metastasized pancreatic cancer*	later diagnosis on pancreas and bone lesion	metastasized pancreatic cancer
On bronchoscopy material suspicion of malignancy	no	No target for TKI detected, reconsider biopsy	*KRAS p.G12D (54%); TP53 p.R181C (54%)*	Lung wedge resection; material was compared to ovary resection >10y earlier → the same entity (*so pulmonary metastasis of ovarian cancer*)	NA
No molecular analysis possible on material bone lesion	yes	No target for TKI detected, consider rebiopsy	NA	NA	NA

Table S1. Continued

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Plasma mutations (VAF)	DNA conc.	Mol. Cov.	Reason plasma analysis
14	f	80	3	-	-	TP53 p.Y205* (27.70%); PIK3CA p.E545K (0.06%)	3,43	1551; 5	Patient and/ or physician declined biopsy
15	m	62	-	former	-	TP53 p.G266V (0.67%)	1,37	14	Material unsuitable/ insufficient for analysis
16	f	73	3	former	-	TP53 p.Y234C (0.38%); TP53 ex6 c.672+1G>T; p.? (10.46%)	1,93	12; 299	Material unsuitable/ insufficient for analysis
17	m	77	2	former	-	TP53 p.R249S (0.05%)	2,36	3	Material unsuitable/ insufficient for analysis
18	f	82	3	former	-	TP53 p.R158H (4.44%)	4,56	191; 180	Material unsuitable/ insufficient for analysis
19	m	82	1	former	40	TP53 p.S215R (0.24%)	1,25	7	Material unsuitable/ insufficient for analysis
20	f	76	0	never	0	TP53 p.P278L (7.48%)	1,04	84	Material unsuitable/ insufficient for analysis
21	m	80	3	former	40	TP53 p.R283H (0.10%)	1,59	3	Material unsuitable/ insufficient for analysis
22	m	87	2	former	-	TP53 p.P278S (1.73%); TP53 p.G279E (0.37%); TP53 c.375+3_375+4insG p.? (0.16%)	0,86	28; 6; 3	Patient and/ or physician declined biopsy

Reason explanation	Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
NA	no	No target for TKI detected, reconsider biopsy	NA	NA	NA
No molecular analysis possible on lung biopsy	yes	No target for TKI detected	NA	NA	NA
No diagnosis on bronchoscopy material	no	No target for TKI detected, reconsider biopsy	NA	NA	NA
No diagnosis on bone biopsy	no	No target for TKI detected	NA	NA	NA
No diagnosis possible on bronchoscopy material	no	No target for TKI detected	NA	NA	NA
No diagnosis on material EUS, pleural fluid or lung biopsy	no	No target for TKI detected, reconsider biopsy	NA	NA	NA
No molecular analysis possible on liver biopsy	yes	No target for TKI detected, consider rebiopsy	*ALK rearrangement* (FISH+/IHC+)	later second liver biopsy was performed	patient was successfully treated with alectinib
No molecular analysis possible on material FNA lymph node or pleural fluid	yes	No target for TKI detected, reconsider biopsy	NA	NA	NA
Biopsy considered too high risk	no	No target for TKI detected, reconsider biopsy	NA	NA	NA

Table S1. Continued

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Plasma mutations (VAF)	DNA conc.	Mol. Cov.	Reason plasma analysis
23	m	74	0	former	-	TP53 p.R267Q (0.24%)	1,1	3	Lesions unreachable
24	f	76	2	never	0	TP53 p.C238F (0.86%); TP53 p.C275S (0.13%)	7,55	49; 7	Material unsuitable/ insufficient for analysis
25	f	81	3	current	65	TP53 p.Y163C (54.77%)	45,2	1887	Patient and/ or physician declined biopsy
26	f	74	3	current	60	TP53 p.R248W (0.34%)	1,41	11	Patient and/ or physician declined biopsy
27	m	70	2	never	0	TP53 p.S241F (3.49%)	145	168	Material unsuitable/ insufficient for analysis
28	m	84	1	former	27	TP53 p.V272G (0.17%)	5,18	13	Material unsuitable/ insufficient for analysis

Legend: f=female, m=male, -=unknown, VAF=variant allele frequency, conc.=concentration, mol. cov.=molecular coverage, MTB=molecular tumor board, NA=not applicable.
Possible targets for treatment are indicated in bold understrike. Confirmative tissue results in bold, additional information of tissue compared to plasma is in italic understrike.

Reason explanation	Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
NA	no	No target for TKI detected, reconsider biopsy	NA	NA	NA
No molecular analysis possible on EBUS material	yes	No target for TKI detected, reconsider biopsy	NA	NA	NA
Poor performance score, spinal cord injury	no	No target for TKI detected	NA	*No TP53 in earlier bladder cancer >10y earlier or white blood cells*	NA
Both pulmonary (COPD) and cardial (atrial fibrillation, pulmonary hypertension) comorbidity	no	No target for TKI detected	NA	NA	NA
No diagnosis on material EUS	no	No target for TKI detected	NA	NA	NA
No diagnosis possible on bronchoscopy material	no	No target for TKI detected, reconsider biopsy	NA	NA	patient deceased a month afterwards

Table S2. Patients without detectable aberrations in plasma

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Reason plasma analysis	Reason explanation
29	f	70	3	-	-	Material unsuitable/ insufficient for analysis	Biopsy not feasible due to poor clinical condition
30	f	69	2	-	-	Material unsuitable/ insufficient for analysis	No diagnosis on bronchoscopy material, liver lesion unreachable
31	f	83	-	-	-	Material unsuitable/ insufficient for analysis	No diagnosis on material scapula
32	f	63	1	former	-	Material unsuitable/ insufficient for analysis	No molecular analysis possible on material EBUS FNA
33	f	85	-	-	-	Material unsuitable/ insufficient for analysis	No diagnosis on bronchoscopy material
34	f	65	0	former	30	Material unsuitable/ insufficient for analysis	No diagnosis on EBUS and lung biopsy
35	m	69	1	former	5	Material unsuitable/ insufficient for analysis	No molecular analysis possible on pleural fluid (3x)
36	f	50	-	former	-	Patient and/or physician declined biopsy	No biopsy possible due to respiratory insufficiency
37	f	62	0	current	-	Material unsuitable/ insufficient for analysis	No diagnosis possible on lung biopsy

Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
no	No target for TKI detected, reconsider biopsy	NA	NA	patient deceased two weeks after plasma withdrawal
no	No target for TKI detected, consider rebiopsy	*TP53 p.G154V (83%)*	Liver biopsy; lesion reachable due to progression (5 months after plasma sample)	NA
no	No target for TKI detected, consider rebiopsy	NA	NA	NA
yes	Consider revision NGS on existing material in expert center	NA	NA	NA
no	No target for TKI detected	*KRAS p.Q61H (33%), indication of extra copies of KIT and PDGRA*	EBUS FNA as a last attempt	NA
no	No target for TKI detected	NA	NA	NA
yes	No target for TKI detected	*No aberrations detected*	later another pleural fluid sample and an abdominal biopsy at further progression was possible	NA
no	No target for TKI detected, reconsider biopsy	NA	NA	NA
no	No target for TKI detected	*SCLC*	later another biopsy was possible at disease progression	Because secondary primary stage III sclc for which cCRT with curative intent

Table S2. Continued

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Reason plasma analysis	Reason explanation
38	m	72	1	former	62	Patient and/or physician declined biopsy	Lung biopsy not possible because of high risk of respiratory insufficiency
39	m	82	-	never	0	Patient and/or physician declined biopsy	lung biopsy not possible due to respiratory insufficiency
40	f	60	2	former	-	Material unsuitable/ insufficient for analysis	No molecular analysis possible on pleural fluid, bronchoscopy material and lung biopsy
41	f	77	-	never	0	Material unsuitable/ insufficient for analysis	No diagnosis on bronchoscopy material
42	f	62	2	former	-	Patient and/or physician declined biopsy	Lung biopsy not possible because of high risk of respiratory insufficiency
43	m	87	2	current	35	Material unsuitable/ insufficient for analysis	No diagnosis possible on material EBUS and bronchoscopy
44	f	61	3	former	25	Patient and/or physician declined biopsy	Anticoagulant therapy and unfavorable location of lesions
45	f	67	1	former	-	Lesions unreachable	Former resection material molecular analysis not possible, new metastasis too small to biopsy
46	m	83	1	former	-	Material unsuitable/ insufficient for analysis	No diagnosis on pleural fluid

Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected, reconsider biopsy	NA	NA	Simultaneous laryngeal carcinoma, possible metastasis or primary lung carcinoma?
yes	No target for TKI detected, reconsider biopsy	NA	NA	NA
no	No target for TKI detected, reconsider biopsy	NA	NA	NA
no	No target for TKI detected, reconsider biopsy	*no malignancy*	later FNA lymph node and lung biopsy at progression showed aspergillosis	later FNA lymph node and lung biopsy at progression showed aspergillosis (history 2x NSCLC)
no	No target for TKI detected, consider rebiopsy	NA	NA	NA
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected, reconsider biopsy	NA	NA	NA
no	No target for TKI detected	NA	NA	NA

Table S2. Continued

Case	Sex	Age	WHO PS	Smoking status	Pack-years	Reason plasma analysis	Reason explanation
47	m	84	3	former	45	Patient and/or physician declined biopsy	Poor performance score, lung biopsy not possible due to respiratory insufficiency
48	m	55	2	-	-	Material unsuitable/ insufficient for analysis	No diagnosis possible on bronchoscopy material
49	f	68	3	former	25	Patient and/or physician declined biopsy	Lung biopsy not possible due to respiratory insufficiency
50	m	75	0	unknown	unknown	Patient and/or physician declined biopsy	NA
51	f	73	3	unknown	unknown	Material unsuitable/ insufficient for analysis	No molecular diagnosis possible on bronchoscopy material
52	f	63	1	former	unknown	Lesions unreachable	NA
53	m	78	3	unknown	unknown	Material unsuitable/ insufficient for analysis	No diagnosis on lung biopsy
54	m	75	1	unknown	unknown	Patient and/or physician declined biopsy	NA
55	f	73	1	current	unknown	Material unsuitable/ insufficient for analysis	No molecular analysis possible on lung biopsy material

Legend: f=female, m=male, -=unknown, MTB=molecular tumor board, NA=not applicable.

Tissue confirmed NSCLC at time plasma analysis	MTB advise	Tissue results	Tissue explanation	Clinical course
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected	*NSCLC NOS, KRAS p.G12D 7.7%, SMAD4 p.T349I 43%*	When respiratory condition improved pleural drainage was undertaken	patient deceased a month afterwards
no	No target for TKI detected	NA	NA	NA
yes	No target for TKI detected	NA	NA	NA
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected	NA	NA	NA
no	No target for TKI detected	NA	NA	NA
yes	Doubtful detection of EGFR exon 19 deletion with simultaneous ddPCR at the limit of detection (NGS negative), consider trial of EGFR-TKI when no other systemic options are left	NA	NA	Patient refused chemotherapy, a trial of osimertinib rendered no response after 4 weeks

Chapter 7

Plasma predictive features in treating EGFR-mutated non-small cell lung cancer

Cancers (Basel). 2020 Oct 29;12(11):3179

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Abstract

Although epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are the preferred treatment for patients with *EGFR*-mutated non-small cell lung cancer (NSCLC), not all patients benefit. We therefore explored the impact of the presence of mutations found in cell-free DNA (cfDNA) and TKI plasma concentrations during treatment on progression-free survival (PFS). In the prospective START-TKI study blood samples from 41 patients with *EGFR*-mutated NSCLC treated with EGFR-TKIs were available. Next generation sequencing (NGS) on cfDNA was performed, and plasma TKI concentrations were measured. Patients without complete plasma conversion of *EGFR* mutation at week 6 had a significantly shorter PFS (5.5 vs. 17.0 months, $p = 0.002$) and OS (14.0 vs. 25.5 months, $p = 0.003$) compared to patients with plasma conversion. In thirteen (second line) osimertinib-treated patients with a (plasma or tissue) concomitant *TP53* mutation at baseline, PFS was significantly shorter compared to six wild-type cases; 8.8 vs. 18.8 months, $p = 0.017$. Erlotinib C_{mean} decrease of ≥10% in the second tertile of treatment was also associated with a significantly shorter PFS; 8.9 vs. 23.6 months, $p = 0.037$. We obtained evidence that absence of plasma loss of the primary *EGFR* mutation, isolated plasma p.T790M loss after six weeks, baseline concomitant *TP53* mutations, and erlotinib C_{mean} decrease during treatment are probably related to worse outcome.

Introduction

Non-small cell lung cancer (NSCLC) has the highest mortality among solid tumors and once metastasized, patients have a limited prognosis and depend on palliative treatment.[1] Lung adenocarcinoma comprises some specific subgroups defined by oncogenic driver mutations, including the epidermal growth factor receptor (*EGFR*)-mutation. The outcome of patients with *EGFR*-mutated NSCLC has significantly improved with the introduction of EGFR-tyrosine kinase inhibitors (TKIs), with a median overall survival (OS) of more than 3 years.[2] However, up to 10% of patients have an initial lack of response (primary resistance) and 10 to 30% of patients have early progressive disease within 6 months.[3, 4] Therefore, it is important to identify these patients early, in order to implement close monitoring and immediate switch to a next line of treatment.

Nevertheless, predictive biomarkers for *EGFR*-mutated NSCLC are scarce. Some studies have observed that concomitant mutations are associated with worse clinical outcome.[5, 6] Especially, the presence of concomitant *TP53* mutations was associated with shorter progression-free survival (PFS) and OS.[7-10]

Next to tumor mutational characteristics, treatment outcome might be dependent on the actual TKI exposure. For multiple TKIs relationships have been found between pharmacokinetic parameters (e.g., minimal drug concentration or total exposure) and OS or PFS.[11] Variability in TKI exposure is high and can be influenced by drug–drug interactions, genetic variations in drug metabolizing (CYP) enzymes, lifestyle (e.g., smoking), and concomitant intake of food or herbs.[12, 13] This is relevant, since higher exposure to a drug is thought to increase toxicity, while a lower exposure may lead to pharmacokinetic resistance, resulting in a lower survival. For several TKIs exposure-response or exposure-toxicity relations have been found.[11] In the case of erlotinib and osimertinib, no definite target plasma concentrations have been described to optimize their efficacy.[11] Concerning toxicity, pharmacologic modelling has shown that osimertinib concentrations have a positive relationship with occurrence of skin rash and diarrhea, and increase in cardiac QTc time.[14] For erlotinib, there is evidence that its main toxicity (diarrhea and skin rash) is correlated with dosage and drug concentrations.[15-17] In addition to TKI exposure, the possibility of penetration of the blood-brain barrier is of interest as brain metastases are a frequent site of metastasis and progression in NSCLC. Of all EGFR-TKIs, drug penetration across the blood-brain barrier is highest for osimertinib.[18] This explains the lower incidence of central nervous system (CNS) progression for osimertinib compared to other EGFR-TKIs.[19]

Although tissue biopsy is still considered the gold standard in defining the histological diagnosis and enabling extensive molecular investigation, the possibilities of plasma investigations for cell-free DNA (cfDNA) from a blood sample are increasing.[20, 21] Besides the benefit of being a less invasive procedure with negligible risks, the cfDNA is likely

to represent the full spectrum of clonal variation in the cell-free tumor DNA (ctDNA) as opposed to tissue obtained from just one tumor lesion/region.[22] Detection of primary activating *EGFR* mutations and resistance mechanisms, e.g., p.T790M development in plasma, have shown to be adequate and effective for directing therapy.[23]

Our study aimed to explore the predictive value of blood-based biomarkers including cfDNA plasma mutation detection and drug level monitoring in patients with *EGFR*-mutated NSCLC treated with a first or second line EGFR-TKI.

Results

Between March 2017 and May 2019, a total of 41 unique patients with *EGFR*-mutated NSCLC, treated with a 1st generation EGFR-TKI in first line or 3rd generation EGFR-TKI in second line for a *EGFR* p.T790M resistance mutation, were included. Five patients were enrolled twice, both at first line and second line treatment. Hence, 46 observed treatment lines were available. Minimal follow-up was six months. Median follow-up of patients still alive at data cut-off at December 1st 2019 was 14.9 months (range 6.4–34.0 months).

Baseline Characteristics

Baseline characteristics are presented in **Table 1**. In the total cohort of individual patients (*n*=41), the median PFS in the first line cohort (13.6 months (*n*=14, 95% confidence interval (CI) 3.2–23.9 months)) was comparable to the PFS in the second line cohort (11.5 months (*n*=27, 95% CI 3.2–19.8, *p* = 0.768)), see Supplementary Data **Figure S1**.

Table 1. Baseline characteristics

Baseline Patient Characteristics	***n*=41**
Gender	
Male	18 (44%)
Female	23 (56%)
Age (median, range)	62 (42–83)
Ethnicity	
Caucasian	36 (88%)
Asian	4 (10%)
Unknown	1 (2%)
Smoking status	
Current	2 (5%)
Former	24 (58%)

Table 1. Continued

Baseline Patient Characteristics	***n*=41**
Never	15 (37%)
Former pack years (median, range)	6 (0–40)
0	15 (37%)
1–15	11 (27%)
15–30	9 (22%)
≥30	1 (2%)
Unknown	5 (12%)
Histology	
NSCLC; adenocarcinoma	39 (95%)
NSCLC NOS	1 (2.5%)
Unknown	1 (2.5%)
Type of primary *EGFR* mutation	
Exon 19	28 (68%)
Deletion	23 (56%)
Deletion-insertion	4 (9.5%)
Other (VUS)	1 (2.5%)
Exon 21	13 (32%)
p.L858R	12 (29.5%)
Other	1 (2.5%)
Exon 20 concomitant mutation	28 (68%)
p.T790M	27 (66%)
Exon 19 + exon 20	1 (2.5%)
Plasma available at baseline	**38 (93%)**
Tissue available at baseline	**32 (78%)**

At baseline, samples for NGS were available in all patients: tissue samples were available in 32 patients (78%) and plasma in 38 (93%) patients of the total population (*n*=41). In 31 out of 38 plasma samples ctDNA was detected (82%). There was no significant difference in PFS in patients with or without detectable ctDNA at baseline (see Supplementary Data **Figure S2**). However, the patients without baseline *EGFR* mutations in plasma (*n*=7) did not have any radiological progression events, compared to 22 events in patients with detectable *EGFR* mutations at baseline (*n*=31). At data cut-off, five patients were still on treatment and two died of other reasons (one patient due to multi-organ failure with empyema after chest tube placement, the other patient suddenly passed away at home after sudden onset of dyspnea, presumably because of pulmonary embolism or a cardiac event, see also Supplementary Data **Table S1**). In one patient a *MAP2K1* mutation was

found in absence of *EGFR* mutations in plasma at baseline, which was not detected in the tumor tissue, this patient was still on treatment at data cut-off. Treatment during study and best response are summarized in **Table 2**.

Table 2. Treatment lines during study

Available Treatment Lines during Study	Total Cohort * (*n*=41)	1st Line Cohort (*n*=19)	2nd Line Cohort (*n*=27)
Best response on treatment			
PR	25 (61%)	13 (68%)	15 (56%)
SD	12 (29%)	4 (21%)	10 (37%)
PD	3 (7%)	2 (11%)	1 (3.5%)
Unknown	1 (3%)	0 (0%)	1 (3.5%)
Progression or death event	28 (68%)	15 (79%)	18 (67%)
Radiological progression	24 (59%)	13 (68%)	16 (59%)
Death without radiological progression	4 (10%)	2 (11%)	2 (28%)
Progression free survival category			
≤6 months	13 (32%)	4 (21%)	9 (33%)
>6 months	28 (68%)	15 (79%)	18 (67%)

** To prevent bias, we only included the most recent treatment lines of the 41 individual patients, 5 treatment lines from the 1st line cohort were excluded in analyses of the total EGFR cohort. Abbreviations: PR = partial response, SD = stable disease, PD = progressive disease.*

Presence of Concomitant Mutations (besides EGFR Primary Mutation and p.T790M)

At baseline, 26 patients harbored concomitant mutations (63%, *n*=41), including *TP53*, *ERBB2, CTNB1, MTOR, CDKN2A, ARAF, PIK3CA, PTEN, MAP2K1*, and *APC* mutations. In 11 patients (27%) more than one concomitant mutation was detected and all of them included a *TP53* mutation. One patient had more than one concomitant mutation found in plasma, with a *PIK3CA* mutation besides *EGFR* and *TP53* mutations, and an additional PTEN mutation in tissue. There was no significant difference in PFS in patients with no, one or more concomitant mutations, see Supplementary Data **Figure S3**.

Presence of TP53 Mutations

A *TP53* mutation was detected in 23 patients (56%); seven times a *TP53* mutation was detected in plasma, compared to 22 in tissue. In one case, the *TP53* mutation in plasma did not correspond to the mutation in tissue at baseline, but did agree with the tissue mutation at radiologic progression. *TP53* mutations were most common in exon 7 and exon 5, the majority were missense mutations, see **Table 3**. In the total *EGFR* cohort, median PFS in patients without a *TP53* mutation at baseline (*n*=10, 18.8 months, 95% CI 13.5–24.1) was not significantly longer than median PFS in the *TP53* mutated group

(*n*=23, 13.1 months, 95% CI 4.1–22.1, *p* = 0.068). In the second line cohort, the PFS was significantly shorter in thirteen patients with a *TP53* mutation at baseline than in six wild-type patients (8.8 (95% CI 0.7–16.9) vs. 18.8 (95% CI 13.3–24.3) months, *p* = 0.017), see **Supplementary Data Figure S4C**. In one patient a new *TP53* mutation was detected in tumor tissue at time of progressive disease (PD). At baseline however, no tissue was available for this patient. No other new *TP53* mutations were demonstrated over the course of treatment.

Table 3. TP53 status at baseline

Baseline *TP53* mutation present (*n*=41)	**23 (56%)**
Present in plasma (*n*=23)	6 (26%)
Present in tissue (*n*=23)	22 (96%)
No plasma sample baseline	2 (9%)
No ctDNA baseline	4 (17%)
Not covered by cfDNA panel	6 (26%)
Covered by cfDNA panel	4 (17%)
Location of mutation	
Exon 5	6 (26%)
Exon 6	3 (13%)
Exon 7	8 (35%)
Exon 8	2 (9%)
Other	4 (17%)
Type of mutation	
Missense	15 (65%)
Nonsense	1 (5%)
Other	7 (30%)

Resistance Mechanisms

At radiologic progression, in 19 patients (83% out of 23 patients with available samples) at least one new concomitant mutation was detected, of which 12 (52%) had more than one concomitant mutation.

First Line Cohort (1st Generation EGFR-TKI)

At data cut-off, 15 of the 19 patients had a PFS event (79%), while 13 showed radiological progression of disease (68%). Plasma samples for analysis at PD were available in 11 patients and tissue samples in seven of these patients. In six patients treated with erlotinib or gefinitib a *EGFR* p.T790M mutation was detected at PD; in two patients *EGFR* p.T790M was detected in plasma but not in tissue (including one missing tissue sample), two cases had *EGFR* p.T790M confirmed in both plasma and

tissue, and in two other patients *EGFR* p.T790M was present in tissue but not in plasma. Two patients also developed an extra possible resistance mutation in tissue samples, a novel *CDKN2A* homozygote deletion and a novel *APC* mutation next to *EGFR* p.T790M.

Second Line Cohort (Osimertinib)
At data cut-off, 18 of 27 patients had a PFS event, of whom 16 demonstrated radiological progression (59%). At PD, plasma samples were collected in all 16 patients with radiologic progression, and a new tumor biopsy was taken in 12 cases. In 14 patients different genetic alterations at PD compared to baseline were detected. These possible resistance mechanisms to osimertinib were divided into on-target and off-target resistance mechanisms. On-target mechanisms were observed in four patients, including three *EGFR* p.C797S mutations. In addition, 21 off target mutations were observed in 11 patients. In three patients, the tumor transformed to a small-cell lung cancer, all having a *TP53* and *PIK3CA* mutation at PD. Loss of *EGFR* p.T790M mutation was observed in nine patients, including all patients with a transformation to small-cell lung cancer. Other emerging mutations were *BRAF* p.V600E, *CDKN2A* homozygous deletions, *MET* amplifications, and *PTEN* mutations. In two patients, the resistance mutation was detected in plasma, as tissue was not available (*EGFR* p.C797S and *BRAF* p. V600E). An extra mutation was detected in two cases, which were not found in tissue (*PIK3CA* mutation and a *BRAF* mutation). In another patient, a *PIK3CA* mutation was detected in both plasma and tissue.

Plasma Conversion

Plasma Conversion to cfDNA Negative for the Primary EGFR Mutation
Plasma conversion status was evaluable in 29 patients at week 6 and 12 after start of treatment. Sixteen and 18 patients had complete plasma conversion at week 6 and 12 respectively. Patients with complete plasma conversion had a significantly longer PFS compared to patients without complete plasma conversion at either week 6 (17.0 (95% CI 9.7–24.2) vs. 5.5 (95% CI 4.7–6.2) months, $p = 0.002$; **Figure 1a**), and week 12 (17.0 (95% CI 11.7–22.3) vs. 5.1 (95% CI 3.7–6.6) months, $p < 0.001$; **Figure 1b**). Illustrative, both patients who reached complete plasma conversion at week 12 already had an almost complete conversion of the primary *EGFR* mutation at week 6 (−80 and −97% respectively). These significant differences in PFS in patients with complete plasma conversion were also present in separate analyses of the treatment cohorts, see Supplementary Data **Table S2**.

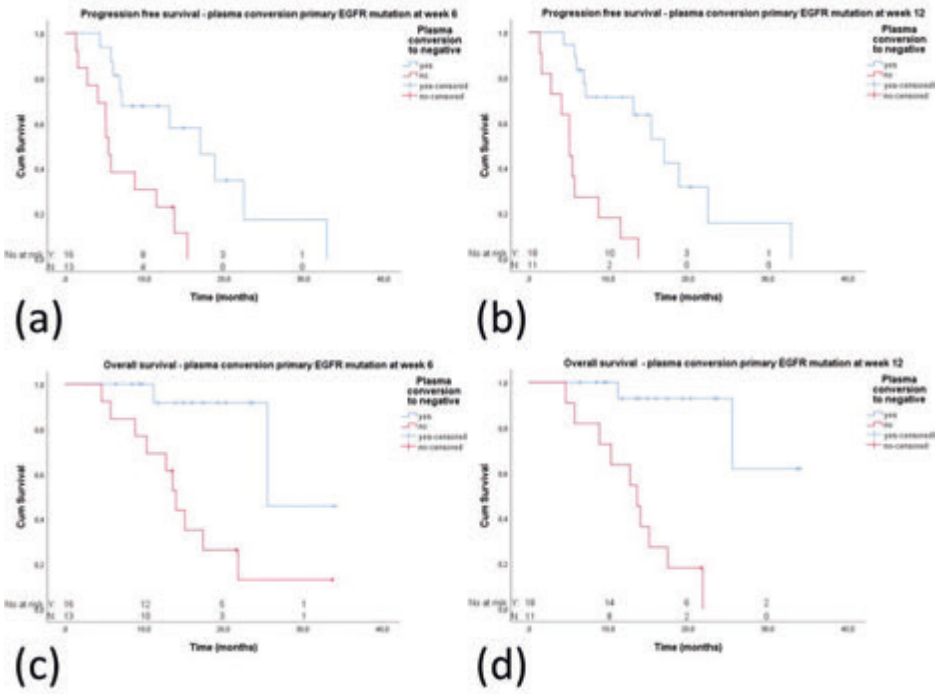

Figure 1. Plasma conversion of the primary epidermal growth factor receptor (EGFR) mutation in relation to progression-free survival (PFS) and overall survival (OS): (***a***) *PFS and plasma conversion at week 6;* (***b***) *PFS and plasma conversion at week 12;* (***c***) *OS and plasma conversion at week 6;* (***d***) *OS and plasma conversion at week 12.*

In addition, OS was significantly longer in patients with plasma conversion at week 6 compared to patients with continuous detection of the primary *EGFR* mutation (25.5 (95% CI could not be calculated) vs. 14.0 (95% CI 12.0–16.0) months, $p = 0.003$). This difference in OS was also present at week 12 (NR vs. 13.6 (95% CI 9.6–17.5) months, $p < 0.001$; **Figure 1c,d**).

Twelve (75%) of the patients with complete plasma conversion at week 6 had a partial response (PR) and four (25%) had stable disease (SD) as best radiologic response. In the case of a lack of complete plasma conversion, only five patients (39%) had a PR, six (46%) had SD, and two (15%) PD as best response (chi square $p = 0.064$). The share of patients with a short and long PFS in relation to plasma conversion is depicted in **Figure 2**.

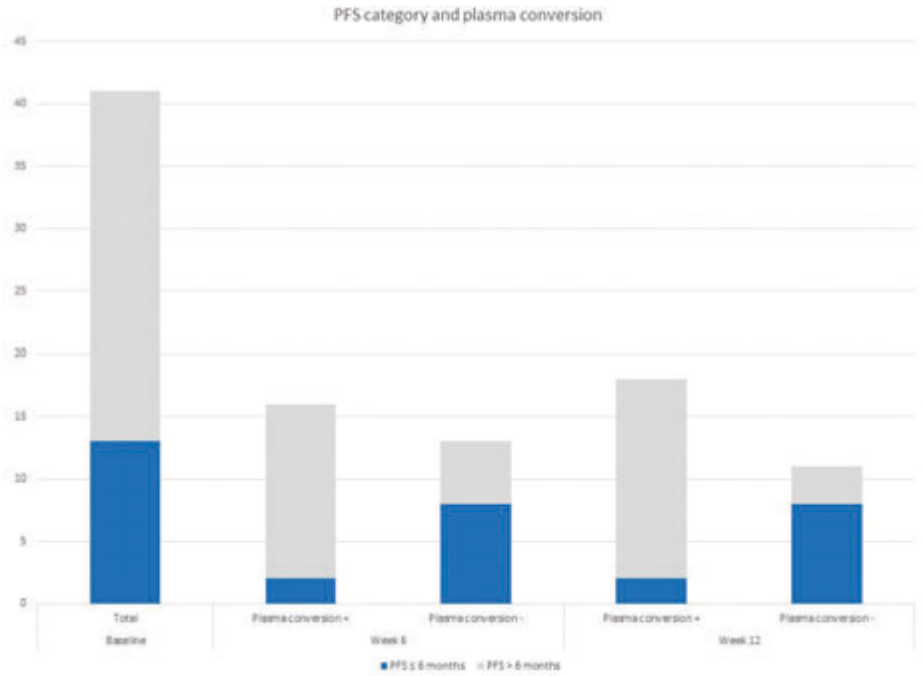

Figure 2. *PFS category distribution and plasma conversion in time.*

Plasma Conversion to cfDNA Negative for the EGFR p.T790M Mutation

In the second line cohort (osimertinib), *EGFR* p.T790M was evaluated in plasma. Ten patients without plasma conversion of the primary mutation, but with loss of *EGFR* p.T790M in plasma at week 6 had a significant shorter PFS of 5.1 months (95% CI 4.6–5.7) compared to 11 patients with plasma conversion for both the primary *EGFR* and the p.T790M mutation with a PFS of 18.8 months (95% CI 7.5–30.1, $p = 0.012$), see **Supplementary Data Figure S5**.

Plasma Mutation Levels during Treatment

There was no correlation between baseline and progression levels of the primary *EGFR* mutation (i.e., variant allele frequency) or cfDNA concentrations in plasma. In most patients in the second line cohort, there was no detectable *EGFR* p.T790M at progression in contrast to baseline, while the primary *EGFR* mutation was detectable in the majority of patients, see Supplementary Data **Figure S6**. The allele fraction of the primary EGFR mutation was widely variable among patients with and without plasma conversion, as shown in **Figure 3**.

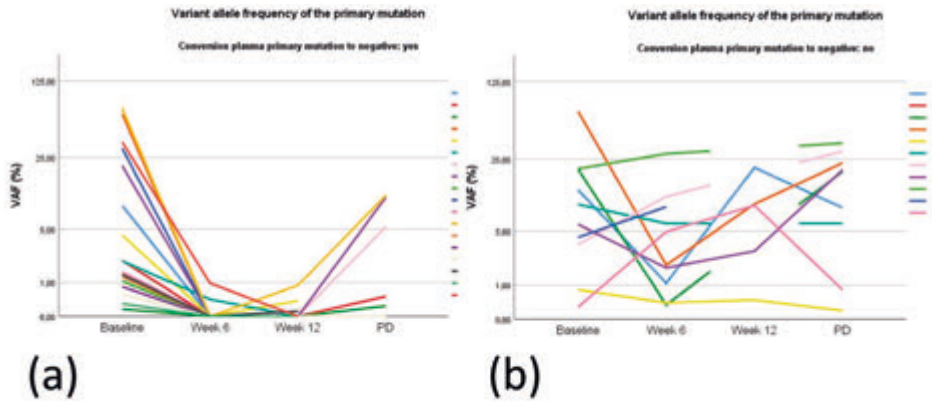

Figure 3. *Variant allele frequency (VAF) of the primary EGFR mutation in time, by plasma conversion status. (a) Patients with complete plasma conversion; (b) patients without complete plasma conversion*

Short Responders

Thirteen patients (32%) met the definition of being a short responder (PFS < 6 months). This group included relatively fewer patients with an exon 19 primary *EGFR* mutation (7 cases, 54%) than in the group with a response of more than 6 months (21 out of 28 cases, 75%, NS). The 2 current smokers at presentation were both short responders, of the 15 never smokers only 2 (13%) were short responders (chi square $p = 0.022$). Of the patients with evaluable plasma and tissue samples (n=33), all short responders had a *TP53* mutation at baseline compared to 58% in the groups with a PFS of more than 6 months (chi square $p = 0.032$). Of the five patients with three other concomitant mutations, besides *EGFR* mutations, three (60%) were short responders, while eleven of the 15 patients without other concomitant mutations (73%) had a response >6 months (NS). Of the patients evaluable for PFS category and plasma conversion at week 6 (n=29), only two patients (13%) reached complete plasma conversion among the short responders, opposite to 88% for patients with PFS > 6 months (chi square $p = 0.009$). The two short responders who did reach complete plasma conversion were a patient with cerebral oligoprogression on erlotinib, and a patient with SCLC transformation during osimertinib as second line treatment.

Plasma Drug Concentrations

In 15 patients treated with erlotinib and 22 treated with osimertinib, a total of 258 samples were drawn for pharmacokinetic analysis. Interestingly, a decrease in erlotinib C_{mean} in the second tertile was correlated with a significantly shorter PFS (median PFS 8.9 (95% CI 3.2–14.6) vs. 23.6 (21.7–25.6) months; n=13; log-rank $p = 0.037$), see **Figure 4**. Additionally, patients treated with erlotinib who experienced a decreased C_{mean} two months prior to PD, compared to C_{mean} until six weeks after treatment initiation, had a significantly lower PFS (4.7 (95% CI could not be calculated) vs. 7.1 (95% CI 6.4–7.8) months; n=5; $p = 0.039$). The average time to second tertile in patients with a decreased

C_{mean} was 20 weeks. Notably, patients treated with erlotinib in whom the C_{mean} decreased in the second tertile compared to the first tertile of treatment, had a significantly shorter time until severe toxicity occurred (median 11.8 (95% CI 10.6–12.9) vs. 23.9 (95% CI could not be calculated) months; *n*=13; *p* = 0.031). Dose reductions were, although non-significant, more frequent in this group with decreased C_{mean} (67% vs. 29%; *p* = 0.17).

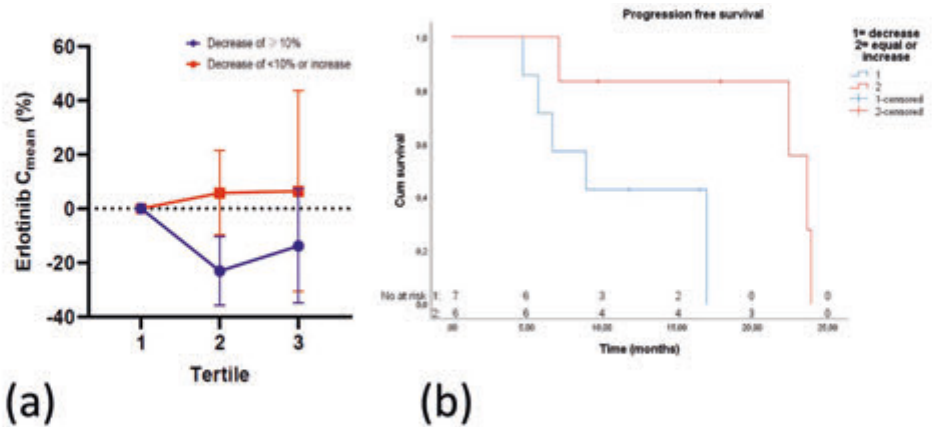

Figure 4. *(**a**) Relative change in erlotinib C_{mean}* during treatment. Treatment period is divided in tertiles; (***b**) progression-free survival based on the erlotinib C_{mean}* in the second tertile compared to the first tertile. The first (red-line) group has a decrease of less than 10% or an increase in C_{mean}. *The second (blue-line) group has a decrease of at least 10% in erlotinib C_{mean}. C_{mean}* = mean plasma concentration.

Median C_{mean} during treatment was 1085 ng/mL for erlotinib and 190 ng/mL for osimertinib. **Table 4** presents an overview of the results of the Kaplan-Meier analysis with the log-rank test to correlate (changes in) C_{mean} during treatment with PFS. The correlations with erlotinib and PFS were not seen in patients treated with osimertinib (all log rank *p* > 0.05; **Table 4**).

Table 4. Kaplan-Meier analysis to correlate (changes in) Cmean during treatment with PFS and toxicity.

Variable	Event	Erlotinib		Osimertinib	
		n (Events)	Log-Rank *p*-Value	*n* (Events)	Log-Rank *p*-Value
C_{mean} of total treatment †	PFS	15 (11)	0.517	22 (13)	0.631
C_{mean} first six weeks †	PFS	12 (8)	0.197	20 (11)	0.972
C_{mean} two months before PD †	PFS	7 (7)	0.779	15 (9)	0.221
C_{mean} two months before PD/C_{mean} first six weeks ‡	PFS	5 (5)	0.039*	7 (7)	0.561
C_{mean} third tertile/C_{mean} first tertile ‡	PFS	8 (8)	0.855	17 (10)	0.821
C_{mean} second tertile/C_{mean} first tertile ‡	PFS	13 (9)	0.037*	17 (10)	0.169
C_{mean} third tertile/C_{mean} second tertile ‡	PFS	9 (9)	0.415	15 (8)	0.517
C_{mean} until severe toxicity or end of study †	Tox	15 (10)	0.430	22 (3)	0.134
C_{mean} third tertile/C_{mean} first tertile ‡	Tox	8 (4)	0.433	17 (2)	0.705
C_{mean} second tertile/C_{mean} first tertile ‡	Tox	13 (8)	0.031 *	17 (3)	0.460
C_{mean} third tertile/C_{mean} second tertile ‡	Tox	9 (5)	0.786	15 (2)	0.564

*† = Variable group deviated by median; ‡ = variable group deviated by a decrease of 10% or more; * = statistically significant ($p < 0.05$). Abbreviations: Cmean = mean plasma drug concentration at 24 h; PFS = progression-free survival; n = number of patients; PD = progressive disease.*

Toxicity

All reported TKI-related toxicities are presented in **Table 5**. Fourteen patients experienced severe toxicity; four (15%) occurred in the osimertinib-treated patients, and ten (56%) in the erlotinib-treated patients.

Table 5. Toxicity

Erlotinib (n = 18)	
Severe toxicity	**10 (56%)**
CTCAE grade ≥ 3	5† (28%)
Hospital admission	2 (11%)
Dose reduction	8 (44%)
TKI interruption/discontinuation	2 (11%)
Erlotinib specific toxicity (all grade)	
Rash	12 (72%)
Alopecia	8 (44%)
Diarrhea	6 (33%)
Sicca (dry eyes, mouth and/or skin)	6 (33%)

Table 5. Continued

Erlotinib (n = 18)	
Paronychia	5 (28%)
Hand-Foot Syndrome	1 (6%)
Hypertrichosis	1 (6%)
Osimertinib (n = 27)	
Severe toxicity	**4 (15%)**
CTCAE grade ≥ 3	4* (15%)
Hospital admission	2 (7%)
Reduction	2 (7%)
TKI interruption/discontinuation	3 (11%)
Osimertinib specific toxicity (all grade)	
CK elevation	6 (22%)
Paronychia	5 (19%)
Diarrhea	4 (15%)
Dry skin	4 (15%)
QTc prolongation	4‡ (17%)
Pneumonitis	3 (11%)
Rash	2 (7%)

*Abbreviations: n = number of patients; CTCAE = Common Terminology Criteria for Adverse Events. † = 1 rash, 1 nausea and vomitus, 1 kidney failure, 1 ALAT increase and 1 hypokalemia and pericarditis. * = 1 pneumonitis, 1 pneumonitis and skin infection and 1 CK elevation. ‡ = calculated for patients which had baseline and follow-up electro cardiograms (n=23).*

The occurrence of severe toxicity was not correlated with C_{mean} divided by median for either drug (**Table 4**). However, all patients who experienced osimertinib-related severe toxicity had a C_{mean} above the median C_{mean} (33 vs. 0%, chi square $p = 0.062$).

Brain Metastasis

At start of treatment, in the first line cohort, the one patient on gefitinib did not have CNS disease, but three patients (17%, *n*=18) treated with erlotinib and five patients (19%, *n*=27) in the second line cohort had intracerebral metastasis. There was no significant difference in *TP53* mutational status in patients with or without baseline intracerebral metastasis ($p = 0.399$). Six patients were evaluable for plasma conversion status, and all six had undetectable primary mutation after 6 weeks of treatment vs. 46% who had no CNS disease at start (chi square $p = 0.017$). If patients had CNS disease at baseline, those treated with erlotinib had a significantly lower PFS compared to patients without CNS disease at baseline: 6.7 (95% CI 5.2–8.2) vs. 17.0 (95% CI 5.2–28.2) months, *n*=18; $p = 0.032$.

From the eight patients with CNS disease at start of treatment, one in each treatment line had intracerebral PD. In total three patients in each cohort had (new) intracerebral disease as site of progression (17% and 11% for erlotinib and osimertinib respectively; chi square $p = 0.591$). Comparing the patients with and without CNS as site of PD, mean TKI concentrations in plasma did not significantly differ for neither erlotinib (1390 vs. 1016 ng/mL; $p = 0.461$) nor osimertinib (206 vs. 188 ng/mL; $p = 0.321$). There was no significant difference in presence of *TP53* mutations at baseline or C_{mean} in the patients with or without CNS progressive disease (see Supplementary Data **Table S3**). Five of the six patients with CNS PD (83%) had concomitant *TP53* mutations at baseline vs. 68% who had no or stable CNS disease (chi square $p = 0.118$). In four patients the primary mutation was detectable in plasma at baseline, but all converted to undetectable after 6 weeks (100% vs. 50%; chi square $p = 0.060$).

Multivariate Analysis

Multivariate analysis with Cox regression was performed for complete plasma conversion of the primary *EGFR* mutation at week 6 and presence of the *TP53* mutation at baseline. In the total cohort, complete plasma conversion was significantly correlated with PFS; HR 0.23, 95% CI 0.07–0.77, $p = 0.017$. However, *TP53* was not significantly correlated with PFS; HR 2.71, 95% CI 0.72–10.23, $p = 0.143$.

Discussion

The emergence of EGFR-TKIs has led to an astounding improvement in survival of patients with *EGFR*-mutated lung cancer. However, a minority of patients do not benefit and have no or just a short-living response. The use of liquid biopsies is promising, as it is a minimal invasive procedure, which is easily performed in clinical practice during routine outpatient clinic visits. Therefore, in this prospective study, both cfDNA and TKI drug concentrations were monitored during EGFR-TKI treatment to identify predictive markers to be used in clinical practice.

First of all, this study shows that absence of complete plasma conversion of the primary *EGFR* mutation at either week 6 or 12 was associated with a significantly shorter PFS and OS. This detrimental effect was demonstrated in the total *EGFR* cohort, as well as in the treatment cohorts. These results therefore do not only support the finding that disappearance of the primary *EGFR* mutation in plasma is associated with a better outcome in first line, but also confirm its predictive value in the second line for osimertinib-treated patients with *EGFR* p.T790M.[24-26] The concept of losing detectable *EGFR* mutations in plasma in association with better outcome was recently also demonstrated at other centers, but often with less sensitive techniques (e.g., Cobas®) than our NGS panel, or cross-sectional at a single time point rather than sequentially analyzed as a change in time.[25-29] In our second line cohort, isolated *EGFR* p.T790M loss in plasma at week 6 was associated with decreased PFS (5.1 vs. 18.8

months). This has been previously observed and may be explained by the emergence of a pre-existing resistant sub-clonal population.[30, 31]

Second, this study shows strong signs of the detrimental effect of a baseline concomitant *TP53* mutation, despite the limited size of the study population. In patients treated with osimertinib, cases who harbored a *TP53* mutation at baseline had a significantly shorter PFS than *TP53* wild-type patients (8.8 vs. 18.8 months). *TP53* mutations were also more common among the short responders in the total cohort. As one of the main functions of *TP53* is the prevention of accumulation of genetic defects, a dysfunctional p53 could result in increased genomic instability and faster development of resistance mechanisms.[32] *TP53* mutations in our cohort, as in general, do not seem to develop during treatment, for they are described to occur early in oncogenesis.[33] Worse outcome associated with *EGFR*-and *TP53*-mutated NSCLC has been well established and our findings are in line with these results.[5, 34] Besides *TP53* mutations, other concomitant mutations were also found. There was a relative increase in concomitant genetic aberrations between baseline and radiologic progression (more than one concomitant mutation in 27% and later 52% of samples). However, because of limited coverage of the cfDNA panel used, it was not possible to prove a relation between multiple concomitant mutations or *TP53* mutational status and PFS based on plasma analysis, although this association has been previously described in tissue.[5, 6] Next, the *Rb1* gene, associated with SCLC histological transformation, was not covered by the NGS panel. Nevertheless, in this study the patients with SCLC transformation had a distinct mutational profile, which included *TP53* and *PIK3CA*, already at baseline, suggesting *PIK3CA* might also play a role in this dedifferentiation.[35]

Third, this study found evidence for a relationship between erlotinib C_{mean} during treatment and PFS. Patients with a decrease of C_{mean} of 10% or more 2 months prior to PD compared to first 6 weeks had a significantly shorter median PFS (4.7 vs. 7.1 months). This result should be interpreted with caution though, since only five patients were included in this analysis. Additionally, patients with a decrease of C_{mean} of 10% or more in the second tertile compared to the first tertile had an significantly lower PFS compared to patients with an equal or increased C_{mean} (8.9 vs. 23.6 months). This decrease could be caused by multiple factors, i.e., dose reductions due to intolerable toxicity, increase in smoking behavior, decrease in therapy adherence, or use of concomitant interacting medication.[13] The contribution of dose reductions is illustrated by a higher prevalence of dose reductions and a significant shorter median time to severe toxicity in the C_{mean} decreased group. Before extrapolation to clinical practice, these results should be validated in a larger cohort.

The same percentage of patients had CNS disease at start of therapy in both cohorts. However, when CNS disease progression occurred, this percentage was almost twice as high in the first line cohort compared to the second line cohort where all patients

were treated with osimertinib (19% vs. 10%). These results are in line with the FLAURA study results, that conclude that patients with *EGFR*-mutated NSCLC and CNS disease should preferably be treated with osimertinib.[3]

Additional to the advantage of being a non-invasive procedure, the difference in the number of plasma cfDNA samples vs. tissue samples at baseline (92% vs. 78%) and at progression (82% vs. 54%) in our study reflects the feasibility of blood sampling. Moreover, plasma sampling reflects the total of genomic aberrations and heterogeneity of the disease. When in the near future reliable detection of translocations and amplifications in cfDNA is possible, plasma diagnostics could replace tissue-based investigation in current daily practice in some cases.

Limitations of this work are, first the limited number of included patients which limits the power for some of the analyses. Moreover, ctDNA could not be detected in all patient samples (ctDNA detected in 31 of 38 patients). In theory, this limits the sensitivity of mutation detection in plasma. However, patients without detectable ctDNA at baseline had a lower rate of radiological progression during follow-up compared to patients with detectable ctDNA baseline. The association between detectable ctDNA at baseline and shorter PFS and OS was previously reported by Buder et al.[26] This could be explained by a lower tumor load or intrathoracic/-cerebral localization which is associated with less shedding of cfDNA by the tumor.[20, 36] Additionally, the coverage of the mutation spectrum in the Oncomine lung cfDNA panel is a limitation in the detection of the aberrations, specifically in the *TP53* gene in our study. At this point, the NGS panel is more extensive for tissue than for plasma, which mostly explains the difference in detection of *TP53* mutations between tissue and plasma, see Supplementary Data **Table S4**. Other reasons could include limited input of DNA, or absence of cfDNA in plasma, excreted by the tumor. As false negative results are an important limitation, the occurrence of "false" positive mutations derived from cfDNA is also a realistic limitation. Plasma cfDNA analysis cannot make a distinction between mutations originating from the tumor or somatic mutations from nonmalignant peripheral blood cells, known as clonal hematopoiesis.[37, 38] One possibility to overcome this limitation is to concurrently sequence peripheral blood cells.[37] In our study, *TP53* mutations found in plasma were matched with tissue samples. One *TP53* mutation detected in plasma did not match the *TP53* mutation observed in tumor tissue and was thus labeled as clonal hematopoiesis. When interpreting plasma cfDNA analysis clonal hematopoiesis should be considered in order to prevent misdiagnosis of malignancies. However, when plasma cfDNA analysis is used as a predictive and follow-up tool in *EGFR*-mutated NSCLC, we believe this is less relevant as mutations can be matched to tumor tissue.

This study shows multiple predictive features in plasma to identify patients with less benefit of EGFR-TKI treatment. The absence of complete plasma conversion of the primary *EGFR* mutation is associated with significant shorter PFS and OS, as well

as *EGFR* p.T790M loss alone in plasma in patients treated with osimertinib in second line. Decrease of C_{mean} in time is also associated with shorter PFS in erlotinib treatment. The presence of TP53 mutations at baseline was associated with a shorter PFS.

After validation of these results in a larger and independent cohort, implementation to clinical practice seems practically possible when at start of EGFR-TKI treatment, cfDNA and PK samples are taken. At baseline, patients with *TP53* mutations on second line osimertinib treatment could already be considered in danger of progressing early. If cfDNA levels of the primary mutation are still detectable after 6 weeks of treatment, patients are at high risk of a short PFS, which could guide the treating clinician to more close follow-up or consideration of more extensive treatment options. For erlotinib-treated patients, monitoring of plasma drug concentrations is feasible. When either a 10% or more decrease in erlotinib C_{mean} occurs after 20 weeks, or when treatment conditions change (for example when C_{mean} decreases because a dose reduction is necessary) this could indicate to a shorter PFS as well. Altogether, the treating physician could use plasma predictive features to consider if closer follow-up or more extensive treatment might be necessary to personalize treatment of patients with *EGFR*-mutated NSCLC.

Materials and Methods

The START-TKI study is a prospective, observational multicenter study in which additional blood samples are drawn to standard outpatient visits in patients treated with a TKI for driver mutation driven NSCLC. The study was approved by the medical ethical committee of the Erasmus MC, Rotterdam, The Netherlands (MEC 16-643), and initiated at two sites; Erasmus MC, Rotterdam and Amphia hospital, Breda, The Netherlands. Written informed consent was obtained from all patients prior to study enrollment.

Study Population

Patients were eligible for the *EGFR* cohort when harboring an activating *EGFR* mutation for which first or second line EGFR-TKI treatment was initiated.

Blood Collection and Processing

Blood samples were collected at baseline, prior to start of EGFR-TKI, and at every outpatient clinic visit following standard of care (i.e., every six weeks) until progressive disease (PD) or end of treatment due to toxicity. For cfDNA isolation, two Cellsave preservative 10mL vacutainer tubes (CellSearch, Menarini Silicon Biosystems, Castel Maggiore, Italy) were drawn. The cfDNA handling has been previously described.[39] Additionally, a 4.0 mL lithium-heparin tube was taken for pharmacokinetic analysis.

Next Generation Sequencing (NGS)

NGS was performed at baseline, at week 6 and 12 and at PD on isolated cfDNA from plasma and tissue if available.[39, 40] NGS was performed by semiconductor sequencing with the Ion S5 System (Thermo Fisher Scientific) with the supplier's materials and protocol. Libraries from tissue DNA were prepared with a custom-made primer panel. cfDNA library preparation was performed using the Oncomine Lung cfDNA Assay v1 (Thermo Fisher Scientific), coverage is available in the Supplementary Data **Table S5**. Standard depth of sequencing was 25.000× on average. Our NGS process including the customized tissue panel has been described in more detail earlier.[39, 40] NGS on tumor tissue at diagnosis was done as part of routine clinical care.

Plasma Drug Concentrations

Patients were asked to delay the intake of TKI tablet until after blood withdrawal. All samples were analyzed with validated liquid chromatography-tandem mass spectrometric assays.[41, 42] Additionally, samples from patients included in this study who also participated in a previous pharmacokinetic study with erlotinib were integrated.[43] Time between last intake and blood withdrawal was calculated with the patient reporting time of last intake. Concentrations from samples collected after the label's time to reach maximum concentration (T_{max}; *c.q.* 4 h for erlotinib and 6 h for osimertinib) were extrapolated to the trough concentration at 24 h ($C_{24\,h}$) after drug intake with the following equation:

$$C_{24\,h} = C_{sample} * e^{(-T_{to\ 24h} * 24 * \frac{0.693}{T_{half}})}$$

$C_{24\,h}$ data for each patient were used to calculate the mean plasma concentration (C_{mean}). The C_{mean} of total treatment duration was analyzed with Cox regression to study the relation between drug exposure and PFS. The C_{mean} for the first six weeks of treatment and two months before PD were calculated. Hypothetically, a decrease in C_{mean} during treatment would be an explanation for PD. To further investigate whether changes in C_{mean} could have an influence on PFS, C_{mean} was calculated for every third of total treatment duration per patient (i.e., tertiles). Changes in C_{mean} during treatment were subtracted from these data. To compare the forthcoming changes in C_{mean} between tertiles, two groups have to be defined; one in which C_{mean} decreases and one in which no change or even increase in C_{mean} occurs. Additionally, C_{mean} was calculated for time until the occurrence of severe toxicity.

Toxicity

Toxicity was scored by the treating physician according to the Common Terminology Criteria for Adverse Events (CTCAE) grading system version 4.03.[44] Severe toxicity was defined as CTCAE grade ≥ 3 or hospital admission, dose reduction, and treatment discontinuation or stop because of TKI-related toxicity.

Brain Metastasis

Patients who had CNS metastasis at baseline and at PD were identified. Mean TKI concentrations were compared for CNS metastasis at PD. Also the presence of *TP53* mutations and complete plasma conversions was compared between patients with and without CNS metastasis.

Objectives

Main objectives were exploration of the predictive value of the presence of concomitant mutations in cfDNA at baseline and TKI plasma trough concentrations (C_{min}) during treatment for PFS. In addition, the associations between change in plasma mutation levels over time and PFS and OS were analyzed. Plasma and tissue mutations were analyzed and compared between different time points.

Additionally, the correlation between TKI plasma trough concentration and occurrence of severe toxicity and the relationship between brain metastasis and pharmacokinetic parameters was explored.

Statistical Analysis

PFS was defined as time from start TKI until radiologic progression or death, OS as time from start TKI until death. Short responders were defined as a PFS < 6 months. We defined plasma conversion as the shift from detectable to undetectable mutation status in plasma. Patients who were enrolled twice (both in first and second line treatment) were included for separate analyses in treatment cohorts (1st line cohort and 2nd line cohort). For analysis of the total population, from these patients only data from the second line cohort were used.

The relationship between PFS and presence of concomitant mutations, *TP53* mutations specifically, plasma mutation conversion and changes in C_{mean} was explored by the log-rank test on Kaplan Meier survival analysis. Kaplan Meier analysis was used for estimation of median survival times and 95% confidence intervals. In case of a limited number of events at data cut off the 95% confidence interval could not be calculated. Differences between groups were compared with Pearson chi-square tests (i.e., for prevalences) or the T-test (i.e., for mean concentrations). To correlate the influence of multiple variables on PFS, Cox regression was performed on the variables that were significantly ($p < 0.05$) correlated with PFS in univariate analysis in the total *EGFR* cohort. IBM SPSS Statistics version 25 was used for all analyses.

Conclusions

Not all patients treated with EGFR-TKIs benefit from a durable response, but predictive markers to identify these short responders are lacking. This prospective blood-based biomarker study, START-TKI, in *EGFR*-mutated NSCLC patients reports poor predictive

markers based on cfDNA and TKI drug concentrations during EGFR-TKI treatment which have potential to be used in clinical practice in the future. Absence of complete plasma conversion of the primary *EGFR* mutation at week 6 and 12 was correlated with significantly shorter PFS and OS. Concomitant *TP53* mutations at baseline also showed signs of detrimental outcome. Patients treated with erlotinib who had a decrease in mean plasma drug concentration of 10% or more during treatment had worse PFS, but in a small cohort. Validation in a larger cohort is preferred. Implementation of these plasma predictive features could aid a physician to consider for which EGFR-TKI-treated patients closer follow up or more extensive treatment might be necessary.

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Supplementary Material

Table S1. Causes of death events in cases without radiologic progression:

1. multi-organ failure associated with empyema after chest tube placement
2. hypoxemia in pulmonary embolism
3. hypoxemia due to pneumonitis (probably osimertinib related)
4. sudden onset of dyspnea at home, presumably because of pulmonary embolism or a cardiac event

Table S2A. PFS and plasma conversion at week 6 (treatment cohorts).

Cohort	Plasma conversion	n/events	Median PFS (months)	95% CI	Log rank *p*=
First line	Yes	6/5	7.1	0.0-14.8	0.014
	No	1/1	1.4	NA	
Second line	Yes	11/6	18.8	7.5-30.1	0.011
	No	12/11	5.5	4.4-6.5	

Table S2B. PFS and plasma conversion at week 12 (treatment cohorts).

Cohort	Plasma conversion	n/events	Median PFS (months)	95% CI	Log rank *p*=
First line	Yes	6/5	7.1	0.0-14.8	0.014
	No	1/1	1.4	NA	
Second line	Yes	11/6	15.4	9.3-21.5	0.000
	No	12/11	5.1	4.6-5.7	

Table S3. PK: Cmean in patients with CNS progressive disease.

Erlotinib	1390 vs. 1015 ng/mL; p = 0.461
Osimertinib	230 vs. 188 ng/mL; p = 0.097

Table S4. Coverage discrepancies of the detected mutations in our study.

Mutation	Covered by plasma panel	Covered by tissue panel
APC c.4399_4400dupCC; p.K1468Lfs*	No	Yes
ARAF c.558-1G>A;p.? VUS	No	Yes
BRAF c.1799T>A; p.V600E	Yes	Yes
BRAF p.469A	Yes	Yes
CDKN2A c.159G>A; p.M53I	No	Yes
CDKN2A c.163G>T; p.G55C	No	Yes
CDKN2A c.250G>A; p.D84N VUS	No	Yes
CDKN2A c.355G>T; p.E119*	No	Yes
CDKN2A homozygote deletie	No	Yes

Table S4. Continued

Mutation	Covered by plasma panel	Covered by tissue panel
CTNNB1 c.94G>T; p.D32Y	No	Yes
CTNNB1 c.110C>T; p.S37F	No	Yes
EGFR c.2170G>A; p.G724S	No	Yes
EGFR p.C797S (c.2389T>A, in CIS)	Yes	Yes
EGFR p.C797S (c.2390G>C, in CIS)	Yes	Yes
EGFR p.V769M, c.2305G>A	Yes	Yes
ERBB2 c.1963A>G, p.(Ile655Val)	No	Yes
ERBB2 c.2066G>A, p.(Arg686),	No	Yes
MAP2K1 p.E203K	Yes	Yes
MET amplificatie	No	Yes
MTOR c.7291C>A; p.L2431M	No	Yes
NTRK1 amplificatie	No	Yes
PIK3CA c.1636C>G; p.Q546E	Yes	Yes
PIK3CA c.3145G>C; p.G1049R	Yes	Yes
PIk3CA p.E542K	Yes	Yes
PIK3CA p.E545K	Yes	Yes
PIK3CA p.E545Q	Yes	Yes
PTEN c.388_400del; p.R130*	No	Yes
PTEN p.L320S, c.959T>C	No	Yes
PTEN c.131G>A; p.G44D	No	Yes
TP53 c.243_244dup; p.P82Hfs*42	No	Yes
TP53 c.536A>G;p.H179R	Yes	Yes
TP53 c.395A>T; p.K132M	No	Yes
TP53 c.469G>T; p.V157F	Yes	Yes
TP53 c.673-1G>T; p.?	Yes	Yes
TP53 c.713G>T; p.C238F	Yes	Yes
TP53 c.733G>A; p.G245S	Yes	Yes
TP53 c.797G>T; p.G266V	Yes	Yes
TP53 c.892G>T; p.E298*	No	Yes
TP53 c.646G>A; p.V216M	Yes	Yes
TP53 c.97-1G>T, p.?	No	Yes
TP53 c.339_341del;p.F113del	No	Yes
TP53 c.560delG; p.G187Vfs*60	No	Yes
TP53 p.C135Y, c.404G>A	No	Yes

Table S4. Continued

Mutation	Covered by plasma panel	Covered by tissue panel
TP53 p.C176Y, c.527G>A	Yes	Yes
TP53 p.C238Y	Yes	Yes
TP53 p.C242*, c.726_744del	Yes	Yes
TP53 p.K132E, c.394A>G	No	Yes
TP53 p.M237Ifs*9, c.711_714del	Yes	Yes
TP53 p.N310Tfs*35, c.927_928delinsG	No	Yes
TP53 p.P223L, c.668C>T	Yes	Yes
TP53 p.R248L, c.743G>T	Yes	Yes
TP53 p.R273H	Yes	Yes
TP53 p.R337C, c.1009C>T	Yes	Yes
TP53 p.S241F, c.722C>T	Yes	Yes

Table S5. Coverage of the Oncomine Lung cfDNA assay v1.

Gene count	11
Gene names	ALK, BRAF, EGFR, ERBB2, KRAS, MAP2K1, MET, NRAS, PIK3CA, ROS1, TP53
Amplicons	35
Hotspots	169

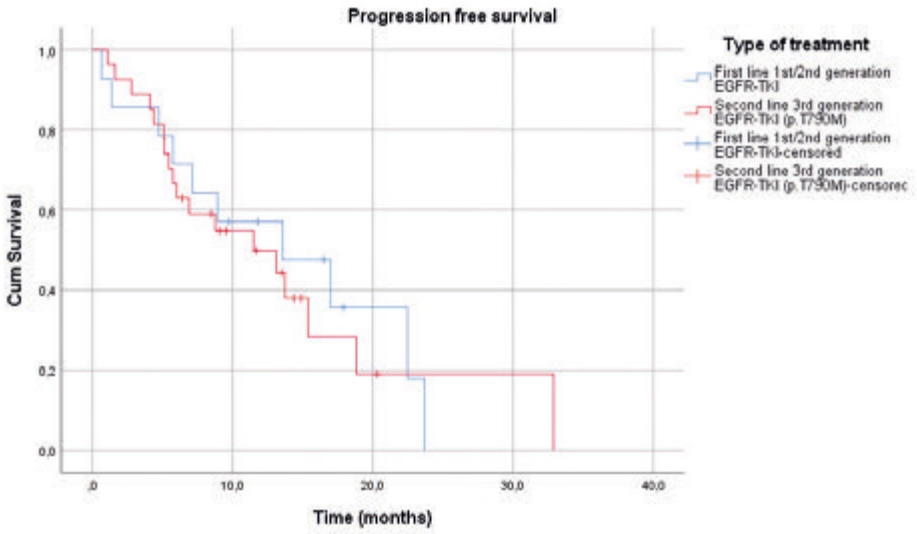


Figure S1. PFS in the total *EGFR* cohort according to treatment line. *n*=41, *p* = 0.768.

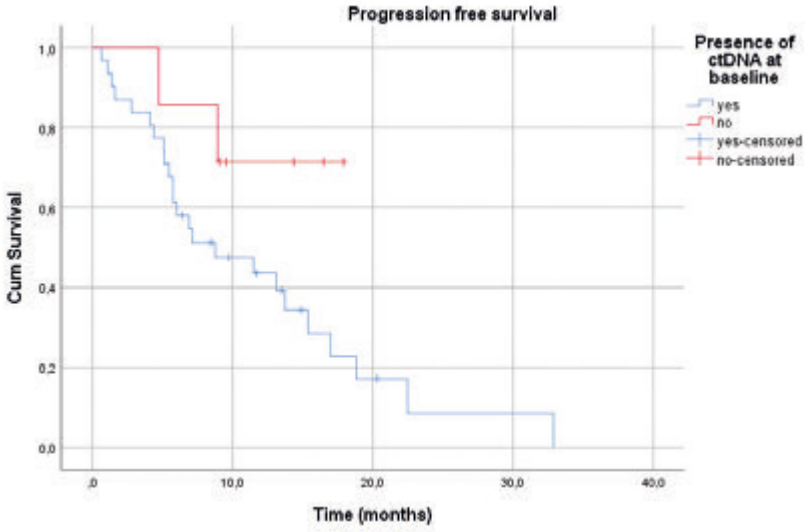


Figure S2. PFS in presence or absence of detectable ctDNA (*EGFR* mutations in plasma). *n*=38, *p* = 0.108.

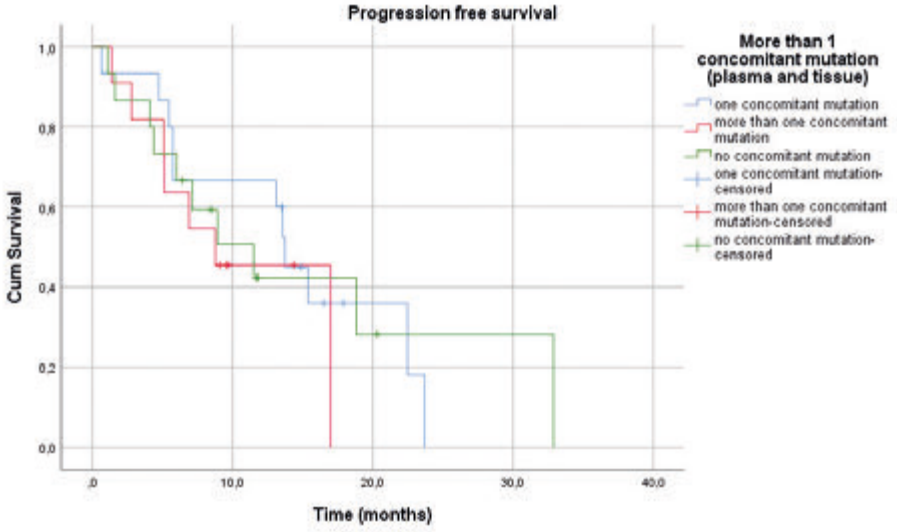


Figure S3. PFS in patients with concomitant mutations (besides *EGFR*). *p* = 0.734.

Concomitant mutations	*n*=	Events	Median PFS (months)	95% CI
0	15	10	11.5	4.6-18.4
1	15	11	13.7	12.7-14.8
>1	11	7	8.8	3.3-14.3

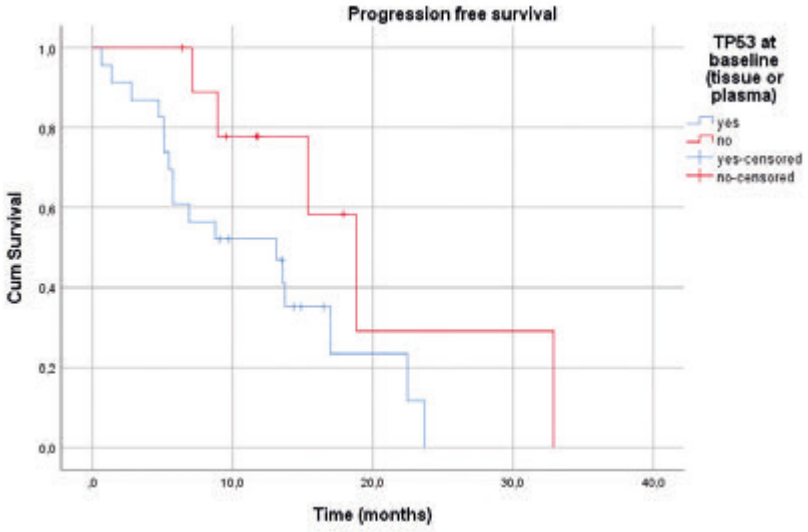


Figure S4A. PFS and *TP53* mutational status in the total *EGFR* cohort. *n*=33, p=0.068.

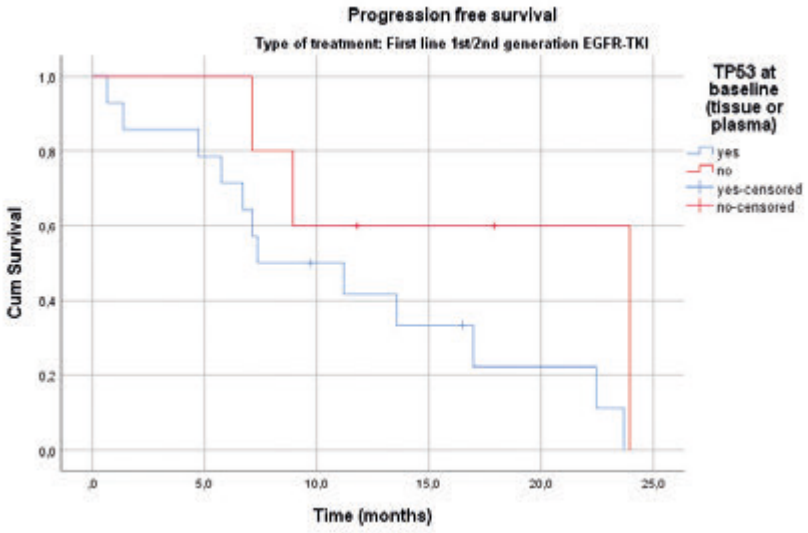


Figure S4B. PFS and *TP53* mutational status in the first line cohort. *n*=19, *p* = 0.116.

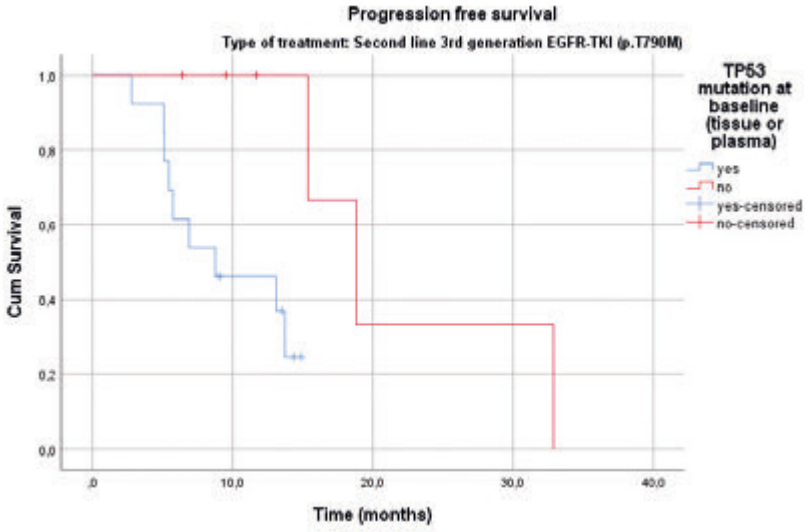


Figure S4C. PFS and *TP53* mutational status in the second line cohort. *n*=19, *p* = 0.017.

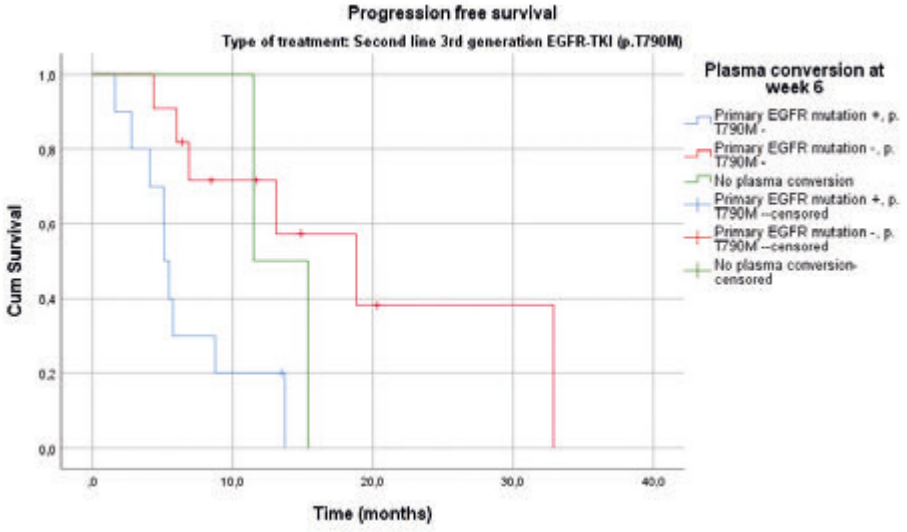


Figure S5. PFS and plasma conversion in the second line cohort. *n*=23, *p* = 0.012.

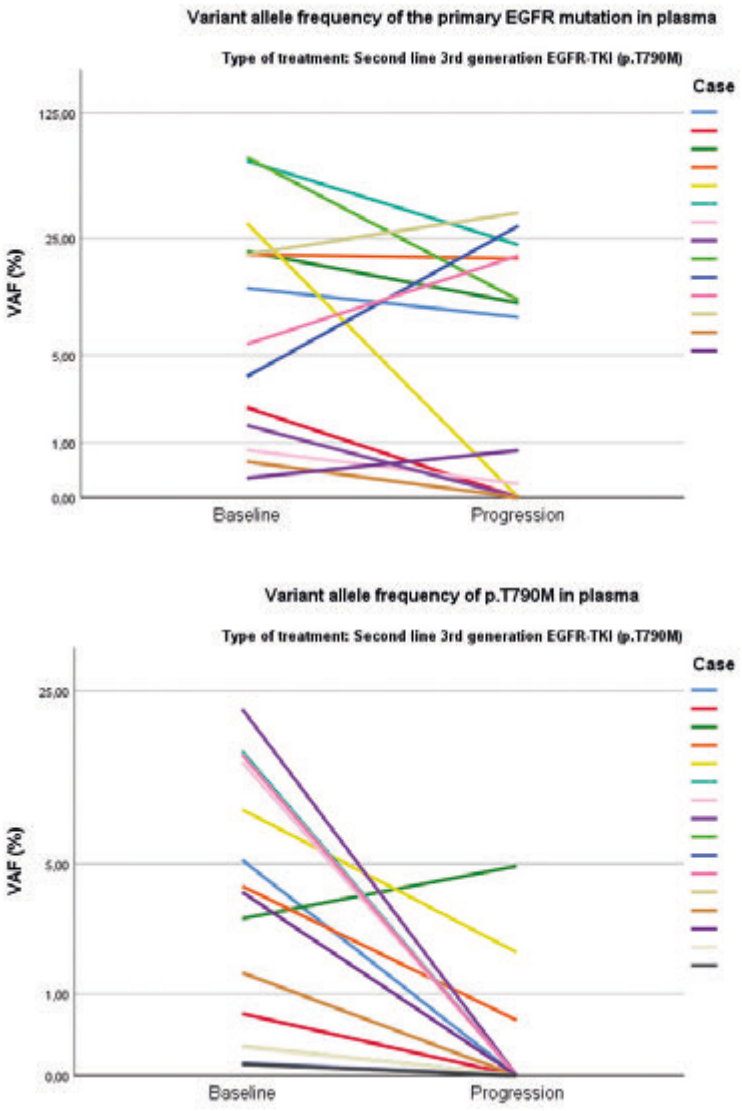


Figure S6. Plasma mutation levels in the second line cohort.

Chapter 8

Summary, General Discussion and Future Perspectives

Summary

The introduction of this thesis (**Chapter 1)** describes the burden of lung cancer, and the evolvement of identification of EGFR as a target of treatment. In this thesis we searched for innovations in optimizing treatment of NSCLC with EGFR-TKI, and along the journey we gained insights which will be discussed below.

Part A Treatment strategies in clinical practice

Based on earlier signs of clinical activity of the 1st generation EGFR-TKI in combination with docetaxel in patients with *EGFR*-WT NSCLC, among others in the phase II NVALT10 trial, a beneficial effect of intercalated erlotinib to docetaxel treatment was hypothesized.[1]

In **Chapter 2** we describe the results of the randomized phase III NVALT18 trial. The study was terminated prematurely due to slow accrual, mainly because of the introduction PD-(L)1 inhibitors as second line treatment. Forty-five patients with *EGFR*-WT NSCLC were randomized between docetaxel plus intercalated erlotinib (*n*=22) or docetaxel monotherapy (*n*=23) after progression on a platinum based chemotherapy regimen.[2] The detrimental effect of the combination was evident with shorter PFS (1.9 vs 4.0 months, p=0.01) as well as OS (4.7 vs 10.6 months, p<0.001) compared to the control docetaxel monotherapy arm. Toxicity was also higher with toxicity ≥CTCAE grade 3 in 77% of the patients treated with the combination in contrast to 26% in the control arm. Although reasons for this are largely unknown, there are some possible hypotheses. One implies that an antagonistic effect may still occur despite the intercalated scheme due to inadequate wash out in the long run because of continuous administration of treatment cycles and persistent activity of erlotinib at the intracellular level. This is based on earlier observed persistent erlotinib detection in plasma after the 5 day wash out period and in tissue specimen after resection and neoadjuvant erlotinib treatment.[1, 3] The other possible explanation is the suspicion of a relevant pharmacokinetic interaction of erlotinib and docetaxel which can lead to higher docetaxel exposure, similar as was noted for the combination of pazopanib with docetaxel.[4] These detrimental results strongly discourage use of combination therapy with docetaxel and intercalated erlotinib in further research or clinical practice.

The exploration of the toxic limit of the 3rd generation EGFR-TKI osimertinib in patients with *EGFR*-mutated NSCLC is found in **Chapter 3**. Additional blood samples were taken every outpatient visit in the real-world prospective cohort START-TKI study. Plasma was analyzed on osimertinib concentration, and patients were followed for severe toxicity (CTCAE grade 3 or higher, leading to drug discontinuation, dose reduction or hospital admission). The correlation between osimertinib exposure (defined as clearance) and severe toxicity, as well as the exposure-efficacy relationship were investigated. A total of 159 patients donated 819 samples. Osimertinib clearance (exposure) was

significantly correlated with severe toxicity in multivariate competing risk analysis (HR 0.91, 95% CI 0.83-0.99), with an optimal toxic limit of 259 ng/mL determined by ROC-curve. A dose reduction to 40mg QD from 80mg QD in the high exposure group would reduce the risk of severe toxicity from 34% to 14%. This effect was preserved when concentrations available in the first two months of treatment (*n*=90) were analyzed, with a risk reduction of severe toxicity from 31% to 17%. There was no correlation between osimertinib exposure and OS or PFS. Patients with an osimertinib concentration >259ng/mL could benefit from a preventive dose reduction, these results should be prospectively validated.

The results of chemotherapy regimens after progression on EGFR-TKI in patients with *EGFR*-mutated NSCLC are presented in **Chapter 4**. Although this study was retrospective and the cohort rather heterogeneous, the efficacy of two commonly used chemotherapy regimens in first line chemotherapy, platinum/pemetrexed and carboplatin/paclitaxel/bevacizumab/atezolizumab (according to the IMPOWER150) respectively, were quite similar and provided substantial benefit for patients.[5] For platinum/pemetrexed as first line chemotherapy, PFS was 5.1 months and OS 15.2 months, for carboplatin/paclitaxel/bevacizumab/atezolizumab this was 5.8 months and 14.9 months respectively.

Furthermore the PFS of patients treated with weekly paclitaxel and bevacizumab as used in the ULTIMATE trial, mostly prescribed in second line and further, resembled the PFS in the original trial (4.9 vs 5.4 months respectively) despite the real world setting, and even in patients with lack of response on a platinum/pemetrexed regimen.[6] This suggests that even in patients pretreated with chemotherapy, the paclitaxel/bevacizumab regimen can be of added value.

The number of patients with carboplatin/paclitaxel/bevacizumab treatment was small (*n*=8), as this regimen is less often prescribed in the Netherlands, and therefore bias is probably coming into play.

In conclusion, patients do benefit substantially from chemotherapy after progression on EGFR-TKI in our real-world study, albeit shorter than the duration of response of first line targeted therapy.

Part B Focus on plasma

In **Chapter 5** plasma ddPCR (Biorad) and NGS (Oncomine cfDNA lung assay v1) are compared to tissue NGS results in patients with *EGFR*-mutated NSCLC. The plasma results showed high agreement on the level of a specific mutation like p.T790M and p.L858R, but NGS provides a broader coverage and is therefore able to detect more different driver mutations.[7] Concordance of plasma with tissue is high, but as to be expected as a biopsy shows the genetic status of a specific clone while plasma represents the genomic overview of the disease as a whole, plasma and tissue showed

to be complementary. Although plasma detection is limited by the ability of a tumor to shed ctDNA as shown by the patients in whom no mutations were found in plasma (6/36), the additional value of plasma analysis is evident from the patients where the p.T790M resistance mutation was detected in plasma but not in tissue (3/36).

Taking the plasma cfDNA NGS a step further, in **Chapter 6** we searched for targetable mutations in a population with suspected metastasized lung cancer when molecular analysis of tissue was not feasible.[8] In a period of 2 years, a total of 55 plasma samples from patients in the Comprehensive Cancer Network Southwest (Erasmus MC) were analyzed. A possible target for treatment was detected in 7 patients. In 1 patient targeted treatment was successfully started upon detection of an *EGFR* exon 19 deletion. One patient with an *EGFR* exon 20 insertion was referred and informed on targeted therapy in a clinical trial but waived this opportunity. For the patients with *KRAS* p.G12C and *BRAF* non-V600E mutations targeted treatment in first line was not yet available at the time of detection. Although the coverage of the Oncomine V1 panel was a limiting factor in the ability to detect all possible targets for treatment which are expanding rapidly, we still were able to identify patients with a possibility of targeted treatment who were otherwise not detected.

Chapter 7 outlines the course of mutations in cfDNA upon start of targeted therapy, and identifies predictive features in plasma during treatment with EGFR-TKI in patients with *EGFR*-mutated NSCLC.[9] Additional blood was drawn at outpatient clinic visits, and analyzed for mutations with NGS and drug concentrations. Plasma clearance of the primary *EGFR* mutation, and of p.T790M when applicable, after 6 and 12 weeks of therapy was evaluated. The absence of this so called plasma conversion correlated with a shorter PFS (5.5 vs 17.0 months, p=0.002) and OS (14.0 vs 25.5 months, p=0.003). Furthermore, presence of TP53 mutations at baseline in the second line osimertinib group correlated to a shorter PFS (8.8 vs 18.8 months, p=0.017). In addition, a decrease of the erlotinib concentration in the second tertile of treatment showed a correlation to a shorter PFS (8.9 vs 23.6 months, p=0.037).

General discussion and future perspectives

Targeting EGFR has been in the spotlights for the past two decades, with impressive progress from identification of patients who benefit from this treatment to the development of new agents. However, there is still room for improvement in terms of identifying those patients with less benefit or more toxicity who will need review of systemic options at short notice compared to those with long clinical benefit. This thesis can provide suggestions for tools to optimize the current practice of radiological and clinical evaluation, and for future directions in research and clinical practice.

EGFR-WT NSCLC

Although the EGFR pathway plays a role in cell proliferation and division in general and in carcinogenesis in particular, the role of EGFR-targeted therapy in patients with *EGFR*-WT NSCLC appears to be limited. Erlotinib is approved as second line (and beyond) therapy after progression on platinum based regimens based on an earlier phase III trial, where erlotinib showed improved OS compared to placebo (6.7 vs 4.7 months, $p<0.001$).[10] However, research thereafter showed no superiority to chemotherapy, and even shorter PFS for erlotinib than chemotherapy in case of *EGFR*-WT NSCLC.[11-16] It seems that EGFR is not the main driving force of the disease in absence of an oncogenic driver mutation, and targeting the receptor in combination with other systemic therapy does add toxicity and possible drug interactions with other agents. Therefore the focus in this patient group should be on optimizing outcomes with current available first line (chemo-immunotherapy) regimens and finding alternatives for treatment in further lines that outshine docetaxel monotherapy in means of response rate, toxicity, PFS and OS.

EGFR-mutated NSCLC

Osimertinib

As osimertinib is the current preferred EGFR-TKI for first line treatment of *EGFR*-mutated NSCLC, finding the right dose in the right patient seems the logical next step. Our findings provide a practical tool: a toxic limit to identify those patients at risk of severe toxicity. At the time of response evaluation, determining the osimertinib concentration at the standard-of-care blood draw and applying a dose reduction in patients with a plasma concentration ≥259 ng/mL could significantly reduce risk of severe toxicity. As our prospective cohort study had an observational study design, it would be good to confirm these results prospectively and evaluate the incidence of severe toxicity and efficacy in terms of PFS and OS in clinical practice. A randomized trial design could be proposed, with inclusion of patients at the start of osimertinib treatment 80mg QD and subsequent blood sampling at every outpatient visit for osimertinib concentration measurement, with half of the patients treated according to standard-of-care and the

other half of the patients treated with a dosing advice at week 4 based on the measured osimertinib concentration and our defined toxic limit of 259 ng/mL.

Although therapeutic drug monitoring (TDM) provides a tool for optimizing toxicity and efficacy balance in patients, and can be able to decrease the financial toxicity of these expensive targeted drugs by reducing the total amount of drug needed, the implementation in clinical practice is not on track with current insights. This might partly be due to lack of knowledge on the subject by some treating physicians and missing optimal logistic routing to access TDM. There is a spectrum of advanced implementation in specialized tertiary centers focusing on pharmacokinetic monitoring, to just following the pharmaceutical registration prescription information in the smaller regional centers. In the case of osimertinib, the blood sample should be processed on ice for a reliable measurement of the drug concentration since the stability of osimertinib is poor at higher temperatures.[17] These challenges emphasize the importance of sharing knowledge on TDM and collaboration in comprehensive cancer networks to optimize the possibility of applying TDM within all associated smaller and larger medical centers in close collaboration.

Chemotherapy

In our real world retrospective study of chemotherapy regimens in patients with *EGFR*-mutated NSCLC after progression on EGFR-TKI, PFS was substantial for platinum/pemetrexed, carboplatin/paclitaxel/bevacizumab/atezolizumab and weekly paclitaxel/bevacizumab, but not as long as we are used to in targeted treatment. On our retrospective heterogeneous cohort we cannot draw conclusions on which regimen seems most effective. The choice of agents will also be related to specific patient situations, such as the physicians assessment whether it is necessary to continue osimertinib along the chemotherapy because of its CNS activity in case of cerebral metastasis. Nevertheless, chemotherapy remains a useful tool for palliative treatment in cases with progressive disease on TKI without a detected targetable resistance mechanism. It is also observed that after a pause in the targeted therapy ('drug holiday') and treatment with chemotherapy in the meantime, a rechallenge with EGFR-TKI upon new progression can result in new responses.[18, 19]

In case of oligoprogression, local ablative therapy is often used to treat the site of progression in order to be able to continue the current line of treatment. This strategy is applied in clinical practice during treatment with EGFR-TKI as well as during other systemic therapy.[20]

Treatment strategies

Open questions remain about the treatment strategies in advanced or metastasized *EGFR*-mutated NSCLC in first line. Although third generation EGFR-TKI osimertinib is considered the current standard treatment, there is also evidence of combining first

generation EGFR-TKI with anti-angiogenic or cytotoxic agents to increase mPFS and still preserve the possibility of treatment with osimertinib in a later line when *p.T790M* develops as a resistance mechanism.[21-26] Another explored strategy is adding intercalated EGFR-TKI to chemotherapy to overcome the possible antagonistic effect of concomitant administration. Although the earlier trials in unselected patient populations showed variable results, this approach showed beneficial PFS and OS in *EGFR*-mutated NSCLC compared to *EGFR*-WT in a phase III trial where patients were randomized to platinum and gemcitabine plus intercalated erlotinib or placebo in the induction phase, followed by erlotinib or placebo maintenance treatment.[27, 28] A phase III trial in the Netherlands randomizing patients with *EGFR*-mutated NSCLC to induction cisplatin and pemetrexed plus intercalated erlotinib or daily erlotinib was ended prematurely due to low accrual and failed to show significant benefit in the low number of included patients (*n*=22).[29]

Combining agents goes at the cost of additional intravenous administration of therapy and increased toxicity. Therefore it is important for future therapeutic strategies to elucidate which patients could benefit most from a different approach than monotherapy with osimertinib. There is also ongoing research on combination therapy added to osimertinib as a new treatment strategy, which will have to be awaited before change in clinical practice could be anticipated.[30, 31] Furthermore, the question remains if all patients with *EGFR*-mutated NSCLC could have additional benefit from immunotherapy in a combination setting when immunosuppressive factors are positively influenced by concurrent therapy with carboplatin, paclitaxel and bevacizumab. Although this is suggested by the subgroup analysis of the IMPOWER150 study, the mPFS and mOS do not appear very different from platinum/pemetrexed in our retrospective cohort study.[5, 32] Investigation of efficacy of this regimen in a larger group in a prospective setting would be valuable, as additional translational exploration of the immune response in these patients could possibly shed light on this matter. If differences between responders and non-responders could be elucidated and probable non-responders could be identified, this would improve the effective use of this regimen. Although checkpoint inhibitors did not show benefit when administered as monotherapy, and two retrospective series did not show benefit of chemo-immunotherapy without anti-angiogenesis in patients with *EGFR*-mutated NSCLC, the latest ESMO consensus statement does leave room to consider the IMPOWER150 regimen as an alternative to platinum containing chemotherapy.[33-38] However a remaining point of attention are the signs of a higher risk of toxicity, in particular of pneumonitis, when treating patients with EGFR-TKI after use of checkpoint inhibitors, especially in case of osimertinib.[39-41]

Plasma cfDNA analysis

The detected aberrations in plasma represent an overview of the whole disease in the body, as opposed to a biopsy which represents the genomic profile of a specific location.[42, 43] In that respect, plasma testing complements the tissue analysis. On the other side, when no mutations are detected in plasma, the need for additional tissue

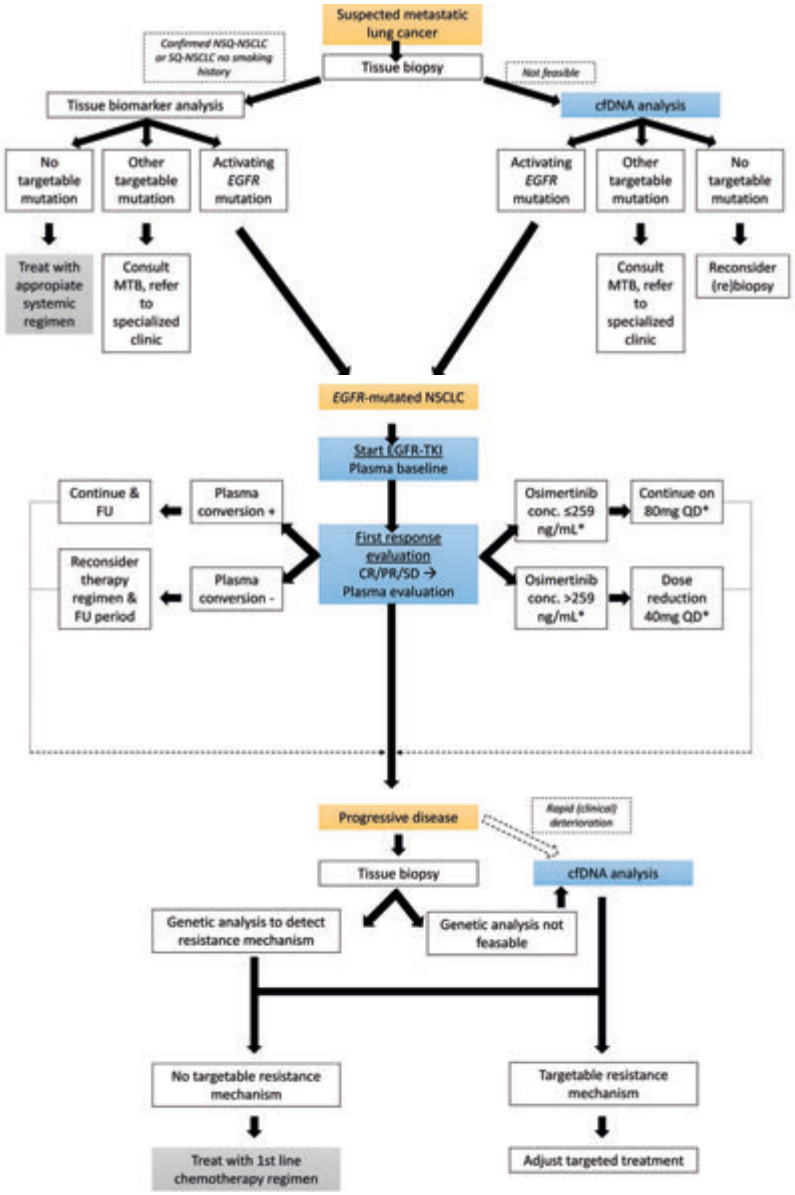
analysis in order to track down targetable (resistance) mutations remains. The amount of ctDNA that is shedded by tumors is dependent on tumor load and the location of disease.[44] Localization of tumor in the thorax or central nervous system is correlated to less detectable ctDNA in plasma.

Although different techniques have been developed over time to detect genetic aberrations of ctDNA in plasma, not all are equally performing around the lower limit of detection. At the point of disease progression, we found ddPCR and NGS performed similar.[7] However, the consumption of the available DNA isolate and therefore the absolute amount of analyzed molecules differs. Although NGS needs a lot of input to be able to reliably detect the aberrations, in the order of magnitude of 30-50ng in total, this technique is able to sequence a lot of different hotspots at once. The needed input for ddPCR is lower per specific probe (4uL of the 50uL isolate in total), but that also results in a lower absolute number of available molecules to detect and lower sensitivity in the lower VAF ranges. Although multiplex ddPCR panels are able to detect more than 1 specific aberration in a single run, the lack of ability to detect as much different genetic aberrations as NGS remains an important disadvantage. Nevertheless, as knowledge on possible targets for treatment and resistance mechanisms is expanding quickly, the swift implementation of comprehensive plasma NGS panels including amplification and fusion detection is warranted.

In our study on patients with suspected metastasized lung cancer the complementary character of the value of plasma analysis is emphasized by the patients in whom tissue analysis was performed at a later point in the disease course and proved to be other histology than lung cancer. Although plasma cfDNA analysis can be of value in detection of targetable driver and/or resistance mechanisms, for now it remains complementary to tissue analysis in case of diagnosing a driver or resistance mutation at the time of diagnosis or progression on current therapy. When looking at the course in time of the level of plasma mutations, the presence or absence of plasma conversion correlates to longer or shorter PFS and OS. This was also observed by multiple similar clinical studies.[45-49] We performed our study with plasma evaluation on week 6 and 12, but others also observed that plasma mutation levels decrease rapidly in case of response with long clinical benefit.[50] It therefore seems logical to follow the level of the primary *EGFR* mutation at the moment of the first response evaluation on EGFR-TKI in those patients with a detectable mutation level at baseline, and to reconsider the therapy regimen and follow up interval in those individuals with lack of plasma conversion based on radiological and clinical assessment.

Concluding remarks and future directions

To integrate the insights of this thesis in current clinical practice, they are summarized and schematically depicted in the flow diagrams below.



*Suggested flow diagram of use of genetic analysis of tissue and plasma in the setting of suspected metastasized NSCLC, use of plasma diagnostics after start of EGFR-TKI treatment and when eventually expected progression of disease occurs. Yellow boxes: clinical setting; blue boxes: plasma analysis; grey boxes: current advise following findings in this thesis.*prospective validation warranted*

At this moment, plasma cfDNA analysis has a complementary role to tissue analysis, but definitely has additional value in clinical practice. When detection of complex aberrations, like fusions and amplifications, and the lower limit of detection are further optimized in the future, this role will become even more solid.

With an expanding interest in artificial intelligence and mathematical models to transcend the limitations of the human mind, possibly this will contribute to further optimization of therapeutic strategies in the future. One of the issues to investigate is if the current strategy to treat metastasized cancer with the maximum tolerated dose of anticancer therapy until progression is indeed the optimal strategy to achieve the maximum gain in life expectancy and quality of life for those patients. Physicians are used to sticking to a treatment regimen until progression or intolerable toxicity occurs, and aim to maintaining the treatment line as long as possible, sometimes even with local treatment of oligoprogression to be able to continue the same agent thereafter. However in patients with *EGFR*-mutated NSCLC clinicians are also aware that targeted treatment puts pressure on a cell to develop genetic resistance mechanisms in the drugged target, bypass signaling tracks or downstream signaling pathways.[51] After progression on EGFR-TKI patients are often treated with non-selective chemotherapy in case of lack of a targetable resistance mechanism. When patients develop further progression after discontinuation of chemotherapy, it is a known phenomenon that after this so-called 'drug-holiday' new responses on restart of the earlier EGFR-TKI do occur.[18, 19] As we know that in case of advanced or metastasized NSCLC all patients develop resistance to systemic treatment irrespective of the mutational status of the tumor or the type of systemic therapy, it could be logical to discontinue systemic therapy at a point in time where the disease is partially controlled but before all cells develop resistance, in order to 'outgrow' subclonal resistance mechanisms and to preserve the option to use the agent again later in the course of the disease. In *BRAF*-mutated melanoma, a phase II trial with a time dependent intermittent dosing schedule of BRAF and MEK inhibition showed a shorter PFS according to radiological evaluation following RECIST v1.1 than continuous administration, but no difference in OS was observed.[52] However, the study had some issues with performed radiologic imaging during the off-treatment period despite the protocol instructions to only perform imaging in the on-treatment period, and no other factors were taken into account besides the set time frame of 5 weeks on- and 3 weeks off-treatment. In the future it may be necessary to adjust our tactics and apply game theory models in combination with plasma and radiological monitoring to modify our treatment plans to optimize outcomes.[53] Game theory is a mathematical frame where strategies, payoffs and dynamical interactions are modelled in search for the optimal strategy to reach the best possible outcome, with the knowledge that the desired outcome (cure) is not feasible.[53] The next challenge will be to explore this approach in metastasized lung cancer, and to integrate radiological, plasma ctDNA and drug concentration parameters in a model to customize treatment to specific disease characteristics in the individual patient.

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Appendices

Nederlandse samenvatting

De introductie van dit proefschrift in **Hoofdstuk 1** bespreekt het veelvuldig voorkomen van de ziekte longkanker wereldwijd en de ontwikkelingen rondom de identificatie van de epitheliale groeifactor receptor (EGFR) als doelwit voor therapie. In Nederland zijn er in 2021 ruim 14.000 patiënten met longkanker gediagnosticeerd volgens de Nederlandse Kanker Registratie, waarbij in ongeveer de helft van de gevallen sprake was van uitzaaiingen. Genezing is dan niet mogelijk, wel kan met remmende therapie een verbetering van levensverwachting bereikt worden. De meest voorkomende vorm van longkanker (85%) is niet-kleincellige longkanker (NSCLC). De mogelijkheden van remmende therapie zijn afhankelijk van de eigenschappen van de kankercel, waarbij er de laatste jaren naast chemotherapie ook immuuntherapie en doelgerichte therapieën zijn ontwikkeld. De groeisignalen van EGFR spelen een belangrijke rol bij longkanker, waarbij patiënten met een mutatie in het *EGFR* gen in de kankercel (ongeveer 10% in Nederland) door aanhoudende signalen vanuit de receptor, een vooral EGFR-afhankelijke tumorgroei hebben. In dit proefschrift zoeken we naar innovaties om de behandeling van NSCLC met EGFR-tyrosine kinase inhibitoren (TKI) te optimaliseren. De verkregen inzichten worden hieronder verder besproken.

Deel A Behandelstrategieën in de klinische praktijk

Op basis van eerdere signalen van klinische activiteit van de 1e generatie EGFR-TKI in combinatie met docetaxel bij patiënten met *EGFR*-wild type (WT) NSCLC, onder andere in de NVALT10 studie, werd de nationale gerandomiseerde NVALT18 studie ontwikkeld. In deze studie werd de standaard tweedelijns behandeling, docetaxel, vergeleken met docetaxel met tussenvoeging (intercalatie) van erlotinib.

In **Hoofdstuk 2** beschrijven we de resultaten van deze gerandomiseerde fase 3 NVALT18 studie. De studie is vroegtijdig beëindigd vanwege trage inclusie, vooral door de introductie van programmed death (ligand) 1 (PD-(L)1) remmers als tweedelijns behandeling in de klinische praktijk. Vijfenveertig patiënten met *EGFR*-WT NSCLC zijn gerandomiseerd naar docetaxel plus tussengevoegde erlotinib (*n*=22) of docetaxel monotherapie (*n*=23) bij progressie na platinumbevattende chemotherapie (en eventueel ook een PD-(L)1 remmer). Het nadelige effect van de combinatie docetaxel-erlotinib is evident, met zowel een significant kortere progressie vrije overleving (PFS) (1,9 versus 4,0 maanden (p=0,01)) als overleving (OS) (4,7 versus 10,6 maanden (p<0,001)) in vergelijking met de controle arm, docetaxel monotherapie. Tevens is er meer toxiciteit gezien, met Common Terminology Criteria for Adverse Events (CTCAE) ≥ graad 3 toxiciteit bij 77% van de patiënten in de experimentele arm versus 26% in de controle arm. Hoewel de reden hiervan grotendeels onduidelijk is, zijn er wel een aantal mogelijke verklaringen. Eén hiervan impliceert dat er sprake kan zijn van een antagonistisch effect, ondanks het doordachte alternerende schema. Dat zou kunnen door inadequate uitwassing op de langere termijn door de voortdurende toedieningen

van behandel cycli en persisterende intracellulaire activiteit van erlotinib. Dat wordt ondersteund door de eerdere observatie van persisterende erlotinib spiegels van wisselende hoogte in plasma na de periode van 5 dagen uitwassing, bij een reguliere halfwaardetijd van 36 uur. Ook zijn persisterende erlotinib spiegels gezien in een studie op weefsel na resectie en neo-adjuvante behandeling met erlotinib. Een andere mogelijke verklaring is een farmacokinetische interactie van erlotinib en docetaxel die kan leiden tot een hogere blootstelling aan docetaxel, zoals eerder is opgemerkt bij de combinatie van pazopanib en docetaxel. Vanwege de inferieure activiteit van de combinatie docetaxel-erlotinib wordt verder gebruik van de combinatie in onderzoeksverband of de klinische praktijk ontraden.

De verkenning van de toxische limiet van de 3^{e} generatie EGFR-TKI osimertinib bij patiënten met *EGFR*-gemuteerde NSCLC is terug te vinden in **Hoofdstuk 3**. In de prospectieve 'START-TKI' cohort studie is bij elk polibezoek extra bloed afgenomen. De osimertinib concentraties zijn in het plasma bepaald, en ernstige toxiciteit (CTCAE ≥ graad 3, of leidend tot medicatie staking, dosis reductie of ziekenhuis opname) is opgevolgd. De correlatie tussen blootstelling aan osimertinib (gedefinieerd als klaring) en ernstige toxiciteit, evenals de relatie tussen blootstelling en werkzaamheid is onderzocht. In totaal zijn 819 bloedmonsters van 159 patiënten onderzocht. Er is een significante correlatie tussen blootstelling aan osimertinib en ernstige toxiciteit in de multivariaat concurrerende risico analyse (Hazard Ratio (HR) 0,91; 95% Confidence Interval (CI) 0,83-0,99), met een optimale toxische limiet van 259 ng/mL bepaald door de Receiver Operating Characteristic (ROC)-curve. Een dosisreductie van 80 mg naar 40 mg per dag in de groep met hoge blootstelling zou het risico op ernstige toxiciteit kunnen terugbrengen van 34% naar 14%. Dit effect persisteert bij analyse van de beschikbare concentraties in de eerste twee maanden, met een risicoreductie op ernstige toxiciteit van 31% naar 17%. Er is geen correlatie aangetoond tussen blootstelling aan osimertinib en overall survival (OS) of progression free survival (PFS). Patiënten met een osimertinib concentratie boven 259 ng/mL kunnen profijt hebben van een dosisreductie, deze resultaten dienen prospectief gevalideerd te worden.

De resultaten van chemotherapie schema's bij progressie na behandeling met EGFR-TKI bij patiënten met *EGFR*-gemuteerde NSCLC worden gepresenteerd in **Hoofdstuk 4**. In deze retrospectieve studie blijkt de werkzaamheid van twee veel gebruikte chemotherapie schema's in de eerste lijn (platinum/pemetrexed en carboplatin/paclitaxel/bevacizumab/atezolizumab) redelijk vergelijkbaar. Bij platinum/pemetrexed is de PFS 5,1 maanden en de OS 15,2 maanden, bij carboplatin/paclitaxel/bevacizumab/atezolizumab is dit respectievelijk 5,8 maanden en 14,9 maanden. Bovendien is de PFS van patiënten die zijn behandeld met wekelijks paclitaxel en bevacizumab in deze klinische setting in de praktijk vergelijkbaar met die van de ULTIMATE studie (respectievelijk 4,9 vs 5,4 maanden), zelfs bij patiënten met eerder een gebrek aan respons op platinum/pemetrexed. Dit suggereert dat voor patiënten die eerder

behandeld zijn met platinum bevattende combinatie chemotherapie, het paclitaxel/bevacizumab schema aanvullende waarde kan hebben. Het aantal patiënten dat is behandeld met carboplatin/paclitaxel/bevacizumab is erg klein (*n*=8), een selectie bias kan hier dan ook niet uitgesloten worden.

Concluderend hebben patiënten baat bij chemotherapie in geval van progressie na EGFR-TKI in onze dagelijkse klinische praktijk, al is de responsduur korter dan die van de eerdere lijn doelgerichte therapie.

Deel B Focus op plasma

In **Hoofdstuk 5** vergelijken we plasma cell-free DNA (cfDNA) droplet-digital polymerase chain reaction (ddPCR) (Biorad) en next generation sequencing (NGS) (Oncomine cfDNA lung assay v1) met weefsel NGS resultaten bij patiënten met *EGFR*-gemuteerde NSCLC. De resultaten van ddPCR en NGS in plasma laten een hoge mate van overeenstemming zien op het niveau van een specifieke mutatie zoals p.T790M of p.L858R, maar NGS heeft een bredere dekking qua hotspots in het panel en is daardoor in staat om een breder spectrum van mutaties te detecteren. De concordantie van plasma resultaten met weefsel is hoog. Hoewel plasmadetectie afhankelijk is van de mate van cell-free tumor DNA (ctDNA) verspreiding vanuit de tumor, zoals getoond door de patiënten bij wie geen mutaties zijn gedetecteerd in plasma (6/36), is de aanvullende waarde van plasma analyse duidelijk voor de patiënten bij wie de p.T790M resistentie mutatie wel wordt gedetecteerd in plasma maar niet in weefsel (3/36).

In **Hoofdstuk 6** gaan we een stap verder met plasma cfDNA NGS en zoeken we naar behandelbare mutaties in een populatie met verdenking op gemetastaseerde longkanker wanneer moleculaire analyse op weefsel niet haalbaar is. In een periode van 2 jaar zijn 55 bloedmonsters van patiënten in het longkankernetwerk Zuidwest (Erasmus MC) geanalyseerd. Bij 7 patiënten is een mogelijk doelwit voor therapie vastgesteld. Eén patiënt is succesvol behandeld met doelgerichte therapie na detectie van een *EGFR* exon 19 deletie. Een andere patiënt met een *EGFR* exon 20 insertie is verwezen en geïnformeerd over mogelijke doelgerichte behandeling in studieverband, maar heeft van deze mogelijkheid afgezien. Voor de patiënten met Kirsten rat sarcoma virus (*KRAS)* p.G12C en B-Raf proto-oncogen (*BRAF)* non-V600E mutaties is er nog geen doelgerichte therapie in de eerste lijn beschikbaar op het moment van detectie. Hoewel de dekking van het Oncomine V1 panel een limiterende factor is in het vermogen van detectie van alle mogelijke doelwitten voor therapie, die ook snel in aantal toenemen, zijn we nog steeds in staat om patiënten te identificeren die een mogelijkheid tot doelgerichte therapie hebben die anders niet ontdekt zou zijn.

In **Hoofdstuk 7** wordt het beloop van mutaties in cfDNA vanaf start van doelgerichte therapie gevolgd, en worden predictieve kenmerken in plasma tijdens behandeling met EGFR-TKI bij patiënten met *EGFR*-gemuteerde NSCLC geïdentificeerd. In de START-TKI

studie wordt bij poliklinische bezoeken extra bloed afgenomen waarin mutaties en medicatie concentraties worden bepaald. Plasmaklaring van de primaire *EGFR* mutatie, en van p.T790M wanneer van toepassing, is na 6 en 12 weken therapie geëvalueerd. De afwezigheid van deze zogenoemde plasmaconversie correleert met een kortere PFS (5,5 vs 17,0 maanden, p=0,002) en OS (14,0 vs 25,5 maanden, p=0,003). Bovendien zien we een correlatie tussen de aanwezigheid van een tumor proteïne p53 (*TP53)* comutatie en een kortere PFS bij patiënten met tweedelijns osimertinib (8.8 vs 18.8 maanden, p=0.017). Verder is een daling van de erlotinib concentratie in het tweede tertiel van behandeling ook gecorreleerd met een kortere PFS (8,9 vs 23,6 maanden, p=0,037).

De samenvatting, algehele discussie en toekomstige perspectieven in **Hoofdstuk 8** plaatsen de eerdere bevindingen verder in context, waarbij verbanden worden gelegd tussen de klinische praktijk en plasma analyse. Plasma kan een aanvullende rol hebben bij patiënten bij wie het niet mogelijk is een moleculaire analyse op weefsel te verrichten, om toch doelgerichte behandeling te kunnen geven wanneer er sprake is van een aandrijvende mutatie. Verder kan bij patiënten met een *EGFR*-gemuteerde NSCLC gebruik worden gemaakt van het bepalen van de mate van plasmaconversie als predictie van een grotere kans op langdurige respons. Ook kan de osimertinib concentratie worden bepaald, waarbij er boven de toxische limiet een grotere kans is op ernstige toxiciteit. Bovendien kan plasma in geval van resistentie van aanvullende waarde zijn om resistentie mechanismen op te sporen. Wanneer er progressieve ziekte is na behandeling met EGFR-TKI zonder behandelbaar resistentie mechanisme, hebben patiënten profijt van cytotoxische chemotherapie.

Er is steeds meer interesse in ontwikkelingen op het gebied van artificiële intelligentie en wiskundige modellen. Wellicht is het mogelijk om in de toekomst, op basis van parameters op radiologisch, (plasma) genomisch en farmacokinetisch gebied, behandelstrategieën nog meer gepersonaliseerd toe te passen dan momenteel mogelijk is.

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PhD Portfolio

PhD period: 2017-2022

Courses

2016 October	Dutch Lung Cancer Course – Mount Sinaï Hospital NYC
2016 November	NFU BROK exam
2017 March	Wengen op de Wadden 2017
2018 October	Course Basic and Translational Oncology
2020 January	Teach the teacher (training docentprofessionalisering basis)
2020 October	NFU BROK recertification
2021 May	Liverpool goes Dutch (palliative care)
2021 October	Scientific Integrity

Teaching

2017 March	Case presentation WodW: targeted therapy resistance and possible approaches in diagnostics and therapy
2017 December	CATT theme unit diagnostics Erasmus MC: cfDNA and lung cancer
2017 November	Case presentation Lung Preceptorship Copenhagen: Whole Genome Sequencing in lung cancer related to response on immunotherapy
2018 March	KMBPio teaching session: Lung cancer
2018 September	Oncology nurse education Amphia: Lung Cancer
2018 September	WCLC 2018 Poster Presentation: P2.13-28 Comparison of ddPCR and NGS in Liquid Biopsy to Pathology Results in *EGFR*-Mutated NSCLC
2019 September	WCLC 2019 Poster Presentation: P1.14-23 Resistance Mechanisms to Osimertinib Treatment in *EGFR*-Mutated Lung Cancer in a Real Life Cohort
2021 September	WCLC 2021 Poster Presentation: P24.08 Lung Cancer Diagnosis in Absence of Adequate Tissue Molecular Analysis in Metastatic Disease by NGS Analysis of Plasma cfDNA

2021 December	NVALT studiedag: Oral presentation NVALT18 results
2021 Oct – 2022 Apr	Supervising research internship medical master student; daily mentor
2022 July	Presentation CCN meeting Guideline NSCLC – session mutations: MTB or not MTB that's the question

Conferences

2017 June	ASCO Telereview 2017
2017 June	Rotterdam ademt Chicago (ASCO) 2017
2017 November	WCLC & ESMO Telereview 2017
2018 April	AACR in review 2018
2018 June	Rotterdam ademt Chicago (ASCO) 2018
2018 September	WCLC 2018
2019 June	Rotterdam ademt Chicago (ASCO) 2019
2019 September	WCLC 2019
2020 September	ESMO 2020 Virtual Congress
2021 Februari	WCLC & ESMO Telereview 2020
2021 June	ASCO Telereview 2021
2021 September	WCLC 2021
2021 September	ESMO 2021 Virtual Congress
2022 June	ASCO Telereview 2022

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Curriculum vitae

Christi Steendam werd op 17 november 1983 geboren te Dordrecht. In 2002 voltooide ze het eindexamen van het Gymnasium aan het Dr. Mollercollege te Waalwijk, waarna ze aan de studie Geneeskunde in Maastricht begon. In Maastricht ontmoette ze Sander, met wie ze later een gezin stichtte met hun twee dochters Robin en Doris. Na het behalen van het basisartsexamen startte ze in 2009 met de specialisatie Longgeneeskunde in het St. Antonius ziekenhuis te Nieuwegein. Na voltooiing van de specialisatie in 2015, begon ze aan een fellowship Thoracale Oncologie in het Erasmus MC Rotterdam en Amphia ziekenhuis Breda. Dit mondde uit in een promotietraject in het Erasmus MC in combinatie met klinisch werk als longarts in het Amphia ziekenhuis van 2017 tot 2021. Na nog een half jaar fulltime in het Erasmus MC gewerkt te hebben maakte ze in 2022 de overstap naar het Catharina ziekenhuis te Eindhoven. Als longarts met aandachtsgebied thoracale oncologie combineert ze hier topklinische patiëntenzorg met verdere ontwikkeling in research, met de voltooiing van dit proefschrift als bijzonder mooie start.

Dankwoord

Hoewel op het omslag van een proefschrift altijd een enkele naam staat, weet iedereen dat het onmogelijk is om alleen tot dit resultaat te komen.

Allereerst veel dank aan alle patiënten en hun naasten, die in een periode van ziekte en kwetsbaarheid bereid zijn onderzoekers te helpen tot nieuwe inzichten te komen. Zonder jullie medewerking is dat niet mogelijk, dit waardeer ik zeer.

Ik ben mijn beide promotoren dankbaar voor het scheppen van kansen en de begeleiding in het gehele traject. Anne-Marie, je hebt me bij je komst naar het Erasmus liefdevol geadopteerd als promovendus en stond altijd open voor begeleiding. Dank voor al je feedback, het delen van je scherpe geest en je continue streven naar een hoger niveau. Joachim, tijdens mijn fellowship kon ik dankzij jouw begeleiding proeven van de wereld van klinisch onderzoek, en dat smaakte naar meer! Dank voor het vertrouwen en de ruimte die je me gaf bij het opstarten van de START-TKI studie, en het daarbij opzetten van een promotietraject. Dankzij deze start heb ik onderzoek als onderdeel van mijn verdere werk omarmd.

Ook alle mensen die in het Erasmus en het Amphia hebben bijgedragen aan de opzet, het verkrijgen en analyseren van data en verwerking tot dit eindresultaat ben ik dankbaar. Bedankt voor de prettige samenwerking op het AKC Ron en Evert, bij de PA Erik Jan, Winand, Jan en Peggy, bij de medische oncologie Marijn, Ron, Stijn, Daan en Esther en vele anderen. Ook aan de medewerkers van het researchbureau Erasmus MC en Amphia; bedankt voor jullie ondersteuning.

Tijdens mijn promotietraject was ik het grootste gedeelte klinisch werkzaam in het Amphia. De vakgroep longziekten Amphia heeft me destijds voor onbepaalde tijd welkom geheten in hun team, bedankt voor de leuke tijd bij jullie! Ik waardeerde naast de topklinische zorg vooral de alledaagse overpeinzingen bij de koffie en de lunch, en jullie pogingen me alsnog voor voetbal te interesseren.

In de oncokamer op de longafdeling van het Erasmus zijn de momenten van discussie, het delen van casuïstiek, inzichten en bijzondere muzieksmaken op vrijdagmiddag in vele opzichten ook verrijkend geweest. Bedankt Daphne, Robin, Marthe en Anne-Marie.

Ook ben ik dank verschuldigd aan de vakgroep longartsen Catharina ziekenhuis, voor jullie vertrouwen en de ruimte die jullie me gaven om eerst nog zaken in het Erasmus af te ronden voor mijn komst naar Eindhoven. Jullie zorgden ervoor dat ik me snel thuis voelde en ik een ideale plek heb gevonden om topklinische zorg met research te combineren.

Tijdens een promotietraject is het fijn om op gezette tijden in goed gezelschap te verkeren en de zinnen te verzetten. Dank vrienden en familie voor het aanhoren van mijn verhalen, maar vooral voor het delen van dat van jullie, dat houdt de blik op de wereld breed.

Ook de steun van mijn ouders Paula en Lieuwe heeft me erg geholpen. Ze leerden me dat je heel veel kunt bereiken als je doelen met overtuiging en doorzettingsvermogen benadert. Daarnaast zitten er ook vele uren in onvoorziene kinderopvang door mijn ouders en schoonouders Loes en Piet; bedankt dat we in noodgevallen altijd een beroep op jullie mogen doen.

Ook wil ik hier graag mijn intervisie/supervisie groep noemen. Tijdens de bijeenkomsten met Femke, Nicole en coach Bert was er altijd ruimte voor inbreng, verdieping en mentaal werk en dat doet goed.

Mijn paranimfen ben ik zeer erkentelijk voor hun ondersteuning. Maya, we hebben inmiddels menig levensfase samen doorgemaakt en hopelijk volgen er ook nog velen. Bij jou voel ik sinds het gedeelde stapelbed tijdens het KoKo introductieweekend altijd een bepaalde gedeelde golflengte en ik ben blij dat jij je ook nog steeds zo kunt verwonderen over de bijzondere loop des levens en het spectrum der mensheid. Nicole, we hebben de weg van beginnend assistent naar medisch specialist gedeeld, waarbij ik je naast collega ook als mens heel erg waardeer buiten werktijd. Ook van gezamenlijk congresbezoek inclusief altijd succesvolle shopsessies kan ik altijd genieten, met nog speciale dank voor die vroege borrel die je me gaf voor mijn allereerste postersessie voor nog vloeiender Engels.

Maar bovenal bedankt Sander. Ik waardeer al je bemoedigende woorden en al je geduld, tot zelfs een keer samen met de oppas wachtend op de bank met een uitgestelde reservering in een goed restaurant omdat ik nog een stuk wilde indienen op de deadline. Ook je tolerantie naar mijn vriend De Laptop in de vrije tijd, tijdens uitstapjes of op vakantie heeft dit proefschrift mede mogelijk gemaakt. Je relativeringsvermogen is van onschatbare waarde geweest om me waar nodig weer even het grotere plaatje te laten zien. De tijd die ik doorbreng met jou en onze meiden is me alles waard. Bedankt dat onze ambitie altijd gelijkwaardig is, we elkaar hierin ondersteunen en proberen om het beste bij elkaar naar boven te brengen. Zonder jou was het niet gelukt!