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ANTIANGIOGENIC THERAPY IN ADVANCED LUNG ADENOCARCINOMA: EFFICACY AND KRAS MUTATION AS A PROGNOSTIC BIOMARKER

PhD thesis

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1. Abbreviations

AE	*adverse event*
AIS	*adenocarcinoma in situ*
ALK	*anaplastic lymphoma kinase*
ARID1A	*AT-rich interactive domain-containing protein 1A*
BEV	*bevacizumab*
BRAF	*B-Raf proto-oncogene*
CDKN2A	*cyclin-dependent kinase Inhibitor 2A*
CHT	chemotherapy
CI	*confidence interval*
CK7	*cytokeratin 7*
COPD	*chronic obstructive pulmonary disease*
CR	*complete response*
CRC	*colorectal cancer*
CRF	*case report form*
CT	*computer tomography*
DLCO	*diffusing capacity of the lungs for carbon monoxide*
DNA	*deoxyribonucleic acid*
ECOG	*Eastern Cooperative Oncology Group*
EGFR	*Epidermal Growth Factor Receptor*
EMA	*European Medicines Agency*
ERBB2	*v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2*
ERK	*extracellular regulated kinases*
ESMO	*European Society for Medical Oncology*
FDA	*Food and Drug Administration (U.S.)*
FEV1	*forced expiratory pressure in 1 second*
FVC	*forced vital capacity*
GABA	*gamma-aminobutyric acid*
GWAS	*genome wide association studies*
HIV	*human immunodeficiency virus*
HLA-A	*human leukocyte antigens on the A locus*

HRAS	*Harvey rat sarcoma viral oncogene homolog*
KEAP1	*Kelch-like ECH-associated protein 1*
KRAS	*Kirsten rat sarcoma 2 viral oncogene homolog*
LA	*Locally advanced*
LADC	*lung adenocarcinoma*
LCC	*large cell carcinoma*
LCNEC	*large cell neuroendocrine carcinoma*
LDCT	*low dose computer tomography*
MAP2K1	*mitogen-activated protein kinase kinase 1*
MEK	*mitogen-activated protein kinase*
MET	*met proto-oncogene (hepatocyte growth factor receptor)*
MGA	*MAX gene associated*
MIA	*minimally invasive adenocarcinoma*
MLL2	*histone lysine methyltransferase gene KMT2D*
NA	*not applicable*
nAChR	*nicotinic acetylcholine receptors*
NF1	*neurofibromatosis type I*
NFE2L2	*Nuclear factor erythroid 2-related factor 2*
NLST	*National Lung Screening Trial*
NNK	*4-(methylnitrosamino)-1-(13-pyridyl-1-butanone)*
NOTCH1	*neurogenic locus notch homolog protein 1*
NRAS	*Neuroblastoma rat sarcoma viral oncogene homolog*
NSCLC	*non-small cell lung cancer*
nsNSCLC	*non-squamous non-small cell lung cancer*
OCS	*observational cohort study*
OS	*overall survival*
PAH	*polycyclic aromatic hydrocarbons*
PD	*progressive disease*
PDYN	*prodynorphin*
PET	*positron emission tomography*
PFS	*progression-free survival*

PIK3CA	*phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha*
PR	*partial response*
PS	*performance status*
PTEN	*phosphatase and tensin homolog*
RAF	*rapidly accelerated fibrosarcoma, receptor tyrosine kinase effector*
RAS	*rat sarcoma viral analog*
RB1	*retinoblastoma protein 1*
RBM10	*RNA-binding protein 10*
RCT	*randomized clinical trial*
RECIST	*response evaluation criteria in solid tumors*
RET	r*earranged during transfection*
RIT1	*Ras-like without CAAX 1*
ROS1	*ROS proto-oncogene 1*
RTK	*receptor tyrosine kinase*
SAE	*serious adverse event*
SBRT	*stereotactic body radiotherapy*
SCLC	*small cell lung cancer*
SD	*stable disease*
SD	standard deviation
SETD2	SET domain containing 2
SMARCA4	*SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4*
SqCC	*squamous cell carcinoma*
STK11	*serine/threonine kinase 11*
TB	*tuberculosis*
TCGA	*The Cancer Genome Atlas Program*
TNM	*Tumor, node, metastasis - Internationally accepted classification of malignant tumors;*
TP53	*tumor protein p53*
TSNA	*tobacco-specific N-nitrosamine*
TTF1	*thyroid transcription factor 1*
TTP	*time-to-progression*

U2AF1	*U2 auxiliary factor 1*
VATS	*video-assisted thoracic surgery*
VEGF	*vascular endothelial growth factor*
WHO	*World Health Organization*
WT	*wild type*

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2. Introduction

2.1. Lung cancer

2.1.1. Epidemiology

More than 8 million cancer deaths and 14 million new cancer cases were documented worldwide in 2012. Lung and breast cancer were diagnosed most frequently among these, and they represent the leading causes of cancer death overall. However, in more developed countries, lung cancer represents the leading cause of cancer death in women(1) and prostate cancer accounts for the most diagnosed malignancy.

Lung cancer became the second most common malignant tumor in the last century. In the 19th century and even in the beginning of the 20th century lung cancer was a very rare diagnosis. In the 1840s only 22 published cases could be found(2), while in 1912 Adler still only identified 374 published cases(3, 4). Recent statistics show that annually there were 1.825 million new lung cancer cases worldwide in 2012. This is a marked rise from 1.6 million new cases in 2008(5). 409 900 of these cases were reported in Europe which was 13% of all cancer cases(6), however it causes more deaths than breast, prostate and colon cancer combined. It is estimated that the total number of deaths caused by lung cancer was 1.589 million worldwide, which accounts for 17% of all cancer related deaths(7). The incidence of lung cancer still rises worldwide, although it shows great variances between countries.

Hungary has on of highest mortality rates of lung cancer in the world both in men and women. Hungary, unlike other developed countries, records a growing number of new cases. While the incidence hasn't increased over the last few years in men, it continuously does in women(8). In 2014, the registered number of new cases of lung cancer in Hungary was 5189 (60% male, 40% female). Incidence is quite low in the population younger than 40 years (0-12%^{000}), however it rises drastically to 250%^{000} in the male population between 50 and 54 years of age(9).

2.1.2. Etiology

2.1.2.1. Smoking

The use of tobacco attributes to 90% of all lung cancer cases, which makes it the single greatest risk factor regarding lung cancer. Smokers have a 15 fold chance of developing lung cancer compared to non-smoking population. Based on less univoque evidence, they presume that work-associated carcinogens attribute for 9-15%, radon released into air for 10% and pollution for 1-2% of cases.

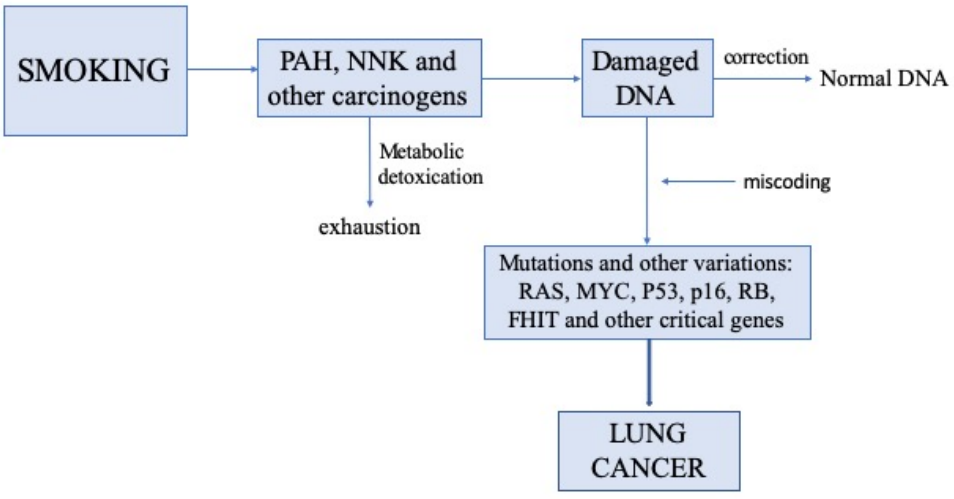
Society-wide understanding of the relationship between smoking and lung cancer was achieved very slowly due to several factors. One of these factors is the long latency period between smoking initiation and the development of lung cancer and another interesting factor was the marketing activity of the tobacco industry(10). Tobacco had been used by people in Europe, America and Asia for centuries without significant incidence of lung cancer. Tobacco was regarded primarily as medicine or was used only in rituals. Tobacco was brought to Europe in the 15th century but cigarettes were only manufactured first in the 19th century. In this era, cigarettes were expensive and hand-rolled and only men used them occasionally(11, 12).

The world wars had a great role in the fact that smoking got popular first in men and later also in women. Smoking increased dramatically after both world wars because soldiers were handed free cigarettes and developed nicotine addiction. At this time the negative effects of smoking were not know to the wide public so soldiers subsequently brought the habit of smoking home. Early reports already suggested a link between smoking and cancer in the 1920s and 1930s but these reports were not perceived widely thus they had no effect on consumption(13-16). The first major epidemiological studies were released in 1950 by Doll and Hill (17) and Wyander and Graham (18). These definitely established the relation between smoking and lung cancer which led to statements Royal College of Physicians in Great Britain in 1962 (19) and the US Surgeon General in 1964 to warn the public about the dangers of smoking.

Smoking a cigar or a pipe is less dangerous than cigarettes because the tobacco smoke exposition is lower and deep inhalation is rare. The chance of developing lung cancer shows an exponential relationship with the number of cigarettes smoked and the total years of smoking. The length of smoking is of greater importance than the age of the patient and no matter how old someone is, quitting decreases the chance of lung cancer.

Passive smoking is also considered a risk for developing lung cancer, however a metanalysis comparing smoking and non-smoking couples found that passive smoking only increased the risk by 20%(20, 21). In Hungary an estimated 34% of the population is smoking (41% in male and 28% in female) which puts Hungary among the countries with the highest risk of lung cancer.

Nicotine, responsible for the addictiveness of cigarettes, is a natural alkaloid acting as an acetylcholine agonist that binds to the nicotinic acetylcholine receptors (nAChR) in the nervous system, causing release of neurotransmitters into the blood stream, including dopamine, serotonin, norepinephrine, endorphins, and gamma-aminobutyric acid (GABA). Nicotine upregulates nicotinic receptors and alters gene expression causing dependence and also helping progression of an existing tumor(22-24). Nicotine itself is not regarded as carcinogen, however there are at least 60 known carcinogens produced at tobacco combustion. The most significant are tobacco-specific N-nitrosamines (TSNAs), such as 4-(methylnitrosamino)-1-(13-pyridyl-1-butanone) (NNK) and polycyclic aromatic hydrocarbons (PAH), including benzo[a]pyrene and nitrates(25). The relationship of NNK to lung cancers, specifically adenocarcinomas have been demonstrated(26). Combustion of tobacco produce a smoke that has a vapor phase that produce 10^{15}, and a particulate phase with 10^{17} free radicals per gram. The damage done by these free radicals is one of the methods of carcinogenesis, the other is DNA adduct and metabolite formation(24, 25). The carcinogenesis of smoking can be seen on Figure 1.



1. Figure – Carcinogenesis of smoking

2.1.2.2. Environmental risk factors

2.1.2.2.1. Radon

It has been known from the 15th century that mining carried the risk of lung disease. It was observed on miners working in the mountains on the German-Czech border that the incidence of "mountain disease" was very high. In these mines they produced cobalt, arsenic, bismuth, iron, silver and later in the 20th century radium. In the 20th century these mining communities had extremely high incidence of squamous cell lung cancer(4). Residential exposition to radon occurs from soil. Radon is a radioactive gas occurring naturally from the earth's crust from natural decay of uranium. Usually radon level can rise to unsafe measures in residential basements. Cigarette smoking in the same time increases the relative risk of lung cancer from radon(27-29).

2.1.2.2.2. Asbestos

Asbestos is the most common occupational exposure to carcinogens but especially in Eastern Europe asbestos can also be still found in residential buildings. Asbestos was used widely in constructions since the 19th century. It contains chrysotile fibers that have been shown to have association with lung disease and thoracic malignancies such as malignant mesothelioma and lung cancer. Residential and continuous occupational exposure to asbestos carries a 5-fold risk of lung cancer and have a synergistic effect with tobacco smoking(30, 31). A closed eternity factory still produce increased risk for asbestos exposure for residents living in nearby villages in Hungary's Selyp.

2.1.2.2.3. Air pollution

An adult inhales approximately 10 000 liters of air per day which means that even a small concentration of carcinogens can cause changes in the cellular DNA with time. Air pollution in big cities consist of carcinogens released from combustion of fossil fuel, arsenic, nickel and chrome. A study conducted in six large cities in the USA found that the incidence of lung cancer was 40% higher in the most polluted city compared to the one with the least air pollution(32). The frequently cited paper of Doll and Petro estimated

that only 1-2% of lung cancer cases was due to air pollution in 1981(20). Their estimation is still most probably correct.

2.1.2.3. Genetic risk factors

The fact that not all smokers develop lung cancer establishes the role of genetic susceptibility to lung cancer. They observed that having a positive family history for lung cancer increases the risk 1.7 fold(33). Genome wide association studies (GWAS) identified the chromosome regions connected to increased risk of lung cancer: 5p15, 15q25-26 and 6q21(34, 35). Telomerase reverse transcriptase (TERT) is involved in cell replication and in the development of lung cancer it is associated with adenocarcinomas. TERT is encoded on the 5p15 region. Chromosome locus 6p21 regulates G-protein signaling, and mutations increase the risk of lung cancer development in non-smokers. The 15q25-26 chromosome locus is known for mutations that are positively linked to nicotine dependence and susceptibility for lung cancer(36, 37).

It is interesting that there have been a change in lung cancer histology throughout the years: in the early 20th century squamous cell lung carcinoma was the most frequent, while since the 1970s the incidence of lung adenocarcinoma rose and incidence of squamous cell carcinomas decreased. The background of this change is not entirely known, however several authors contribute this change to the wide use of filters in cigarettes. The use of filters decreased the amount of inhaled PAHs but on the other hand the deeper inhalation increased the amount of nitrogen-oxides and nitrosamines(32).

2.1.2.4. Coexisting diseases and infections

Previous lung injury caused by chronic obstructive pulmonary diseases (COPD) and lung fibrosis also plays a role in lung cancer development, however it is challenging to study their effect separately because smoking also has a role in COPD. Both diseases can cause lung injury through inflammatory pathways and it was also shown that infections such as tuberculosis (TB) were associated with a 1.7 fold increase in risk for lung cancer(38).

Human immunodeficiency virus (HIV) infection also carries a risk among other diseases for lung cancer. Lung cancer is the most common malignancy in patients with HIV infection and it also accounts for 30% of cancer deaths related with HIV(39, 40). The HIV virus itself has not been connected directly to oncogenesis, however the

immunosuppression caused by it defiantly plays a role since organ transplant recipients and HIV patients have a similar increase in cancer rates(41). Declining CD4 counts were found to be the reason for higher lung cancer rate. 42% of HIV patients also smoke, however HIV-infected patients have a 2.5 fold greater risk of developing lung cancer regardless of the smoking status(42).

2.1.3. Histology and molecular background of lung cancer

The histopathological diagnosis of lung cancer is based on morphologic features. Two main histopathological groups were made: small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). SCLC accounts for approximately 15% of cases, while NSCLC accounts for 85%. NSCLC is usually subcategorized into adenocarcinoma, squamous cell carcinoma and large cell carcinoma, even though more and more evidence suggests that that these are molecularly heterogenous diseases(43).
Histological classification was last updated in 2015 by the World Health Organization (WHO). It recognizes the new molecular discoveries achieved since 2004.

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1. Table – WHO 2015 classification of lung epithelial tumors

Adenocarcinoma	**Large cell carcinoma**
Lepidic adenocarcinoma	Adenosquamous carcinoma
Acinar adenocarcinoma	Pleomorphic carcinoma
Papillary adenocarcinoma	Spindle cell carcinoma
Micropapillary adenocarcinoma	Giant cell carcinoma
Solid adenocarcinoma	Carcinosarcoma
Variants of adenocarcinoma	Pulmonary blastoma
Invasive mucinous adenocarcinoma	Other and unclassified carcinomas
Mixed invasive mucinous and non-mucinous adenocarcinoma	Lymphoepithelioma-like carcinoma
Colloid adenocarcinoma	NUT carcinoma
Fetal adenocarcinoma	Salivary gland-type tumors
Enteric adenocarcinoma	Mucoepidermoid carcinoma
Minimally invasive adenocarcinoma	Adenoid cystic carcinoma
Non-mucinous	Epithelial–myoepithelial carcinoma
Mucinous	Pleomorphic adenoma
Preinvasive lesions	Papillomas
Atypical adenomatous hyperplasia	Squamous cell papilloma
Adenocarcinoma *in situ*	Exophytic
Non-mucinous	Inverted
Mucinous	Glandular papilloma
Squamous cell carcinoma (SqCC)	Mixed squamous cell and glandular papilloma
Keratinizing SqCC	Adenomas
Non-keratinizing SqCC	Sclerosing pneumocytoma
Basaloid SqCC	Alveolar adenoma
Preinvasive lesion	Papillary adenoma
SqCC *in situ*	Mucinous cystadenoma
Neuroendocrine tumors	Mucous gland adenoma
Small cell carcinoma	
Combined small cell carcinoma	
Large cell neuroendocrine carcinoma (LCNEC)	
Combined LCNEC	
Carcinoid tumors	
Typical carcinoid	
Atypical carcinoid	
Preinvasive lesion	
Diffuse idiopathic pulmonary neuroendocrine cell	
Hyperplasia	

2.1.3.1. Adenocarcinoma

Adenocarcinoma is currently the most common malignancy of the lung and it is a heterogenic group of tumors. In Hungary adenocarcinoma accounts for 47% of all lung cancer incidence(44). The common in all adenocarcinomas is the glandular structure and some level of mucin production. The growing pattern can be variable, the most common is lepidic growth that follows the alveolar wall, acinar, papillary or solid. Several of these patterns can be present within one tumor but usually one is dominant. The diagnosis of adenocarcinoma is often proven by immunohistochemistry (TTF1, Napsin-A, CK7) and mucin staining.

Adenocarcinoma in situ (AIS) was introduced as a new precancerous category. These tumors are smaller than 3 cm in diameter, they are solitary, the show clearly lepidic growth pattern and they do not invade the pleura, stromal cells or the vessels.

Minimally Invasive Adenocarcinoma (MIA) was also introduced as a new precancerous category. These tumors are not greater than 3 cm in diameter, they are solitary, they show dominantly lepidic growth pattern and they only show minimal (smaller than 5mm) stromal, vascular or pleural invasion.

The nomenclature do not use bronchiolo-alveolar carcinoma and mixed adenocarcinoma anymore(45).

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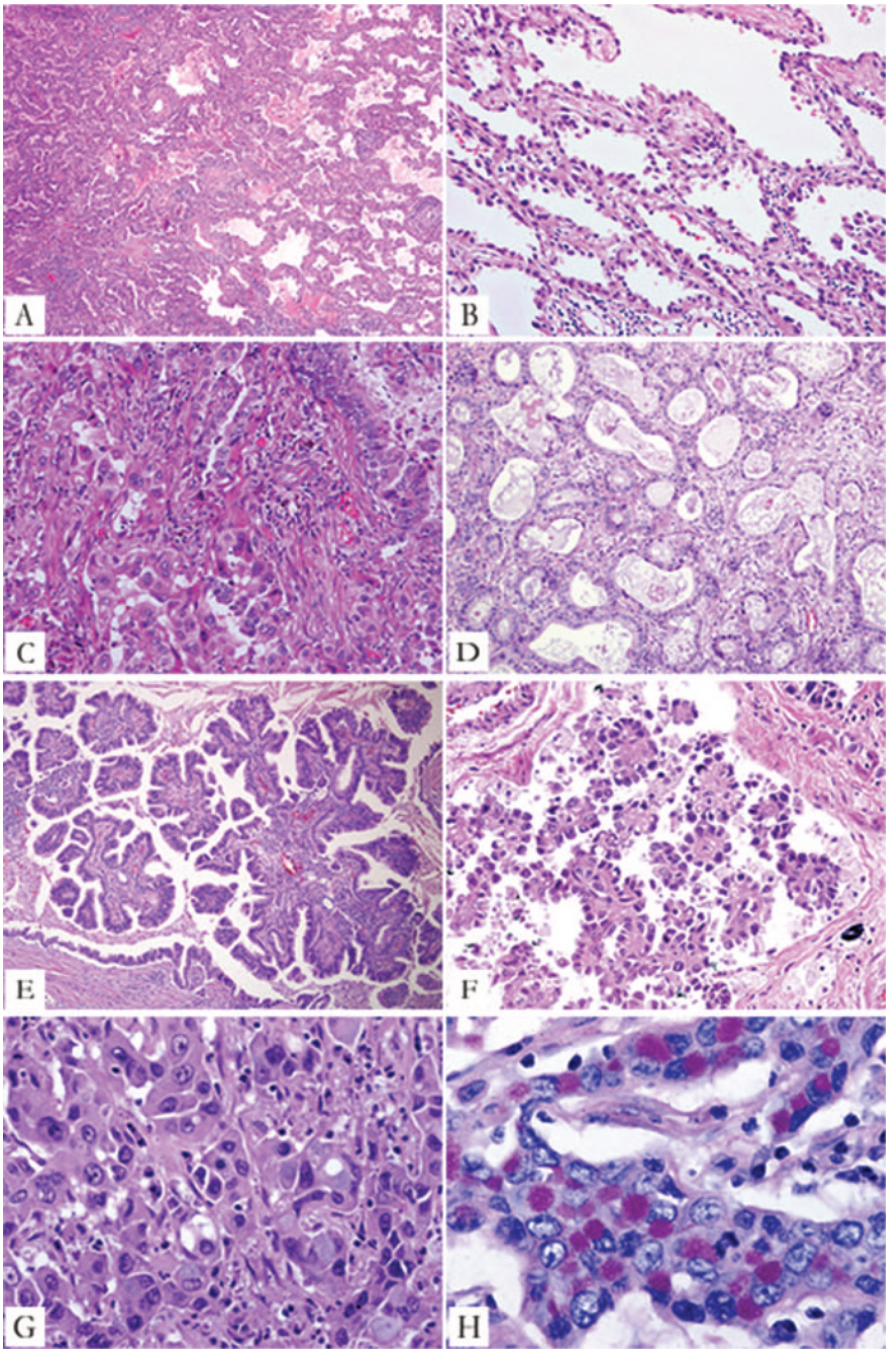
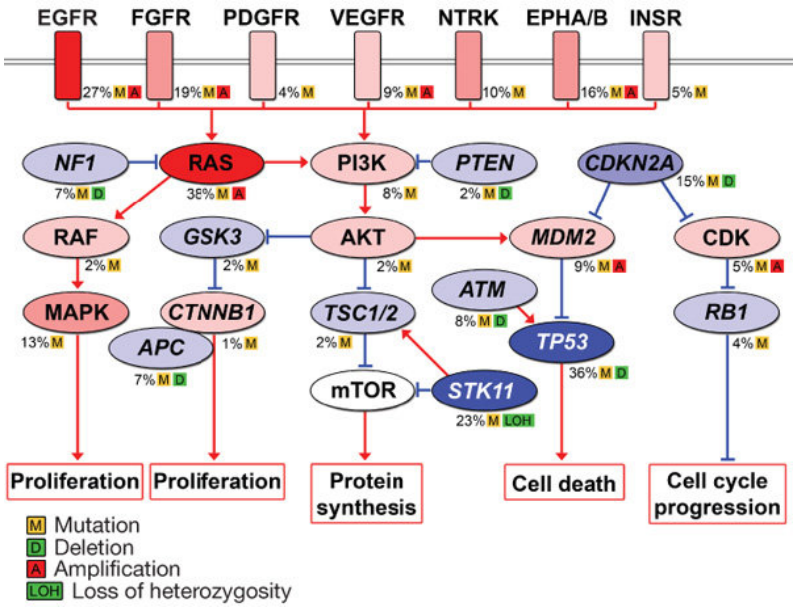


2. Figure – Histologic subtypes of lung adenocarcinoma: A, Predominantly lepidic pattern with lepidic growth on the right with invasive acinar adenocarcinoma on the left; B, Proliferation along the alveolar wall (type II pneumocytes and Clara cells); C, Invasive acinar adenocarcinoma; D, oval-shaped malignant glands invading the fibrous stroma in invasive acinar adenocarcinoma; E, Papillary adenocarcinoma; F, Small papillary clusters of glandular cells in a micropapillary adenocarcinoma; G, Solid adenocarcinoma; H, Solid adenocarcinoma with mucin. Adopted from (46).

The most common significantly mutated pathways include the *EGFR, FGFR, VEGFR, NTRK, EPHA/B* and *INSR* genes as seen in Figure 3.



3. Figure – Significantly mutated pathways in lung adenocarcinoma. Oncoproteins are indicated in pink to red and tumor suppressor proteins are shown in light to dark blue. The darkness of the colors shows the number of tumors with genetic alterations.(47)

18 significant somatic mutations were identified in lung adenocarcinoma by comprehensive molecular profiling of 230 tumors by The Cancer Genome Atlas Program (TCGA) in 2014: *TP53* (46%), *KRAS* (33%), *KEAP1* (17%), *STK11* (17%), *EGFR*(14%), *NF1* (11%), *BRAF* (10%), *SETD2* (9%), *RBM10* (8%), *MGA* (8%), *MET* (7%), *ARID1A* (7%), *PIK3CA* (7%), *SMARCA4* (6%), *RB1* (4%), *CDKN2A* (4%), *U2AF1* (3%), and *RIT1* (2%). A mean of 8.9 mutations were reported per megabase which is considered very high. This widened the possibility of targeted therapy. 75% of the examined adenocarcinomas contained genetic changes to the RTK/RAS/RAF signaling pathway and 62% of them had driver genetic alterations that promote this cascade. The most common driver mutations were *KRAS*, *EGFR* and *BRAF* with 32, 11 and 7% respectively in this study. Other promoter mutations included MET exon 14 skipping (4.3%), *ERBB2* mutation (1.7%), *ROS1* fusion (1.7%), *ALK* fusion (1.3%), *MAP2K1* mutation (0.9%), *RET* fusion (0.9%), *NRAS* mutation (0.4%), and *HRAS* mutation (0.4%).

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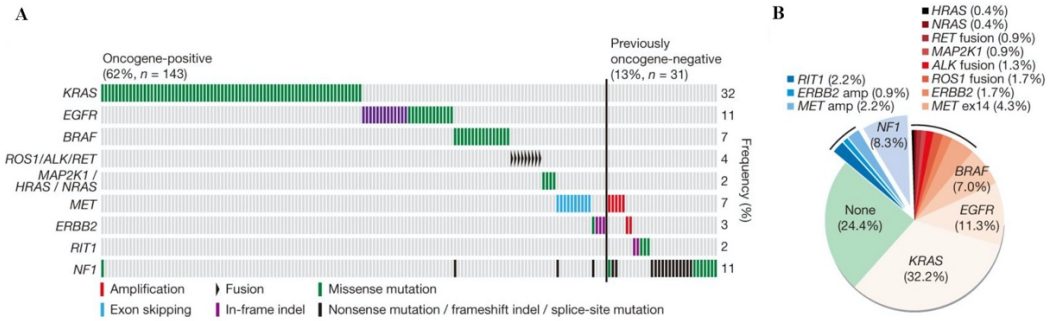


4. Figure – A: Co-mutation plot of variants of known significance within the RTK/RAS/RAF pathway in lung adenocarcinoma. B: New candidate driver oncogenes (blue: 13% of cases) and known somatically activated drivers events (red: 63%) that activate the RTK/RAS/RAF pathway in the majority of the 230 adenocarcinoma cases. Adopted form the TCGA. (48)

The DNA copy numbers were also examined in the remaining 38% of cases were driver mutation was not found. It showed amplification of oncogenes in the RTK/RAS/RAF pathway: *MET* amplification (2.2%) and *ERBB2* amplification (0.9%). New genetic alterations were also identified in this study: *NF1* and *RIT1* mutations, comprising 8.3% and 2.2% respectively. Altogether 75% of lung adenocarcinomas have genetic driver alterations to the RTK/RAS/RAF pathway. This comprehensive study also conducted mRNA profiling, DNA methylation profiling and protein profiling(49).

2.1.3.2. Squamous Cell Carcinoma

In Hungary SqCC accounts for 25% of all lung cancer incidence(50). Squamous Cell Carcinomas are classified as keratinizing SqCC, non-keratinizing SqCC, and basaloid SqCC. Classification is only possible from resected samples. To detect basaloid SqCC immunohistochemistry testing of p63 and p40 is needed.

Comprehensive molecular testing of lung SqCC was performed in 2012 by TCGA. 178 cases of lung SqCC were profiled and complex genetic alterations were identified. Most patients were heavy smokers which caused the high number of genetic changes: a mean of 360 exonic mutations, 165 genetic rearrangements and 323 segments of copy number alterations per tumor. 11 statistically significant mutations were found: *TP53, CDKN2A, PTEN, PIK3CA, KEAP1, MLL2, HLA-A, NFE2L2, NOTCH1, RB1,* and *PDYN*. The incidence of *TP53* was 90%.

This comprehensive study also conducted pathway analysis, mRNA profiling, DNA methylation profiling and protein profiling(51).

2.1.3.3. Large Cell Carcinoma

Large Cell Carcinomas (LCC) are rare, in Hungary LCC accounts for only 2% of all lung cancers. The diagnosis can only be made from resected specimen which can often cause a clinical problem is advanced cases were normally only fine needle or core biopsy would be taken. By definition LCC is a NSCLC that does not show signs of glandular or squamous differentiation(45). Immunohistochemistry has an important role in the diagnosis of LCC because most poorly differentiated tumors can be classified as either adenocarcinoma or SqCC with the right immunopanel (TTF1, Napsin-A, p40, p63, CK5/6).

2.1.3.4. Neuroendocrine tumors

This category "neuroendocrine tumors" was established in the 2015 WHO classification. It consist of three subtypes: SCLC (20%), large cell neuroendocrine carcinoma (LCNEC – 3%), and carcinoid tumors. Carcinoids tumors can be divided in atypical (0.2%) and typical (2%) groups. Clinical behavior of neuroendocrine tumors can greatly vary, thus the distinction between high-grade neuroendocrine tumors, such as SCLC and LCNEC, and a carcinoid tumors is very important. The prognosis of the latter group is usually benign and these tumors frequently occur in non-smoking population, whereas high-grade neuroendocrine tumors are usually the most aggressive and the patients are usually heavy smokers.

In the pathological diagnosis of neuroendocrine tumors chromatin structure, the presence of necrosis and proliferative activity (mitosis number) are important.

2.1.3.4.1. Small Cell Lung Cancer

SCLC consists of small cells with typical dust-like chromatin, decreased cytoplasm and high mitosis number (›11/2mm^2) is seen.

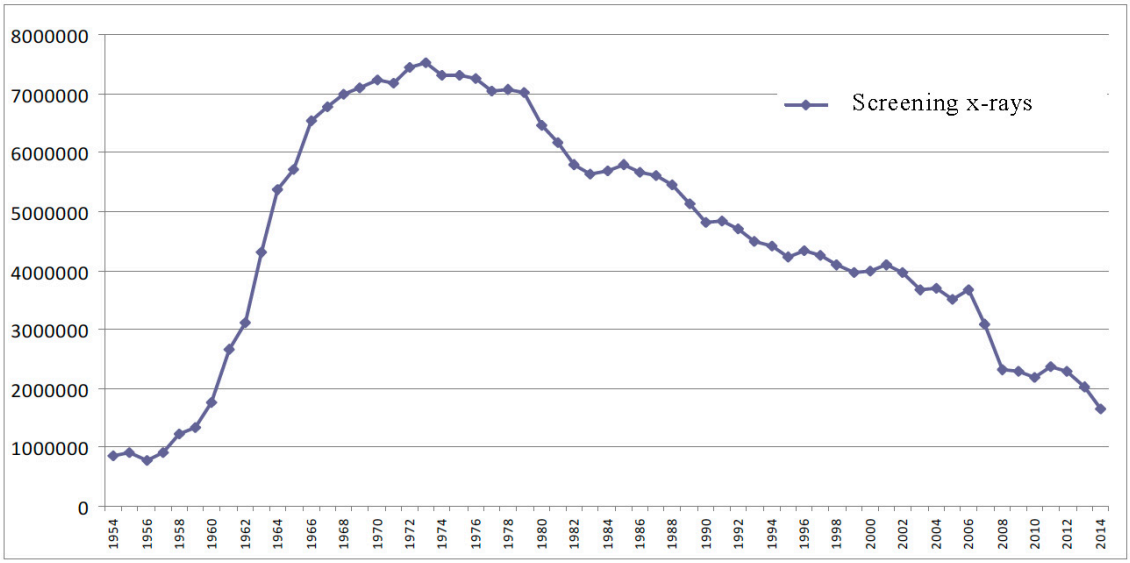
Two groups reported full genomic analysis of SCLC. *SOX2* was reported as a frequently amplified gene in SCLC by Rudin et al(52). It was also shown that *in vitro* suppression of *SOX2* blocked the proliferation of SOX2-amplified SCLC cell lines. Peifer et al. on

the other hand, found mutations in the *CREBBP, EP300*, and *MLL* genes that encode histone modifiers. This suggest that histone modification is an important process in SCLC(53). In 2015, George et al. found complex genetic alterations in SCLC, such as C:G>A:T transversions (in 28% of all cases), inactivation of *TP53*, *RB1* and the *NOTCH* family genes (25% of all cases)(54, 55). These changes are often seen in heavy smokers.

2.1.4. Screening, clinical diagnosis, staging and prognosis of lung cancer

2.1.4.1. Screening

Screening for lung disease traditionally existed in most developed countries for the screening of infectious diseases such as tuberculosis (TB). Radiological screening with roentgen started widely after the II. World War when the price of the radiological examination was lowered(56). In Hungary, yearly lung screening was mandatory for every person of the age of 14 or older form 1960 to 2004. Screening examinations were done in a separate lung-screening network with yearly notifications via post.



5. Figure - Lung screening X-rays conducted in Hungary from 1954 to 2014(57)

Screening included chest x-ray and in some cases cytology and bacteriological culture from sputum. This system was primarily set up to find TB cases, however it helped in the discovery of lung cancer as well. With the notable decrease in TB cases and the worsening

financial system in the Hungarian health care, the screening network has been partially dismantled since 2004 and screening is not mandatory anymore. In the late 1990's lung cancer screening became an important topic in the developed countries. The first country to introduce a modern screening system with computer tomography (CT) was Japan and the first dedicated program was the Early Lung Cancer Action Program in the USA(58, 59).

Several countries, including the USA, Netherlands, Japan, Denmark conducted observational trials and the conclusion was always the same: lung cancer can be found in an earlier stage with low-dose CT (LDCT) screening(60). To acquire reliable mortality data a randomized trial was warranted. The National Cancer Institute (US) founded the National Lung Screening Trial (NLST), a multi-center randomized study that was conducted from 2002 to 2009 and enrolled a total of 53,456 participants (from 2002 to 2004). Only heavy smokers (more than 30 pack year) were included between the ages of 55 to 75. Approximately 50% of cases were randomized to a chest x-ray arm, while the other 50% to a low dose screening arm. 309 deaths per 100,000 persons-year was observed in the chest radiography arm, while 247 in the LDCT arm, which means a 20% relative reduction in lung cancer mortality. Since LDCT screening is a relatively expensive option, population wide LDCT screening is not affordable for most countries. People should be selected on the basis of lung cancer risk assessment.

2. Table - Approximate 10-year risk of developing lung cancer.
Adopted from Bach PB et al. (61)

	Duration of smoking					
	25 years		**40 years**		**50 years**	
Age	**Quit, %**	**Still smoking, %**	**Quit, %**	**Still smoking, %**	**Quit, %**	**Still smoking, %**
			1 pack per day smokers			
55	<1	1	3	5	NA	NA
65	<1	2	4	7	7	10
75	1	2	5	8	8	11
			2 packs per day smokers			
55	<1	2	4	7	NA	NA
65	1	3	6	9	10	14
75	2	3	7	10	11	15

Several models were made to predict the risk of lung cancer development. Bach et al. proposed a risk assessment model which primarily counts smoking history (in Table 2.). Later the Spitz model (62) and the Liverpool Lung project (LLP) (63) model were also published. These models take several factors in consideration: age, age, smoking duration, exposure to carcinogens, diagnosed emphysema, prior diagnosis of pneumonia, prior diagnosis of malignant tumor, family history of cancer in first-degree relatives. D'Amelio et al conducted a validation study including the Bach, the Spitz and the LLP

models. The latter two proved to be more discriminative, accurate and clinically more utilizable(64).

Screening with LDCT after lung cancer risk calculation is an affordable and efficient way of early lung cancer detection and thus a way to decrease lung cancer mortality. It raises some ethical considerations, however. Using the above mentioned risk assessment models mean that non-smokers will most probably be excluded from lung cancer screening. Considering the fact that non-smokers can also develop lung cancer, although the chance is thinner, raises concerns about the above described system. No financially feasible, more effective and ethically non-objectionable method has been developed however. In Hungary, currently no lung screening program is available and this situation must be addressed sooner than later.

2.1.4.2. Clinical diagnosis of lung cancer

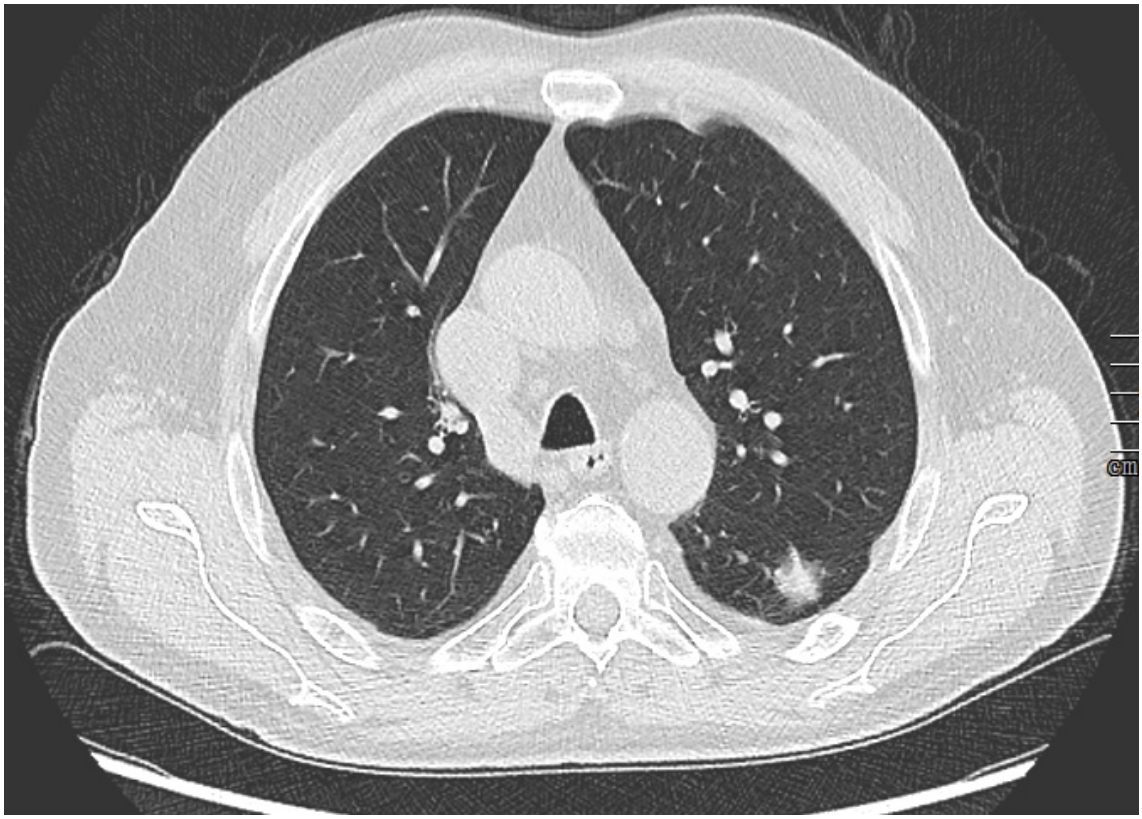
Lung cancer detection in early stage is the most important step to achieve good results with treatment. 85% of the cases however, are only detected when symptoms occur and in 57% the diagnosis is only made when metastases are already present (65). The problem is that in most cases lung cancer does not cause early symptoms. Symptoms can be divided to subgroups (66):

- Symptoms related to endobronchial growth: Cough (8% to 75%), dyspnea (3% to 60%), pain, wheezing (0% to 2%), poststenotic pneumonia, hemoptysis (6% to 35%), stridor (0% to 2%).
- Symptoms related to intrathoracic extension: Chest pain (20% to 49%), hoarseness (recurrent nerve involved), diaphragm paralysis – raised diaphragm (phrenic nerve involved), upper airway inflow obstruction, dysphagia (esophagus involved), pleural effusion (carcinosis pleurae), pericardial effusion (pericardium involved), superior vena cava syndrome (v. cava superior involved), Horner triad, chronic shoulder pain (in Pancoast tumors).
- Systemic cancer symptoms: Weight loss (0% to 68%), fatigue, fever (0% to 20%), night sweats.
- Symptoms of distant metastases: Bone pain (6% to 25%), headache, neurological or psychiatric abnormalities, hepatomegaly, pathological fractures.

- Paraneoplastic syndromes: Cushing syndrome, Lambert-Eaton syndrome, Perre-Marie-Bamberger syndrome, syndrome of inappropriate ADH secretion, etc.

Lung cancer can be detected after the previously mentioned symptoms occur or in asymptomatic changes. Detection is most commonly done by chest x-ray or CT, however MRI (Magnetic Resolution Imaging) and PET (Positron Emission Tomography) are also more frequent.

Lung cancer diagnosis is often made from CT morphology and optionally the level of FDG uptake on PET, when a spiculated mass is seen, however pathological verification is also needed, primarily to distinguish between SCLC and NSCLC because the treatment strategies are very different. SCLC in most cases presents in a later stage with lymph node involvement. Generally small, central lung lesions with extensive lymphatic involvement always raise the concern for SCLC. The pathological diagnosis can be made from biopsies or after operation depending on the stage and treatment plan.



6. Figure – CT axial section of a spiculated mass in the left lower lobe (segment 6). Picture obtained from the Department of Thoracic Surgery, National Institute of Oncology, Budapest.

Lung cancer workup includes flexible bronchoscopy for the intrabronchial evaluation of anatomy and tumor spread. Bronchial washing and brushing can be done, which is ideal for intrabronchial or central tumors. Biopsies can be made through the bronchoscope with x-ray or ultrasound guidance (TBNA – trans-bronchial needle aspiration, EBUS – endo-bronchial ultrasound). The primary lesion and most regional lymph node stations can be reached with EBUS and also endoscopic ultrasound (EUS) guided biopsy can be of help. In case of peripheral lesions, transthoracic fine needle aspiration or core biopsy under image guidance (usually CT) can be performed to get diagnosis and even the molecular characteristics of the tumor. Thoracentesis can also be of help in case of pleural fluid as a palliative and diagnostic tool. When none of the above mentioned methods yield a result, invasive, surgical techniques can be considered like mediastinoscopy, VATS biopsy or thoracotomy.

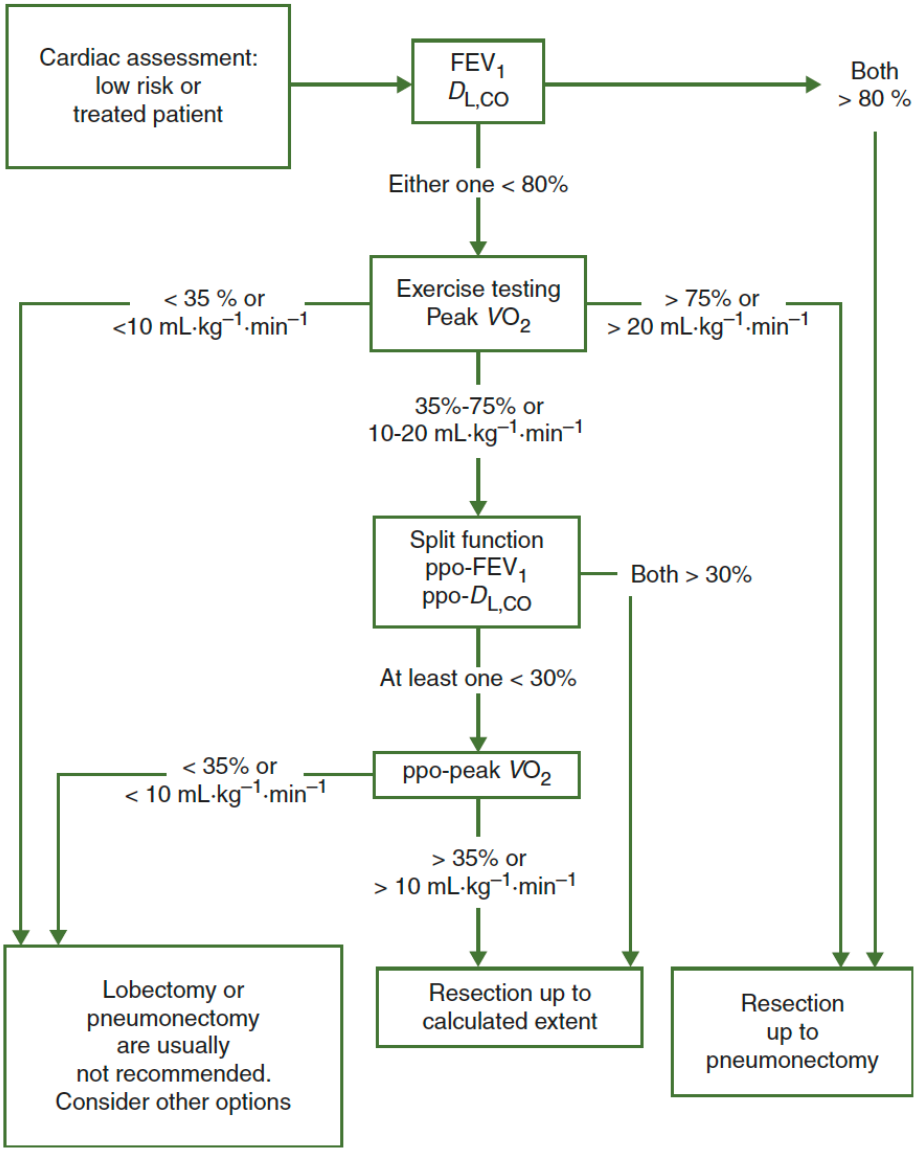
To provide adequate treatment it is important to assess how far the tumor have spread (staging) and to estimate the physical condition of the patient.

2.1.4.2.1. Functional evaluation of patients diagnosed with lung cancer

Physical evaluation of patients have to include the assessment of comorbidities, general performance (Karnofsky-index and the Eastern Cooperative Oncology Group (ECOG) status is most commonly used) and lung function. The most important comorbidities to take into account are cardiac, liver and renal diseases that can affect the feasibility and the result of certain treatment options. Cardiac echographia with the measurement of cardiac output and in selected cases cardiac stress test is often needed. Liver and renal function is tested with laboratory workup.

The evaluation of lung function is most important before surgical treatment, where the risk of the treatment and the quality of life achieved have to be taken into consideration. Generally, Forced Expiratory Volume in 1 second (FEV_1), Forced Vital Capacity (FVC) and Diffusing Capacity (DLCO) are measured. Patients with FEV_1 of more than 80% of the normal (adjusted for size and age) and DLCO more than 80% are suitable for surgical treatment (including pneumonectomy) without further tests. In case either of these values are under 80% spiroergometry is recommended. If the maximum oxygen consumption is above 20mL/min/kg or 75% of normal, the patient can be referred for surgery (including

pneumonectomy). When the maximum oxygen consumption is below 40% of the expected (or ‹ 10mL/min/kg) functional inoperability is established. In case the maximum oxygen consumption is between 40% and 80%, postoperative FEV_1 are calculated on the basis of quantitative ventilation and perfusion on lung scintigraphy. If the estimated FEV_1 after the procedure is higher than 40% of normal, the operation can be performed; if it is below 40% functional inoperability has to be assumed.



7. Figure – Algorithm of functional evaluation recommendation of the European Society for Medical Oncology (ESMO); 2017 (67)

2.1.4.3. Staging of lung cancer

To evaluate the extent of the disease CT, PET CT, bone scintigraphy, brain MRI, abdominal ultrasound and biopsies are used. The staging is made following the actual international guideline. The latest staging system for lung cancer is the 8th tumor, node, metastasis (TNM) classification which was introduced after examining 77.156 cases and their outcome regarding survival and relapse (68).

T stage is determined by the size of the primary tumor and its involvement of the adjacent structures:

3. Table - T value in the 8th edition of TNM staging. Adopted from: International Association for Study of Lung Cancer, 2015. [69]

T – Primary Tumour		
Tx		Primary tumour cannot be assessed
T0		No evidence of primary tumour
T1		Tumour 3 cm or less in greatest diameter surrounded by lung or visceral pleura, without evidence of main bronchus
	T1a(mi)	Mininally invasive adenocarcinoma
	T1a	Tumour 1 cm or less in greatest diameter
	T1b	Tumour more than 1 cm but not more than 2 cm
	T1c	Tumour more than 2 cm but not more than 3 cm
T2		Tumour more than 3 cm but not more than 5 cm; or tumour with any of the following features: Involves main bronchus (without involving the carina), invades visceral pleura, associated with atelectasis or obstructive pneumonitis that extends to the hilar region
	T2a	Tumour more than 3 cm but not more than 4 cm
	T2b	Tumour more than 4 cm but not more than 5 cm
T3		Tumour more than 5 cm but not more than 7 cm or one tha directly invades any of the following: chest wall, phrenic nerve, parietal pericardium, or associated separate tumour nodule(s) in the same lobe as the primary
T4		Tumours more than 7 cm or one that invades any of the following: diaphragm, mediastinum, heart, great vessels, trachea, recurrent laryngeal nerve, oesophagus, vertebral body, carina; separate tumour nodule(s) in a different ipsilateral lobe to that of the primary

N stage determines the lymph node involvement of lung cancer in the hilar and mediastinal regions. Lymph node involvement separates cases by prognosis and thus have a great effect on treatment.

4. Table - N value in the 8th edition of TNM staging. Adopted from: International Association for Study of Lung Cancer, 2015. (69)

N – Regional Lymph Nodes		
Nx		Regional lymph nodes cannot be assessed
N0		No regional lymph node metastasis
N1		Metastasis in ipsilateral peribronchial and/or ipsilateral hilar lymph nodes and intrapulmonary nodes, including involvement by direct extension
N2		Metastasis in ipsilateral mediastinal and/or subcarinal lymph node(s)
N3		Metastasis in contralateral mediastinal, contralateral hilar, ipsilateral or contralateral scalene or supraclavicular lymph node(s)

The N categories were made after the survival was assessed in pathological classified cases. N1 was divided into N1a (single station, ipsilateral hilum) and N1b (multiple stations); and N2 into N2a1 (single N2 station without N1 involvement), N2a2 (single N2 station with N1 involvement) and N2b (multiple N2 stations). The 5-year survival rates were the following: N1a, 59%; N1b, 50%; N2a1, 54%; N2a2, 43%; and N2b, 38%. A single-stage mediastinal nodal disease without N1 disease has the same prognosis as multiple N1 station involvement.

M stage defines distant metastases beyond the regional lymph nodes.

5. Table - M value in the 8th edition of TNM staging. Adopted from: International Association for Study of Lung Cancer, 2015. (69)

M – Distant Metastasis		
M0		No distant metastasis
M1		Distant metastasis
	M1a	Separate tumour nodule(s) in a contralateral lobe; tumour with pleaural or pericardial nodules or malignant pleural or pericardial effusion
	M1b	Single extrathoracic metastasis in a single organ
	M1c	Multiple extrathoracic metastases in one or several organs

M1b was introduced as a separate category for the single extrathoracic metastases, since this patients had a significantly better survival (with a mean survival of 11.4 months instead of 6.3 months)(70). The treatment strategy also differs for these patients, since in

selected oligometastatic cases better survival can be achieved with aggressive local therapy, whereas in M1c cases systemic therapy has better results.

The appropriate stage is given from the TNM values according to the following table:

6. Table – Staging according to the 8th TNM. Adopted from: International Association for Study of Lung Cancer, 2015. (71)

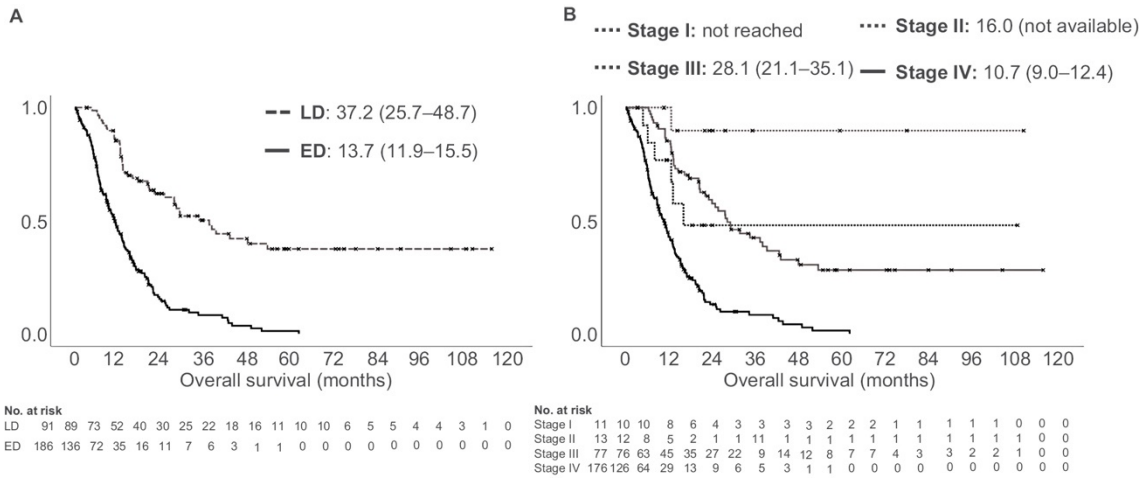
T/M	Subcategory	N0	N1	N2	N3
T1	T1a	IA	IIB	IIIA	IIIB
	T1b	IA	IIB	IIIA	IIIB
	T1c	IA	IIB	IIIA	IIIB
T2	T2a	IB	IIB	IIIA	IIIB
	T2b	IIA	IIB	IIIA	IIB
T3	T3	IIA	IIIA	IIIB	IIIC
T4	T4	IIIA	IIIA	IIIB	IIIC
M1	M1a	IVA	IVA	IVA	IVA
	M1b	IVA	IVA	IVA	IVA
	M1c	IVB	IVB	IVB	IVB

2.1.4.4. Prognosis of lung cancer

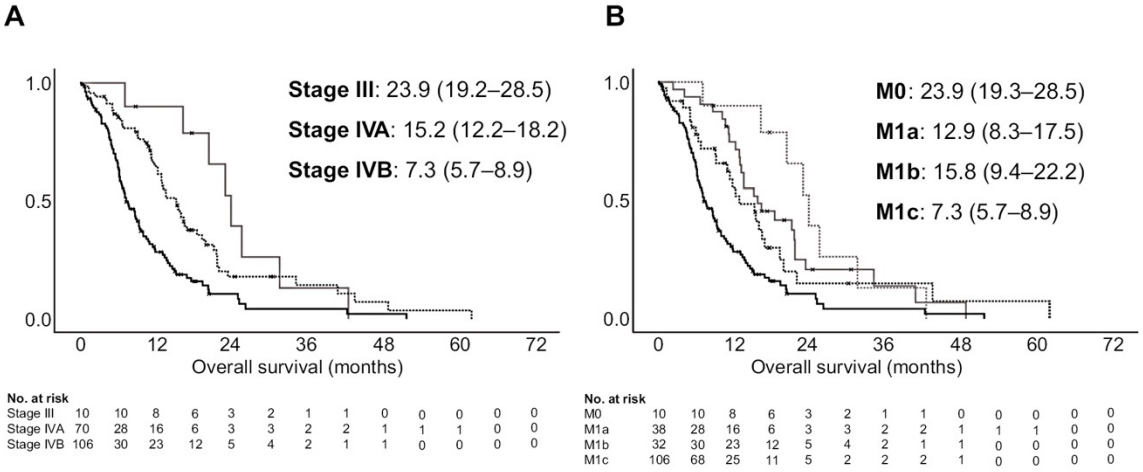
The prognosis depends highly on the histology, most importantly the distinction between SCLC and NSCLC, the biological behavior of the cancer and the stage.

2.1.4.4.1. Prognosis of SCLC

For the staging of SLCL currently the 8th TNM system is used, however another approach classifies SCLC into two groups: Limited Disease (LD) and Extensive Disease (ED). This classification reflects the clinical behavior and the spread of the tumor rather well, since LD patients have significantly superior survival when compared to ED patients. One contributing factor is that often small, accidentally found lesions turn out to be SCLC after final pathological examination. In these cases the surgical removement is done in a very early stage. The TNM system gives a more accurate estimation of the prognosis, however very few cases are found in early stages. When creating the 8th TNM for SCLC, Shirasawa et al. included 277 patients in a retrospective cohort and compared their prognosis. In this cohort 65.7% of the patients were classified as ED and 34.3% as LD, the OS was 37.2 (95% CI 25.7–48.7) and 13.7 (95% CI 11.9–15.5) months, respectively (this can be seen of Figure 8A). On Figure 8B. OS can be seen according to the TNM stages. OS drops markedly between stages, comparison of the curves reveals significant difference between every line (P values were the following: stage I vs II, P=0.04; I vs III, P=0.02; I vs IV, P<0.001; II vs III, P=0.47; II vs IV, P=0.009; III vs IV, P<0.001). It is important to know however, that the distinction between IVA and IB stage is also important because the change of M descriptor was also significantly associated with OS. OS of stage IVB patients with the M1c descriptor was inferior to that of the stage III and IVA patients with the M0, M1a, or M1b descriptors (Figure 9A-B) (72).



8. Figure - Kaplan–Meier analysis-based estimates of survival based on staging system in SCLC patients (n=277). (A) Comparison of survival between LD (n=91; gray) and ED (n=186; black) SCLC patients (P<0.001). (B) Comparison of survival between patients in TNM stages I to IV. Adopted from (72).

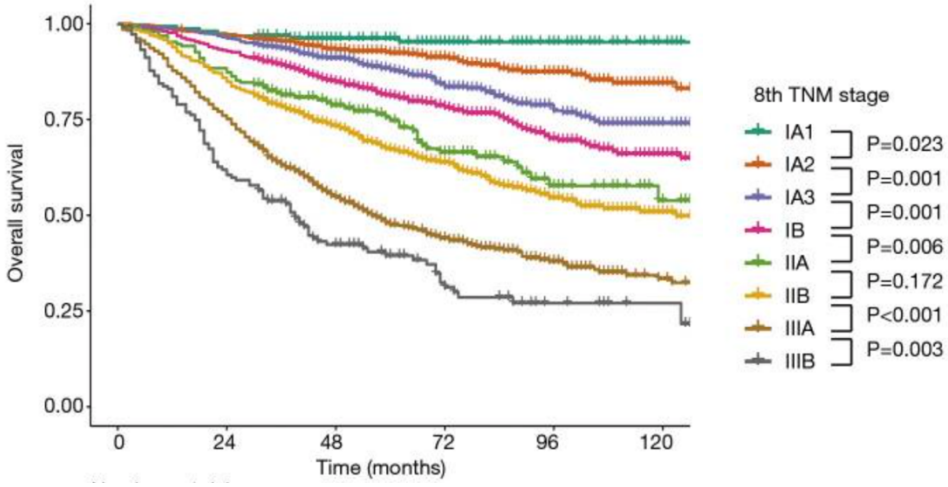


9. Figure - Kaplan–Meier analysis-based estimates of survival for ED SCLC patients. (A) Comparison of survival between patients in stages III (n=10, gray solid line), IVA (n=70, gray dotted line), and IVB (n=106, black solid line) (B) Comparison of survival according to the M descriptor of the 8th TNM. Adopted from (72).

2.1.4.4.2. Prognosis of NSCLC

The average 5-year survival in NSCLC is approximately 15%. This dismal result is due to the fact that most cases are found in latter stages (73) as previously mentioned. When

recently validation the 8th TNM Yun et al. compared the survival of 3950 following lung cancer resection. All TNM stages showed significant differences (except IIA and IIB where the difference was not significant). The 5-year median survival was 90.7%, 79.9%, 68%, 58.7% and 44.9% for IA, IB, IIA, IIB and IIIA stages, respectively according to the 7th TNM, however, reassessing the patients with the 8th TNM the 5 year median survival was quite different: 96.1%, 92.3%, 87.9%, 81.1%, 74.7%, 67.2%, 47.5% and 38.3% for IA1, IA2, IA3, IB, IIA, IIB, IIIA and IIIB stages, respectively. These results can be seen on Figure 10. The 8th TNM distinguishes better between both early and advanced stage NSCLC patients according to Yun et al (74).



10. Figure – Survival curves of OS according to the 8th TNM in the cohort of Yun et al. (74)

The 8th TNM was not yet validated in regards to stage IIIB and IV. However, available survival data shows significantly worse survival in these groups. The 5-year survival is reported to be 19%, while the median survival is 16 months in IIIB stage. The median survival drops to 6 months in IV. stage with the 5-year survival only 6%(75). This is already and improved results as 5-year survival virtually never existed in IV. stage before. Further development and superior survival results are to be expected with the introduction of novel therapies such as immunotherapies.

2.1.5. Treatment of lung cancer

2.1.5.1. Treatment options for SCLC

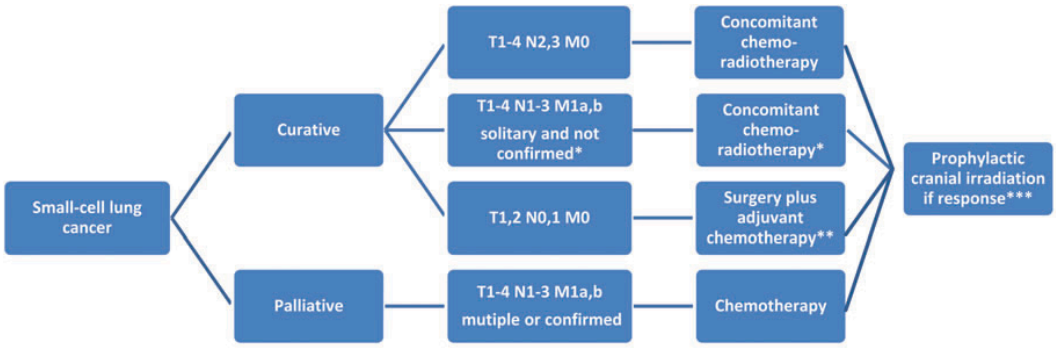
The TNM staging system is useful to select patients for surgical treatment, however only very few cases are suitable for removal, reportedly less than 5%(76). Most of these patients are operated for an unknown lesion in the lung. These patients have superior outcome with 5-year survival up to even 50% is some reports (77, 78). Surgical approach can only be selected in these cases when lymph node involvement is ruled out with adequate mediastinal staging with PET-CT, EBUS and or mediastinoscopy. Postoperative adjuvant chemotherapy (CHT) is indicated even in T1-2,N0,M0 cases. Usually 4 cycles of CHT is administered. In case of unexpected N1 or N2 disease postoperative radiotherapy should be considered depending on the completeness of lymphadenectomy performed by the surgeon.

In cases with N2 disease, even without distant metastases (M0) surgery has no role. Total gross tumor volume proved to be an independent prognostic factor independent on the local therapy(79). N2 patients should undergo combined concurrent chemoradiotherapy. Prophylactic cranial irradiation (PCI) should be considered at al T1-2,N0-1,M0 patients in case they responded to initial treatment.

Patients with T1-4,N0-3, M0 SCLC should be treated with chemotherapy and thoracic radiation concurrently in case the performance status (PS) allows. 70 Gy in daily fractions or 1.5 Gy in 30 fractions twice a day are usually selected options for radiotherapy. For the planning of radiotherapy to choose the target volume a PET CT is usually used.

For patients with metastatic SCLC the treatment is palliative. Most commonly a combination of CHT is used. A big meta-analysis reviewed the data of 19 randomized trials and shown that cisplatin was superior in terms of outcome in combination therapy when compared to other agents(80). Another collecting the data of 36 randomized trials concluded that etoposide, especially in combination with cisplatin showed longer OS than regimens without these two agents(81). The above mentioned results led to the clinical practice of etoposide-cisplatin combination therapy for SCLC as a standard of care. Usually 4-6 cycles are administered in first line treatment.

PCI has led to significantly longer OS and less symptomatic brain metastases in randomized trials (82), thus patients responding well to initial CHT are usually offered PCI as well (25 Gy in 10 daily fractions or 20 Gy in 5 fractions).



***if no confirmation of solitary metastasis is obtained, radiotherapy may be added after first response evaluation and is omitted in case of obvious metastatic involvement**
**** concomitant chemoradiotherapy as an alternative option**
***** or stable disease in case of localised disease**

11. Figure – First line treatment algorithm for SCLC (ESMO 2013). Adopted from (83).

Second-line treatment for SCLC is only recommended in patients who respond to the first-line therapy. In patients who progress during chemotherapy and non-responders (early relapse within 6 weeks) outcomes are poor and further systemic therapy does not yield significant benefit. For these patients best supportive care is offered. Topotecan, a topoisomerase-I inhibitor, is a drug used for previously treated patients with SCLC. Its efficacy in terms of longer OS and better quality of life (better symptom control) was proven in a phase III trial (84). 8 years later the oral version of the drug was also tested in a phase III trial and was found to be equally efficient(85).

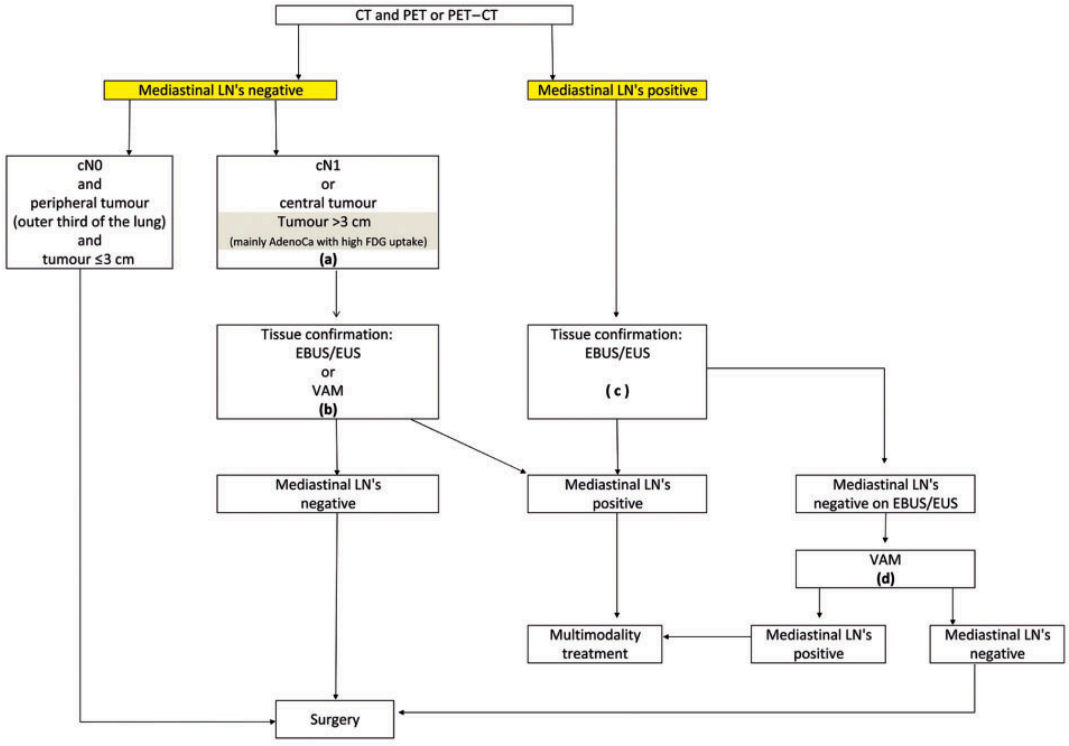
Targeted therapies were studied in extent for SCLC both in combination with chemotherapy and as a single agent, however very few studies managed to meet the primary endpoint. The examined drugs included: bevacizumab (86), vandetanib (87) and aflibercept (88) (anti-angiogenesis agents), panobinostat (89) (histon deacetylase inhibitor), oblimersen (90) (an agent targeting apoptosis) and cixutumumab and vismodegib (91) (agents targeting cell signaling). Alisertib, an aurora kinase inhibitor and sunitinib, a multi-targeted receptor tyrosine kinase inhibitor (TKI; targeting VEGFR, PDGFR and KIT) are the only agents showing preliminary signs of efficacy in SCLC(92,

93). The latter also showed improvements in PFS, however it is still not clear whether they will impact the standard of care.

2.1.5.2. Treatment of NSCLC

2.1.5.2.1. Treatment of early stage NSCLC

Surgical removal remains the first choice of treatment in localized, resectable NSCLC (Stage I and II). First-line surgery provides the best survival. As previously mentioned 5-year survival is between 90.7% (IA) and 58.7% (IIB)(74).
The choice of upfront surgery is made after staging, assessment of technical operability and tissue biopsy when needed. The algorithm of lymph node staging recommended by ESMO can be seen on Figure 15.



(a) : In tumours > 3 cm (mainly in adenocarcinoma with high FDG uptake) invasive staging should be considered
(b) : Depending on local expertise to adhere to minimal requirements for staging
(c) : Endoscopic techniques are minimally invasive and are the first choice if local expertise with EBUS/EUS needle aspiration is available
(d) : Due to its higher NPV, in case of PET positive or CT enlarged mediastinal LN's, videoassisted mediastinoscopy (VAM) with nodal dissection or biopsy remain indicated when endoscopic staging is negative. Nodal dissection has an increased accuracy over biopsy

12. Figure – ESMO 2017: recommended algorithm for staging in patients with M0 NSCLC (FDG - fluorodeoxyglucose; LN - lymph node, NPV - negative predictive value; VAM - video-assisted mediastinoscopy) Adopted from (94).

Surgical techniques have improved significantly in the last twenty years. Most of the traditional thoracic operations were first described between 1900 and 1950. The first anatomical dissection of hilar structures, as lobar vein, artery and bronchus was first done in 1933 by Rienhoff, which was a big step to achieve fewer complications. The techniques improved quickly and anatomical segmentectomy (Churchill and Belsey in 1939) and even sleeve lobectomy was developed by 1947 (Price Thomas) (95). From the 1950s to the 1980s new surgical instruments were developed, but the main types of lung operations were not changed.

Wedge resection is an atypical resection of lung parenchyma most often done by surgical staplers. Atypical in this sense means that the resection line does not follow anatomical structures or borders. It is mainly used for inflammatory, benign, metastatic or unknown lung lesions. Segmentectomy means the selected resection of one or several pulmonary segments. This procedure requires precise surgical technique and was mainly used in situations when the pulmonary function did not allow more extensive parenchyma resection. Lobectomy is the gold standard operation for the removal of primary lung malignancies. Lobectomy means the central dissection and ligation or stapling of the lobar pulmonary artery(s), vein and bronchus. Lobectomy is also often used when the parenchyma of a complete or near complete lobe is damaged by inflammatory diseases or when a secondary tumor is deep in the parenchyma and a wedge resection is not possible. Pneumonectomy is the most extensive operation performed on a single lung, it means the complete removal of the lung on one side by centrally closing the main pulmonary artery, lower and upper lobe veins and the main bronchus.

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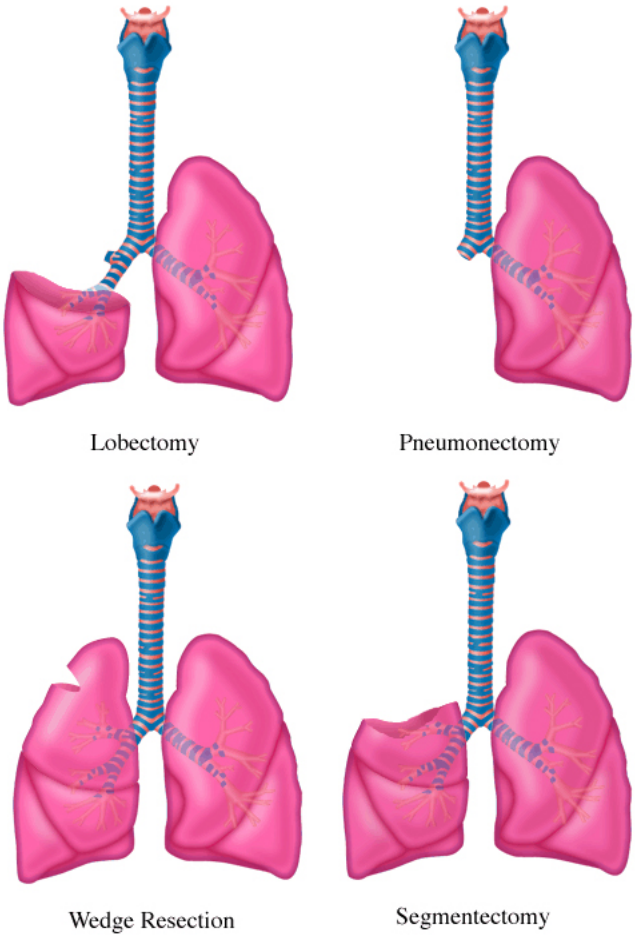

13. Figure – The main four types of pulmonary resections (Education material from the University of South Carolina; access: cts.usc.edu)

All of the above mentioned procedures were done through a thoracotomy, when the thoracic cavity is approached through a rib space. To get appropriate access to preform complicated operations, long wounds had to be made and the ribs had to be either resected or pushed apart from each other. Thoracotomy was and still is a painful operation and it can have significant complications which sour the patients life. Most pain is caused by damaging the intercostal nerves. Neuralgic pain can last for years after the operation. A less invasive way to operate the chest was introduced very early: the first thoracoscopic procedures are attributed to Jacobeus (1910), who used a cystoscope to evacuate pleural fluids and to take pleural biopsies, although Sir Francis Richard Cruise was the first to apply and endoscope in the chest in 1865. Despite the early thoracic application of the technique, major thoracic procedures were only performed in the late 1980s, early ‘90s, after the video camera was invented. Rovario performed and described the first lobectomy

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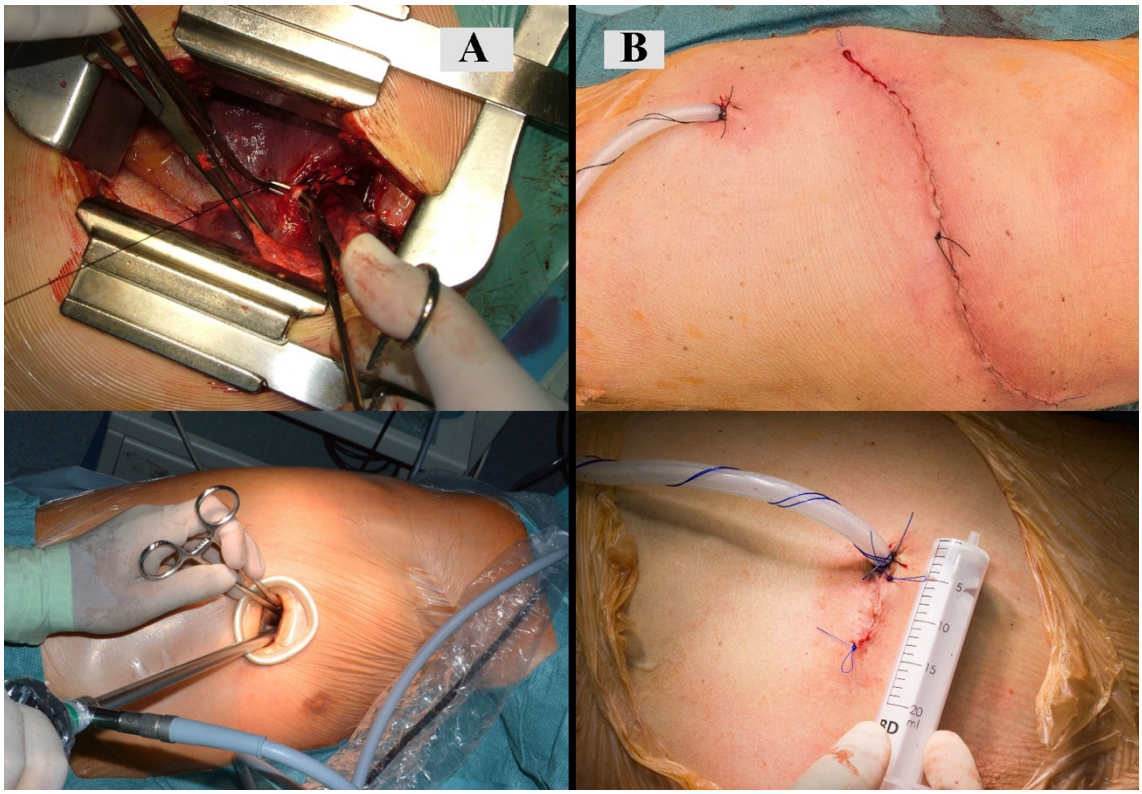
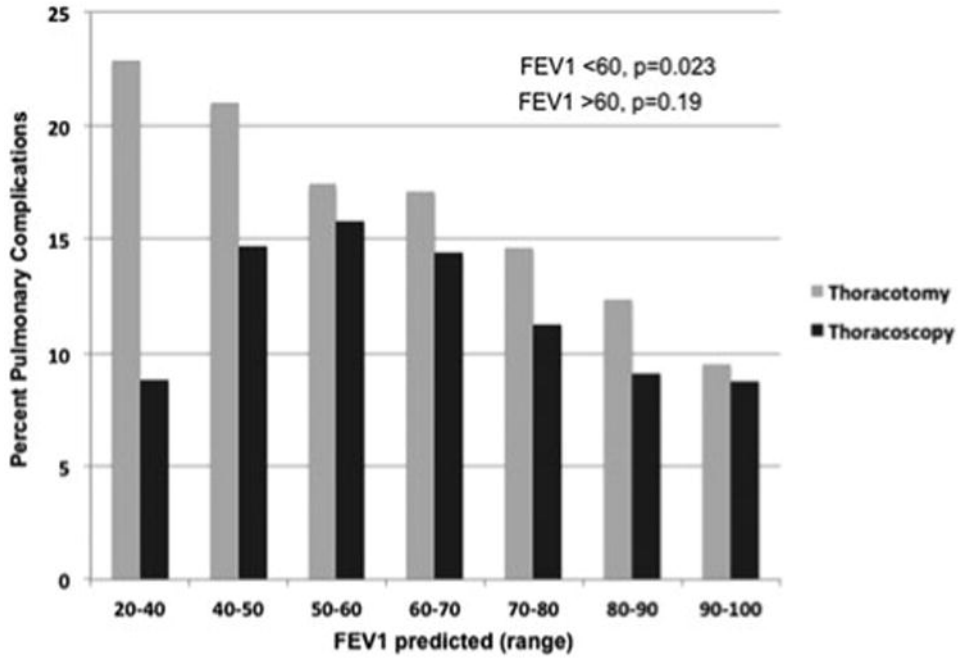
done by Video Assisted Thoracic Surgery (VATS) in 1991 (96). Later McKenna presented already 1000 cases of VATS lobectomy and in 2008 the first VATS sleeve lobectomy as well (97). VATS can be done through several small incisions, most commonly three, however newer techniques emerged: Gonzales-Rivas performed the first lobectomy in 2011 through one port (uniportal or single-port) (98). Ever since there was a shift towards single-port and less invasive surgery. At the beginning VATS was only used for small or benign lesions and there was a general view that VATS is less radical. However, later several study proved that OS was comparable after VATS lobectomy as with an open procedure (99-103) and the amount of lymph nodes taken out was also not different (104). In the US centers often choose another minimal-invasive technique instead of VATS, the Robotic Assisted Thoracic Surgery. Robotic surgery got widely accepted as a form of minimal-invasive surgery first in the US. Postoperative pain is reported to be comparable with VATS and the OS comparable with VATS and thoracotomy (105, 106).



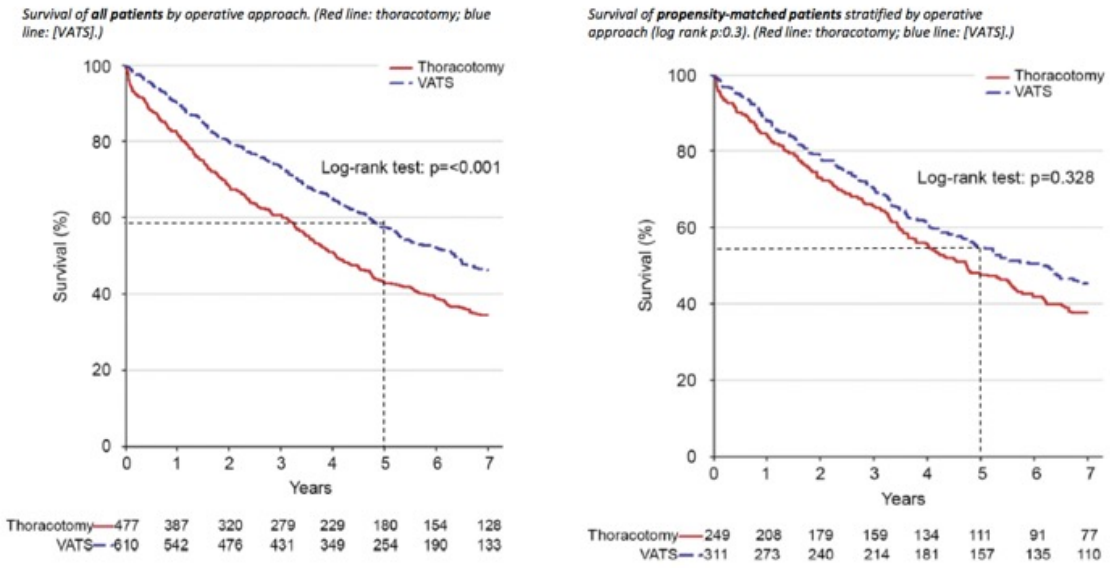
14. Figure – Thoracotomy versus VATS. Comparison of (A) intraoperative access and incision and (B) postoperative wound length (Pictures taken in the Department of Thoracic Surgery, National Institute of Oncology, Budapest)

The real advantage of VATS or robotic thoracic surgery is shown in the early postoperative phase when patients are pain-free and can leave the hospital on the postoperative second day. This leads to less pulmonary complications (107), and some studies suggest that even less immunosuppression and thus better resistance against cancer (108). Ceppa et al. used the national database in the US, in their meta-analysis they showed that the greatest benefit of VATS can be seen in patients with poor pulmonary function. The incidence of pulmonary complications in patients with compromised pulmonary reserves, was higher after thoracotomy than after VATS (109), as can be seen on the figure below.



15. Figure – Pulmonary complications after thoracotomy (grey) and VATS (black). The difference of pulmonary complications after surgery is most significant in patients with poor pulmonary function (FEV1 lower than 50%). Adopted from (109).

It is not yet widely accepted that OS is better after VATS or robotic thoracic pulmonary resection and most guidelines do not differentiate, data suggest that minimal invasive resection should be the gold standard of care for early lung cancer (108).



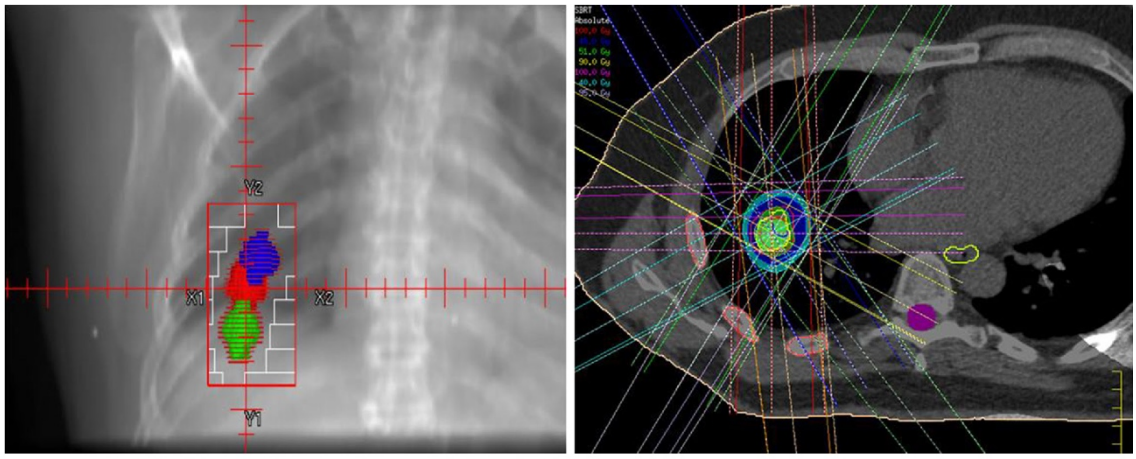
16. Figure – OS is longer in patients who had VATS resection for early lung cancer when compared with those who underwent resection through thoracotomy (left). The difference is still visible after propensity crossmatching, although it is not statistically significant (right). (Figures adopted from Berry et al. (108))

Although imaging, surgical and staging techniques have significantly changed in the last two decades, still the Lung Cancer Study Group (LCSG) 821 trial remains the ground for surgical treatment. According to this trial lobectomy is the cornerstone of surgical therapy for stage I and II NSCLC, as local recurrence was found more frequently after less radical operations (wedge resection and segmentectomy) (110). This data and along that our current practice will most probably be updated as the new TNM distinguishes between several types of stage I NSCLC. Based on large collected cohorts segmentectomy seems equally appropriate for T1a tumors. In squamous cell carcinoma lobectomy was found to be superior even in early stages, however in adenocarcinoma equivalent results were reported after anatomical segmentectomy (111). Of note, in another study anatomical segmentectomy was also found to be equally effective in T1c adenocarcinoma (112). Altogether it can be anticipated that in selected early NSCLC anatomical segmentectomy will be the choice of treatment, however no hard data is available to support this yet. Two major randomized studies are underway (CALGB 140503 and JCOG0802/WJOG4607L) (113, 114). The preliminary results have been published of the latter about the safety and feasibility of segmentectomies (simple and complex as well), their results show that

segmentectomy is not inferior in any of the examined parameters (complications, length of hospital stay, duration of air leak etc.) (115).

Lymph node management during surgery is also controversial. In early stages, the removal of lymph nodes is mainly necessary for staging purposes, to get a guaranteed R0 resection and an accurate mediastinal staging. The latest ESMO recommendation implies surgical evaluation of at least six lymph node stations, three of which are mediastinal, including the sub-carinal station. If all of these are negative on pathological report than R0 and N0 can be safely stated (116). It is widely accepted that in early stages, especially stage I OS and PFS, as well as local recurrence might not be influenced by the type of surgical lymph node assessment, however in stage II and particularly IIIA intraoperative nodal dissection is recommended (117). The extent of dissection is also up to debate, the most common technique is lobe specific lymph node dissection, though perioperative lymph node assessment (EBUS, mediastinoscopy) can also be of effect.

For high-risk patients, or for patients who are unwilling to take the risk of an operation, or patients with unresectable tumors primary stereotactic body radiotherapy (SBRT) can be recommended(67). The reported local control rate of SBRT is between 79-90.9% at 5 years (118, 119). The recommended dose is a biologically equivalent tumor dose of 100 Gy. The toxicity using this dose SBRT for stage I or II peripheral NSCLC is low and acceptable, even in elderly and patients with severe chronic obstructive pulmonary disease (COPD) (120). Data is available on outcomes of SBRT in patients who underwent the procedure for peripheral stage I tumors and are generally fit for surgery, however, there is no evidence to currently to recommend SBRT over up front surgery (121, 122). One problem with SBRT is the lack of pathological confirmation after the procedure. Randomized studies are being conducted, although three have failed to complete its plan (123). Two studies that have concluded reported comparable recurrence-free survival three years after the procedure . Given, that the toxicities are tolerable and the quality of life is good after SBRT, patient opinion should be more included in the discussion to recommend personalized treatment in the future.



17. Figure – SBRT distribution plan for NSCLC. Adapted from (124).

Multiple primary tumors should also be assessed with curative intent and complete surgical resection is recommended when feasible. In patients with borderline risk, combination of surgery and SBRT has been proven efficient(125).

In case a patient has contraindication against both surgery and SBRT radiofrequency ablation (RFA) might be the reasonable choice of treatment, however the level of supporting evidence is from observational cohorts only (126).

Postoperative radiotherapy is rarely indicated in stage I and II cases. It was found detrimental in cases without lymph node invasion (127). Cases with unexpected N1 and N2 disease are not clear. Postoperative radiotherapy (concurrent with chemotherapy) seem a reasonable choice when R0 resection is suspected (mainly because of N1 lymph nodes) or when the N2 lymph nodes are involved. To support this recommendation a large clinical trial is underway (128).

Adjuvant platinum based chemotherapy was found to have beneficial effect on survival for stage II NSCLC patients(129). Altogether a 4-5% improvement in 5-year survival was reported in N1 and N2 disease. The adjuvant regimen used was 300mg/m2 of cisplatin in three to four cycles (130). The best timing of adjuvant chemotherapy is not entirely clear, although most protocols say 6 weeks after surgery, recent data form the National Cancer Database (United States of America – US) show comparable results after longer interval between resection and chemotherapy (131).

Based on this knowledge, patients with completely resected stage II tumors are recommended adjuvant chemotherapy within 6-8 weeks after surgery. On the other hand,

in stage IA, chemotherapy has not been proven to be beneficial in an adjuvant setting(132). In IB a small benefit was seen in OS and an in depth analysis showed that this was due to patients with tumors >4 cm (133).

In stage IA NSCLC is therefore only followed up after resection, in IB, however adjuvant chemotherapy can be recommended in cases when the tumor size is over 3-4cm.

As of today, no evidence suggest that the use of targeted agents, such as TKIs for EGFR mutant cases, yield a benefit in an adjuvant setting. Prospective studies are being conducted, however until their result is presented, targeted agents are not recommended as adjuvant CHT.

There is less experience with neoadjuvant CHT in early NSCLC, most studies found no survival comparing neoadjuvant and adjuvant modalities (134, 135). In some, selected cases when downstaging or size reduction is needed for surgical removal, neoadjuvant regimens can potentially be of use (less extensive surgical procedure is possible after) (136).

Both adjuvant and neoadjuvant trials have been initiated using immunotherapy (anti-PD-1 and anti-PD-L1 checkpoint inhibitors) for early lung cancer (trials NCT NCT02504372, NCT02273375 and NCT02998528) on the basis of the promising results in second line curative therapy. This might change the clinical practice in the near future.

2.1.5.2.2. Treatment of locally advanced (stage III) NSCLC

Stage III or locally advanced (LA) NSCLCs can be divided in two groups: surgically resectable LA NSCLC and unresectable LA NSCLC. The treatment strategy is understandably different.

Resectable LA NSCLC usually occurs in three instances: 1) single-station N2 lymph node involvement is suspected or confirmed by biopsy (other stations were found negative with PET and biopsy). In these cases complete lymphadenectomy and postoperative CHT has shown superior outcome compared to surgery or chemotherapy alone (137). However, it is not clear whether surgery or SBRT is the better option in these cases. In the limited number of studies comparing the two modalities of local control no clear advantage was shown for either (138, 139).

2) T4N0 tumors where R0 resection is considered feasible. In these cases up front surgery (generally open technique) and postoperative CHT is recommended. Potentially

resectable superior sulcus tumors should first be treated with neoadjuvant concurrent radio-chemotherapy and followed by resection. The same can apply to central T4 cases. Surgery should not be more than 4 weeks after radiotherapy in these cases (139).

3) in cases with multiple level N2 involvement who underwent neoadjuvant chemotherapy and regression is confirmed by CT. In these cases surgery is recommended followed by definitive CHT (140).

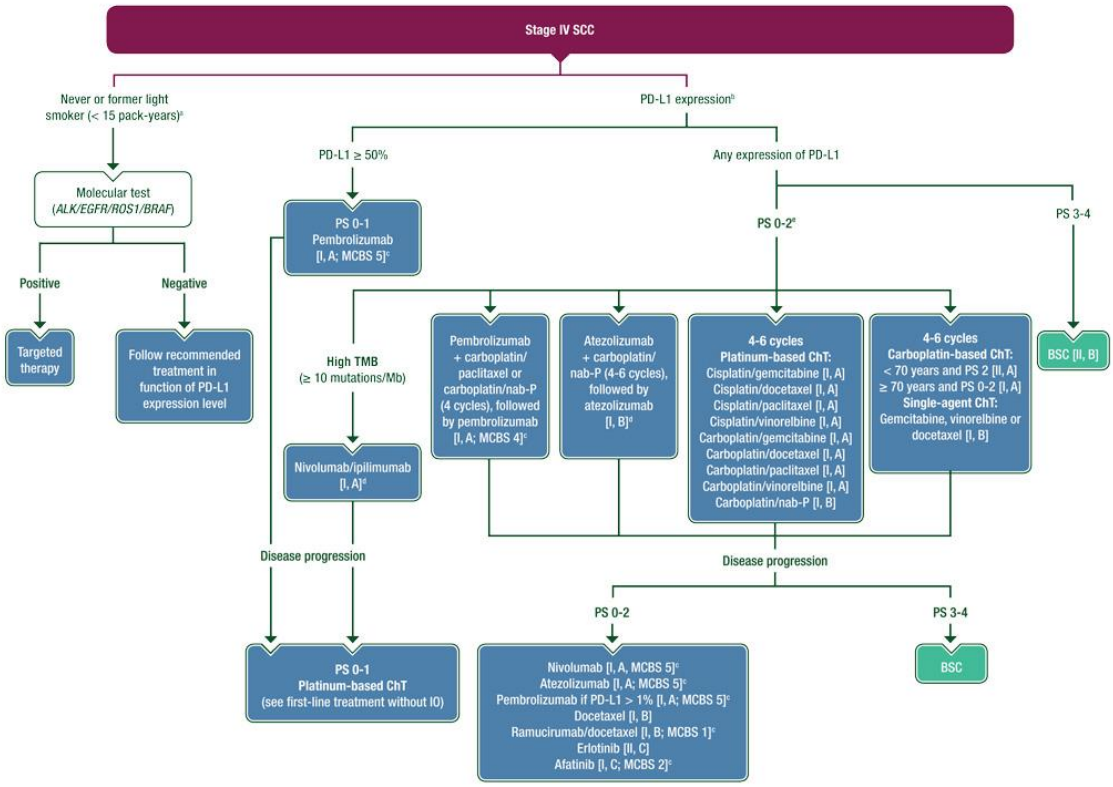
Standard cisplatin based systemic therapies were found to be the most effective in an adjuvant setting in stage III, although immunotherapy is currently being evaluated. Pemetrexed-cisplatin failed to show benefit in OS versus the standard cisplatin-etoposide (141).

In unresectable LA NSCLC (in cases of stage III patients where R0 resection is not considered possible by a multidisciplinary team) concurrent chemo-radiotherapy was shown to have the best results (142, 143). There is no data regarding targeted therapy in stage III patients with driving mutations such as EGFR or ALK. No conclusion can be drawn from the two studies examining this subject (144, 145).

2.1.5.2.1. Treatment of metastatic NSCLC

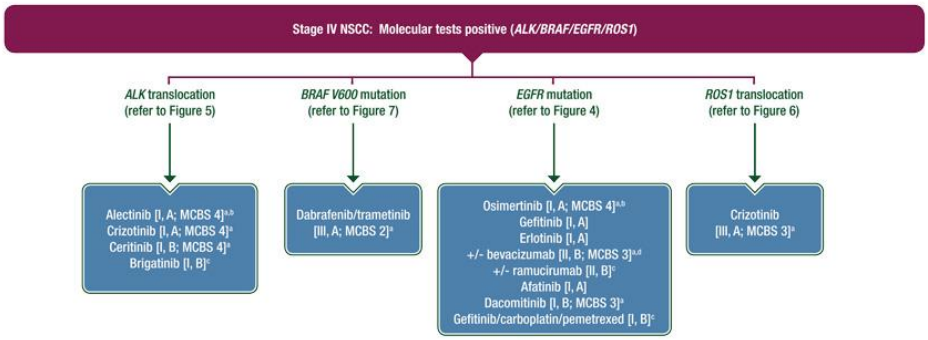
The treatment strategy for stage IV NSCLC varies greatly on histology, molecular pathology, age, comorbidities – performance status (PS) and patient preference. Most cases are offered systemic therapy when the ECOG PS is below 2, with some notable exceptions. Smoking cessation is important in any stage of lung cancer and it should be encouraged in stage IV as well, as it can interfere with some of the systemic treatments (146, 147).

Patients with newly diagnosed metastatic NSCLC should be evaluated by multidisciplinary tumor boards. The first to consider is the PS: PS 0-2 patients are recommended systemic treatment, whereas 3-4 patients are generally offered best supportive care (BSC). Molecular testing should be done in patients, selected for systemic treatment. In case targetable driving mutations are found, target therapy should be offered.



18. Figure – Treatment algorithm of stage IV NSCLC. Recommendation by ESMO, 2018 (148).

The best established oncogenic target in advanced stage NSCLC is *EGFR* mutation (149, 150). First- (erlotinib and gefitinib) and also second generation (afatinib) TKIs were compared with standard CHT and were found superior in terms of disease control, OS and PFS (151-155). Patients with poor PS (3 or 4) can also benefit from TKI therapy when an EGFR mutation is present (156). Clinically stable patients, who have previously benefited from TKI therapy should continue receiving EGFR TKI, even when radiological progression occurs (157). Local control (surgery or radiotherapy) should be considered when radiological progression is only seen in one distant site. It is not clear whether the administration of second generation TKIs yield a significant benefit for patients. Most studies reported improved PFS, however OS was not affected (158-160).Third generation TKIs are being introduced into standard care. Osimertinib is in the most advanced stage, as it was reported to improve both PFS and OS (161). The addition of antiangiogenesis agents, such as bevacizumab was also investigated. Seto et al. reported improved PFS, however the difference was not seen in OS (162). Other studies are also investigating combinations with encouraging early results (163, 164).



19. Figure - Treatment algorithm for stage IV NSCC, molecular tests positive (ALK/BRAF/EGFR/ROS1). ESMO recommendation (148).

ALK rearrangement is also a well-tested target for advanced NSCLC. Crizotinib was the first to be tested against other treatment options. Significant advantage was seen in both objective response rate (ORR) and PFS in two multicenter single-arm studies (165, 166). Second generation ALK inhibitors like alectinib and certinib were found to be more effective in terms of PFS and intracranial activity (167, 168). Patients, treated with crizotibin should be offered next-generation ALK TKIs when they show progression.
Crizotinib and ceritinib were showed to also be effective in ROS1-rearranged NSCLC (169-172), while brigatinib, lorlatinib, repotrectinib and entrectinib were also found to have potential anti-ROS activity in preclinical studies (173).
BRAF mutation was also successfully targeted in advanced adenocarcinoma. *V600E (Val600Glu)* is the most commonly observed mutation with and incidence of 1-2% in lung adenocarcinomas and with a higher incidence in smokers (174, 175). Three agents, vemurafenib, dabrafenib and sorafenib were all found to be effective with a mean ORR of 53% (176).

In patients who do not harbor known targetable genetic changes, immune checkpoint inhibitors showed great promise recently. A phase III study, the KEYNOTE-024 established pembrozilumab as a standard first-line treatment for advanced NSCLC when PD-L1 expression is higher than 50% and EGFR mutation and ALK translocation is absent. ORR, PFS, OS, safety and quality of time all favored pembrolizumab over standard CHT (177-179).

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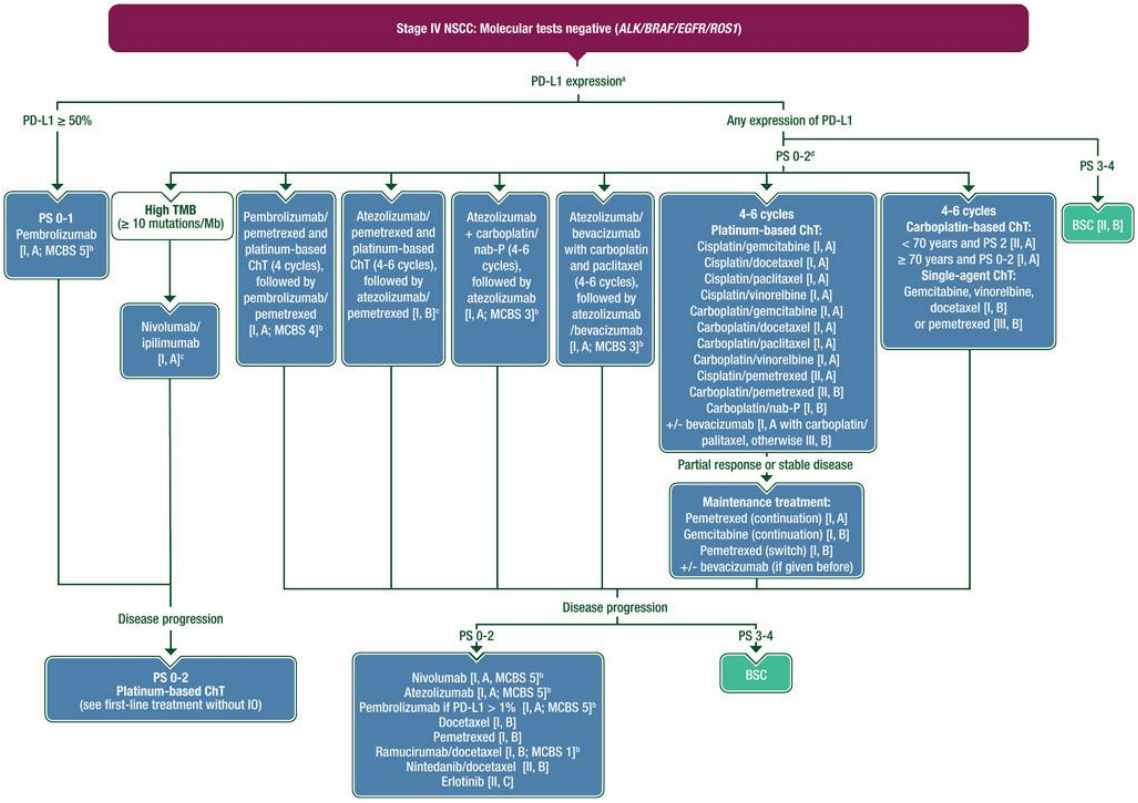


20. Figure - Treatment algorithm for stage IV NSCC, when molecular tests are negative (ALK/BRAF/EGFR/ROS1) (148).

CHT with platinum doublets is still standard therapy in all advanced NSCLC patients who do not harbor targetable genetic changes and do not have major comorbidities. A 23% reduction of risk of death, 9% 1-year survival gain, 1.5 month increase in absolute median survival and improved quality of life were observed in two big meta-analyses (180-182).

Antiangiogenic agents showed efficacy as additional therapy (described in detail later). Most studies in advanced squamous cell carcinoma did not report any benefit from targeted therapy (181), thus platinum-based combinations with the addition of a third-generation cytotoxic agent, such as gemcitabine, vinorelbine or taxanes, are recommended.

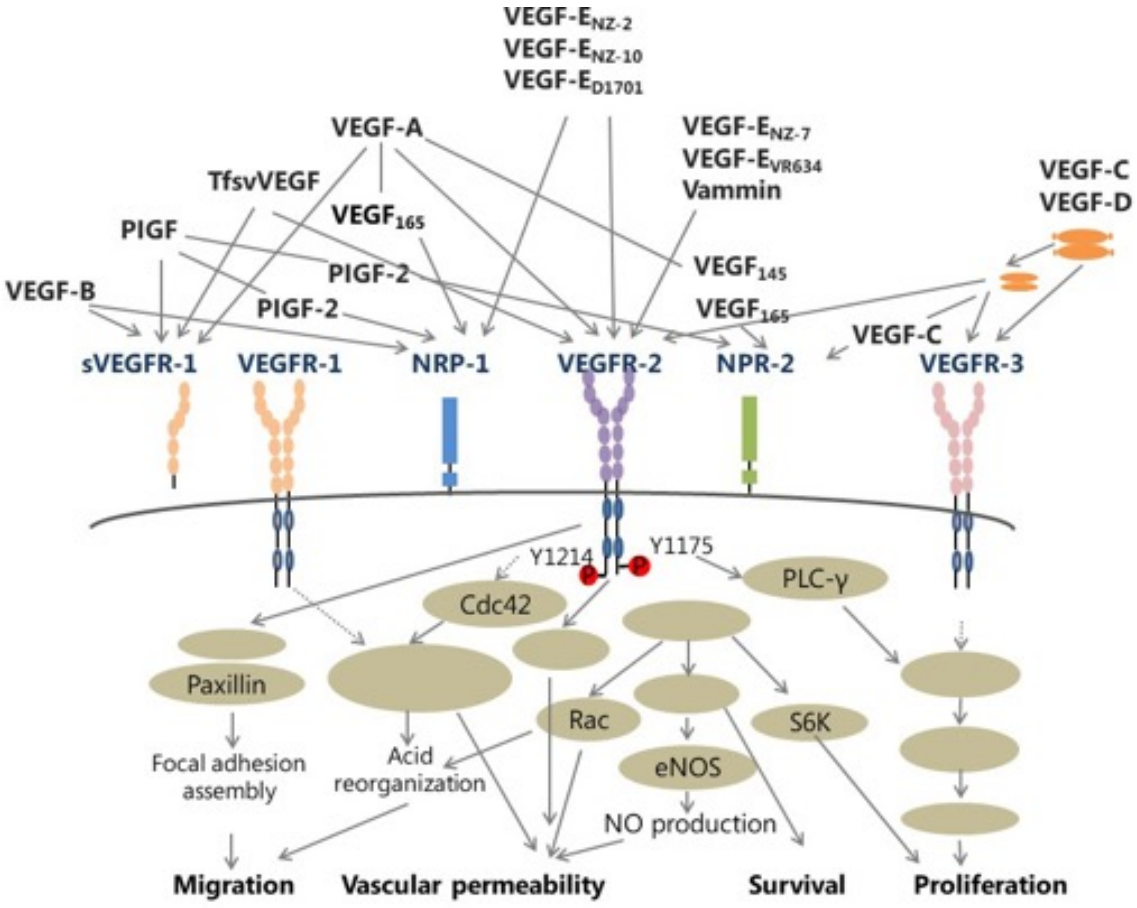
Role of surgery

Oligometastatic disease was introduced as a new subcategory in advanced NSCLC. M1b disease was defined in contrast to patients with multiple metastases (M1c) (183).

Prospective studies suggest that surgery only has a role in oligometastatic disease, when complete (R0) resection can be reached. Nodal disease means poor prognosis (184-186).

2.2. Vascular endothelial growth factor and bevacizumab

Vascular endothelial growth factor (VEGF) is a key factor to endothelial cell growth and one of the most important regulators of angiogenesis. Increased expression of VEGF can be demonstrated in most solid tumors including NSCLC (187). In many cases, VEGF overexpression is associated with an increased risk of relapse and metastasis (188-191). According to preclinical studies, anti-VEGF monoclonal antibodies are capable of inhibiting the growth of human tumor xenografts both in monotherapy and in combination with chemotherapy (192-195).



21. Figure – VEGF signaling pathways and receptor binding specificity. Adopted from: https://www.bocsci.com/vegf-signaling-pathway.html

Bevacizumab (BEV; Avastin®; Genentech/Roche, South San Francisco, CA, USA) is a humanized monoclonal antibody that acts by binding and neutralizing the VEGF-A isoform, thus preventing VEGF ligand-receptor binding. It has demonstrated its efficacy in colorectal (196, 197), ovarian (198), breast(199, 200) and renal cancer (201, 202). This has also been the first and only antivascular drug to be licensed for the treatment of NSCLC so far.

According to a phase II study (203), bevacizumab treatment in combination with chemotherapy in NSCLC was more effective than chemotherapy alone. The combination was also well tolerated, however, the incidence of lung hemorrhage increased. In a post hoc multivariate analysis, squamous cell histology was identified as an independent risk factor for bleeding (204). Consequently, patients with squamous cell histology were excluded from most of the clinical trials of bevacizumab in NCSLC.

Subsequent to the above Phase II study, the Eastern Cooperative Oncology Group (ECOG) E4599 trial was initiated (205). This study, which was the first published Phase III randomized trial of an antiangiogenesis agent in combination with chemotherapy in patients with advanced NSCLC, randomized chemotherapy-naive patients with predominantly non-squamous cell histology were included. In the bevacizumab treatment arm, following completion of chemotherapy, single-agent bevacizumab was continued until disease progression. Results showed that the addition of bevacizumab was associated with a significant improvement in the median overall survival (OS) compared with chemotherapy alone. Progression-free survival (PFS) was also significantly improved.

A second Phase III trial (Avastin® in Lung; AVAiL), evaluating bevacizumab in combination with cisplatin and gemcitabine (206) (another commonly used and efficacious regimen in NSCLC) was originally initiated with a primary end point of OS. However, after the positive OS results of E4599, the study design was amended so as to change the primary end point from OS to PFS. Patients were randomly assigned to receive cisplatin 80 mg/m2 and gemcitabine 1250 mg/m2 for up to six cycles plus low-dose bevacizumab (7.5 mg/kg), high-dose bevacizumab (15 mg/kg) or placebo every 3 weeks until disease progression. PFS was significantly prolonged with bevacizumab. Interestingly, according to the final efficacy analysis, OS was > 13 months in all treatment

groups, which is the longest OS reported for advanced non-squamous NSCLC in a clinical trial setting, although it did not yield a statistically significant prolongation with either bevacizumab dose (207).

As a result of the above trials, bevacizumab in combination with platinum-based chemotherapy was approved for the first-line treatment of patients with advanced NSCLC by the European Medicines Agency (EMEA) in August 2007.

Although bevacizumab was approved with platinum-based chemotherapy in NSCLC in 2007, so far no Hungarian data have been available. The AVALANCHE (ML21783) study was undertaken to assess the clinical outcomes of first-line bevacizumab combined with standard platinum-based regimens in Hungarian clinical practice.

2.3. KRAS mutation and antivascular treatment

KRAS protein, encoded by the KRAS proto-oncogene, is a small GTPase which plays a key role in regulating various cell functions (208). Alterations of the KRAS gene are typically missense mutations that can lead to the oncogenic conversion of KRAS resulting in the constitutive activation of its effector pathways and thus cancer development and progression (209). KRAS is frequently mutated in pancreatic and colorectal cancer (CRC) and in lung adenocarcinoma (LADC). With an incidence of up to 30%, KRAS mutation is the most common driver mutation in LADC. The most prevalent G12C and G12V KRAS mutation subtypes are associated with smoking-, while the G12D subtype has been observed in never-smokers (210, 211). Several other rare mutations of KRAS codon 12, 13 and 61 have also been reported (210).

The prognostic and predictive power of KRAS mutation in non-small cell lung cancer (NSCLC) patients remains controversial. It was first reported in the late 1980s that KRAS mutation is associated with poorer survival (212, 213) and since then several groups confirmed these findings (214, 215). However, most of these studies were rather heterogeneous in terms of histology, tumor stage and methodologies of KRAS mutation detection. Although two different meta-analyses concluded that KRAS mutation is a negative prognosticator in LADC (216, 217), the most comprehensive study of more than 1500 NSCLC patients (including 300 KRAS mutant cases) from four trials of adjuvant chemotherapy (CHT) reported that KRAS mutation had no clear prognostic or predictive relevance with regards to response to CHT (218).

Previously, our group performed a mutation subtype-specific analysis of 505 stage III–IV LADC patients treated with platinum-based CHT and found that there were no significant differences in progression-free survival (PFS) and overall survival (OS) among patients with wild type (WT), codon 12 and codon 13 KRAS mutations. Importantly, however, G12V KRAS mutant patients tended to have a higher response rate and a modestly longer median PFS (219).

The importance of subtype-specific KRAS mutation analysis was further highlighted in the preclinical study of Garassino et al. These authors investigated the role of different KRAS mutation subtypes (G12C, G12V and G12D) in the in vitro chemosensitivity of human NSCLC cells and found that the expression of G12C was associated with a reduced response to cisplatin and an increased sensitivity to taxol and pemetrexed. In the same study, G12D mutation led to resistance to taxol and sensitivity to sorafenib, whereas the G12V mutation sensitized the cells to cisplatin (220).

Increased expression and the negative prognostic role of vascular endothelial growth factor (VEGF, the key angiogenic cytokine) have been reported in most solid tumors including NSCLC (187) (221). Several phase 2 and 3 clinical trials demonstrated that the addition of BEV to CHT improves PFS and OS of NSCLC patients (203, 205, 206, 222, 223). Accordingly, BEV in combination with platinum-based CHT was approved for the first-line treatment of patients with advanced-stage NSCLC by the FDA (U.S. Food and Drug Administration) and the EMA (European Medicines Agency) in 2006 and 2007, respectively. The efficacy of BEV in a real-life setting in Hungary was shown in the Avalanche study (224).

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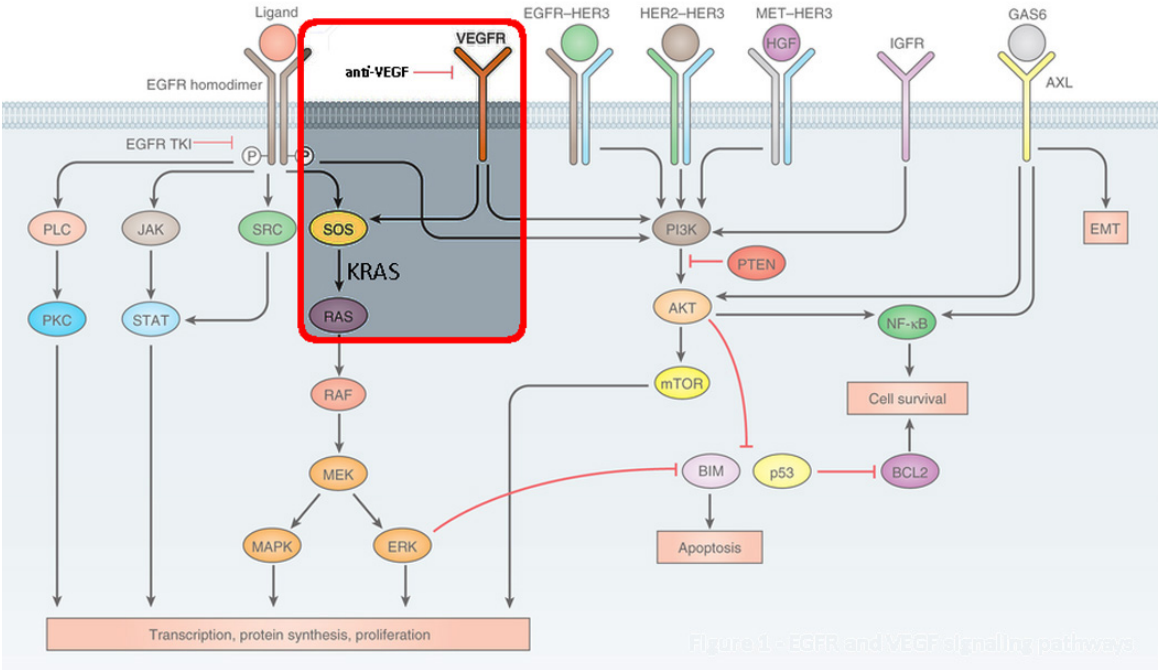

22. Figure – EGFR signaling pathway and the connection to the RAS/RAF/MEK/ERK cascade. Adopted from (225).

Although the RAS/RAF/MEK/ERK signaling pathway has been implicated in the regulation of VEGF expression and angiogenesis (226), only very few studies investigated the effect of KRAS mutation on the efficacy of BEV therapy. Most studies focused on CRC, where the addition of BEV to CHT prolonged survival regardless of KRAS mutational status (227-230). Two different groups, however, demonstrated that G12V, G12A (231) and G12D (232) KRAS mutations are associated with poor outcome in metastatic CRC patients receiving BEV. As for nonsquamous NSCLC, in a phase 2 trial evaluating the addition of neoadjuvant BEV to CHT, Chaft et al. found that no patient (0 out of 10) with KRAS mutation showed pathological response to neoadjuvant BEV/CHT, in comparison to 11 of 31 KRAS WT patients (233). In another small study of stage IV NSCLC, BEV therapy was associated with improved OS and PFS in KRAS WT (n=26) but not in KRAS-mutant (n=16) patients (234). No study was done on a large cohort of patients to examine the amino acid substitution-specific KRAS mutational status of BEV/CHT-treated stage III-IV Caucasian patients.

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3. Objectives

Bevacizumab is a relatively new treatment option in advanced LADC. The addition of BEV to standard platinum based chemotherapy was validated in several international randomized trials, however no Hungarian data was available.
Despite the research effort no validated predictive or prognostic biomarker is known regarding antivascular treatment in NSCLC.

3.1. The safety and efficacy of bevacizumab in addition to platinum based chemotherapy in patients with advanced NSCLC

Although BEV was approved with platinum-based chemotherapy in NSCLC in 2007, so far no Hungarian data have been available. The AVALANCHE study (ClinicalTrials.gov, identifier: NCT03170284) was undertaken to assess the clinical outcomes of first-line BEV combined with standard platinum-based regimens in Hungarian clinical practice. To achieve results that represent the Hungarian practice, a multi-institutional, single-arm observational study was conducted.
The primary endpoint of the study was PFS, the time from first administration of BEV to disease progression. Secondary endpoints included tumor response, OS and safety.

3.2. KRAS mutation as a biomarker for anti-VEGF therapy in NSCLC

Based on international literature and previous findings form a sub-cohort of the AVALANCHE study, anti-VEGF therapy is not effective in all patients. The exact mechanisms of therapy resistance and furthermore, a predictive and prognostic biomarker for antivascular therapy is not yet known.
The molecular connection between the KRAS signaling cascade and the VEGF signaling pathway has been established, however its clinical relevance is not yet studied. The aim of this study was to analyze whether KRAS mutation could be used as a biomarker for anti-VEGF therapy in NSCLC. We examined the amino acid substitution-specific KRAS mutational status in a large cohort of BEV/CHT-treated stage III-IV Caucasian patients and its effect on PFS and OS. Our aim was to show the clinical relevance of subtype

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specific KRAS mutational status in anti-VEGF therapy and furthermore to establish a predictive biomarker that can help identify therapy resistant patients and thus to give individualized therapy.

4. Methods

4.1. General ethical consideration

Patient data, including pathological reports, clinicopathological variables and data regarding outcome was collected in the following institutions:

- Bács-Kiskun County Hospital, Kecskemét, Hungary
- Békés County Institute of Pulmonology, Gyula, Hungary
- Borsod-Abaúj-Zemplén County Szent Ferenc Hospital, Miskolc, Hungary
- Clinical Center, University of Pécs, Pécs, Hungary
- Csongrád County Hospital of Chest Diseases, Deszk, Hungary
- Fejér County Szent György Hospital, Székesfehérvár, Hungary
- Institute of Pulmonology, Törökbálint, Hungary
- Jász-Nagykun-Szolnok County Hetényi Géza Hospital and Clinic, Szolnok, Hungary
- Kaposi Mór Teaching Hospital, Kaposvár, Hungary
- Municipality of the City of Budapest Uzsoki Street Hospital, Budapest, Hungary
- National Korányi Institute of Pulmonology, Budapest, Hungary
- Petz Aladár County Teaching Hospital, Győr, Hungary
- Semmelweis University, Budapest, Hungary
- Szabolcs-Szatmár-Bereg County Jósa András Hospital and Clinic, Nyíregyháza, Hungary
- Szent Borbála Hospital, Tatabány, Hungary
- Tolna County Balassa János Hospital, Szekszárd, Hungary
- Vas County Markusovszky Hospital, Szombathely, Hungary
- Veszprém County Institute of Pulmonology, Veszprém, Hungary
- Zala County Hospital, Zalaegerszeg, Hungary

All data collection and analyzes was approved by the local or national ethical committees. No individual patient data is identifiable in the respective publications or in this thesis. All methods included in this thesis were performed in accordance with the relevant

guidelines and regulations. All experiments that were reported in this thesis were done complying with all mandatory laboratory health and safety procedures.

4.2. The safety and efficacy of bevacizumab in addition to platinum based chemotherapy in patients with advanced NSCLC

4.2.1. Study design

AVALANCHE (ClinicalTrial.gov Identifier NCT03170284) was a multi-center single-arm observational study designed to assess the efficacy and safety of bevacizumab therapy in patients with advanced, unresectable, metastatic or recurrent nsNSCLC (non-squamous, other than predominantly squamous cell histology) in the routine oncology practice in Hungary. Further objective of the study was to assess and identify possible treatment-related prognostic factors.

4.2.2. Patients

This study was originally projected to enroll 150 patients from 40 Hungarian study centers. The planned number of patients was later increased to 300 in order to obtain stronger and more representative real-life efficacy and safety data with bevacizumab therapy in Hungary.

Patients with histology or cytology proven unresectable advanced, metastatic or recurrent (stage IIIB/IV) NSCLC other than predominantly squamous cell histology were included in the present study.

Exclusion criteria were as stated in the Hungarian prescribing information.

The study was done in accordance with the Declaration of Helsinki, Good Clinical Practice International Conference on Harmonization Tripartite Guidelines, laws and regulations of the participating institutes' country. The study was approved by the Hungarian ethics committee and health authority. All patients provided written informed consent.

4.2.3. Treatment

Eligible patients received first-line bevacizumab with cisplatin or carboplatin in accordance to the approved and reimbursed bevacizumab indication in Hungary

(bevacizumab 7.5 mg/kg, every 3 weeks with any platinum-doublet for up to 6 cycles) then non-progressors proceeded to receive bevacizumab until disease progression or unacceptable toxicity. The third component of the combination chemotherapy was one of the following: paclitaxel, gemcitabine, docetaxel or vinorelbine. Based on the therapeutic protocol, patients were followed up until the first progression of their primary disease, or death, or withdrawal of consent, or loss of contact with the patient, or closure of the study, whichever occurred first.

4.2.4. Progression-free and overall survival

Investigators seemed to be frequently using progression-free survival (PFS) and time-to-progression (TTP) interchangeably in clinical trials in the early 2000s (235). The protocol of our study defined TTP as the time elapsed from the date of enrolment until the first documented progression or the death of the patient from any cause which is in accordance with the current definition of PFS. To avoid confusion, PFS will be used hereinafter for the denomination of the primary endpoint of the study. Progression was determined by the investigator at the routine clinical practice follow-up examinations. PFS was calculated from the start of bevacizumab treatment.

Secondary endpoints included best tumor response (complete remission (CR), partial remission (PR), stable disease (SD), progressive disease (PD)), OS (based on retrospective analysis) and indicators of safety (serious and non-serious adverse events). ORR (Objective Response Rate) was calculated from patients experiencing complete or partial remission.

Basic demographic data, basic vital parameters, primary disease-related historical data, ECOG performance status, data related to bevacizumab treatment, results of the staging assessments as well as the patient's comorbidities and concomitant treatments were recorded in an electronic case-report form.

Following the closure of the study, data for the assessment of the primary study parameter (PFS) were available for 252 patients. As per the amended protocol, the secondary endpoint (OS) was retrospectively analyzed based on data from 250 patients.

During the treatment period regular monitoring visits were conducted to ensure high-quality data collection. Data related to bevacizumab treatment, blood pressure, body weight, concomitant treatments and adverse events were registered.

The following data were recorded at the end-of-treatment visit: end date of bevacizumab treatment, reason for ending treatment, ECOG status, best tumor response observed during treatment, concomitant treatments administered during bevacizumab treatment and adverse events observed during bevacizumab treatment.

4.2.5. Statistics

PFS (primary study endpoint) and OS in the total population were analyzed using Kaplan-Meier curves. Log-rank test was used for comparison between groups. When sufficient data were available, an extended analysis using Cox-regression was also performed.

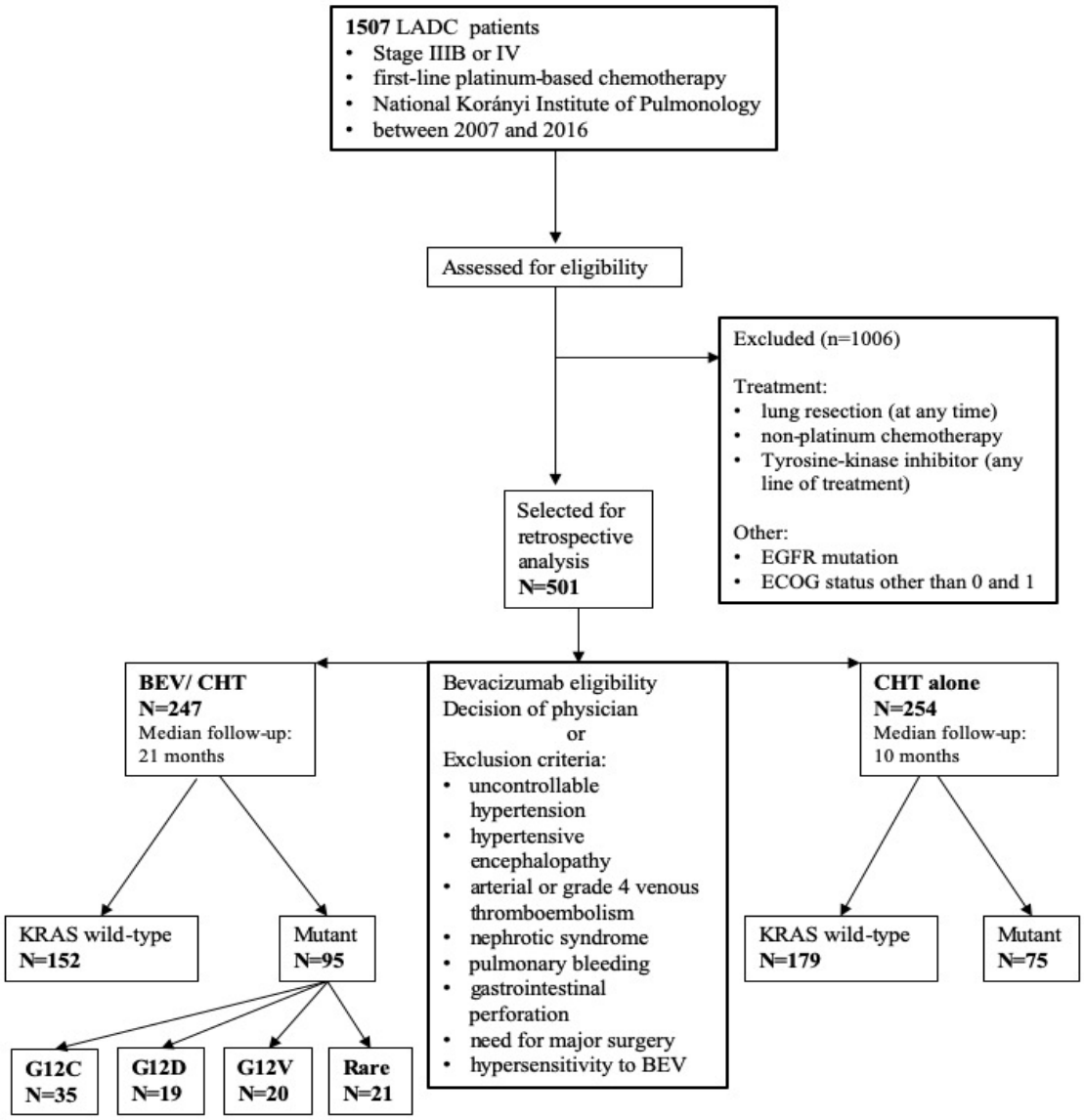
PFS was defined as the time elapsed from the date of enrolment until the first documented progression or the death of the patient from any cause. For study subjects who had not shown progression and had not died by the closure of the study, the data were censored at the date of the last contact.

OS was defined as the time elapsed from the date of enrolment until the death of the patient from any cause. Regarding subjects who had not died by the closure of the study, the OS data were analyzed retrospectively after the end of the study in the knowledge of their dates of death. Otherwise, data were censored at the date of the last contact.

4.3. KRAS mutation as a biomarker for anti-VEGF therapy in NSCLC

4.3.1. Study population

In this single-center, retrospective study, 501 consecutive patients with advanced lung adenocarcinoma (LADC) were included who underwent first-line platinum-based (cisplatin or carboplatin) doublet chemotherapy (CHT) with or without BEV at the National Korányi Institute of Pulmonology, Budapest between 2007 and 2016 (Table 10, Figure 23). The addition of BEV to CHT was individually decided by the treating physician in line with the proof of concept BEV clinical trials [16,18] and with the EMA summary of BEV characteristics. According to our inclusion criteria, cytologically or histologically verified unresectable stage IIIB or IV LADC patients were included. Patients with uncontrollable hypertension, hypertensive encephalopathy, arterial or grade 4 venous thromboembolism, nephrotic syndrome (grade 4 proteinuria), pulmonary bleeding, gastrointestinal perforation, need for major surgery or with hypersensitivity to BEV were considered not eligible for BEV therapy (Figure 23).



23. Figure - Consort diagram for advanced LADC cases. Consort diagram to demonstrate the selection of stage IIIB/IV LADC cases for BEV/CHT or CHT alone in this study. Where patients were excluded, the reasons for exclusion are indicated.

In the BEV/CHT group (n=247), platinum was given together with paclitaxel (84.7%) or gemcitabine (15.3%). In order to rule out the potential confounding effect of different treatment regimens, patients receiving other non-platinum partners, such as pemetrexed or docetaxel, were excluded from the CHT group (n=254). Additionally, all patients receiving tyrosine-kinase inhibitors in any further line of treatment were also excluded. According to the therapy guidelines of the host institute, only ECOG (Eastern

Cooperative Oncology Group) performance status (PS) 0 or 1 LADC patients were included in this study, since higher PS contradicted the use of cytotoxic chemotherapy. Smoking status, TNM stage and molecular tumor characteristics (EGFR and KRAS mutational status) were defined at the time of diagnosis. For the calculation of PFS and OS, date of the first CHT was used. Patients with known EGFR mutations were excluded. Clinical follow-up was closed on the 1st of August, 2017. Median follow up was 21 months in the BEV/CHT group, while 10 months in the CHT group. The study and all treatments were conducted in accordance with the current National Comprehensive Cancer Network guidelines, based on the ethical standards prescribed by the Helsinki Declaration of the World Medical Association and with the approval of the national level ethics committee that included a waiver for this retrospective study (52614-4/2013/EKU). Due to the retrospective study design and the anonymity of the patient records, an informed consent was not recommended.

4.3.2. Molecular diagnosis

All mutational analyses were performed at the time of diagnosis at the 2nd Department of Pathology of the Semmelweis University as previously described (219, 236, 237). DNA isolation was performed from formalin fixed paraffin-embedded (FFPE) tissue blocks or cytological specimens of primary tumors or lymphatic or organ metastases (including pleural effusion).

KRAS exon 2 mutations were identified by microcapillary-based restriction fragment length analysis as described (219, 236). Briefly, tumor-rich microscopic area on H&E staining had been determined by pathologists prior to macro dissection from FFPE tissue or cytological smears. DNA was extracted using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, WI) according to the instructions of the manufacturer. The microfluid-based restriction fragment detection system characterized by 5% mutant tumor cell content sensitivity. Density ratio of the mutated band to the WT one was calculated and samples containing >5% of the non-WT band were considered mutation positive due to the sensitivity threshold. The base-pair substitution in the mutant samples were verified and determined by sequencing on the ABI 3130 Genetic Analyzer System (Life Technologies, Carlsbad, CA) with the BigDye® Terminator v1.1 Kit.

4.3.3. Statistical methods

Categorical parameters, such as gender (male vs. female), smoking status (never vs. ever smoker), ECOG PS (0 vs. 1), and KRAS mutation status (KRAS-mutant vs. WT) were statistically analyzed by Chi-square test or Fisher's exact test. Age as a continuous variable was analyzed in the different KRAS mutational groups by Mann-Whitney U test as the data was not normally distributed in each group (as per the Shapiro-Wilk normality test). Kaplan-Meier survival curves and two-sided log-rank tests were used for univariate survival analyses. Median follow-up time was calculated by using the reverse Kaplan-Meier approach. The Cox proportional hazards model was used for uni- and multivariate survival analyses to detect the impact of both continuous and categorical factors and to calculate the hazard ratios (HR) and corresponding 95% confidence intervals (CI). For multivariate survival analyses, the Cox regression model was adjusted for age (as a continuous variable), gender (female versus male), smoking (never- versus ever-smoker), ECOG PS (0 versus 1) and stage (IIIB versus IV). In order to establish potential predictive factors, interaction terms were calculated between KRAS status and other variables (age, sex, smoking status, ECOG PS and stage) in the adjusted multivariate Cox regression model. P values are always given as two-sided and were considered statistically significant below 0.05. Metric data are always shown as median or mean and corresponding range or, in case of OS and PFS, as median and corresponding 95% CI. All statistical analyses were performed using the PASW Statistics 18.0 package (Predictive Analytics Software, SPSS Inc., Chicago, IL, USA). Graphs were created with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

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5. Results

5.1. The safety and efficacy of bevacizumab in addition to platinum based chemotherapy in patients with advanced NSCLC

5.1.1. Baseline Characteristics of the Patients

A total of 284 patients with corresponding diagnosis were identified at the Hungarian study sites, and were subsequently enrolled into the study between 17th June 2008 and 3rd May 2011, out of which data of 283 patients were evaluable. From among the 41 study centers originally involved, no patients were enrolled at 16 sites, thus in fact 25 centers participated actively. The highest number of patients enrolled at one center was 36, whereas the smallest was 1. One patient did not comply with all the inclusion and exclusion criteria: the patient's histological diagnosis was squamous cell carcinoma; therefore evaluable patient population was 283. Central localization of the primary tumor was reported in 61 patients (21.6%) and cavitated tumor in 4 patients (1.4%) in the total patient population.

The study population had to be reduced to 252 in case of PFS and 250 regarding OS. In case of PFS 31 patients and in case of OS 33 patients had to be excluded from the data assessment due to missing or incomplete information. These information could not be recovered retrospectively.

The demographic characteristics of the enrolled and evaluable patients are summarized in Table 7.

7. Table - Patient demographics and treatment in the Avalanche study

Characteristic	n (%)	Treatment	n (%)
Number of patients		**Previous Treatment**	
Evaluable patient population	283 (99.6%)	Previous surgery	64 (22.6%)
Patient population evaluable in terms of PFS	252 (88.7%)	Adjuvant/neoadjuvant chemotherapy	18 (6.4%)
Patient population evaluable in terms of OS	250 (88%)	Radiotherapy	18 (6.4%)
Age (Year)		**Chemotherapeutic agent during study**	
Mean	58.16 ± 9.032	Paclitaxel	132 (46.6%)
Mean (men)	58.30 ± 8.986	Gemcitabine	111 (39.2%)
Mean (women)	58.02 ± 9.113	Docetaxel	18 (6.4%)
Gender		Vinorelbine	2 (0.7%)
Male	143 (50.5%)	Other	7 (2.5%)
Female	135 (47.7%)	No data	13 (4.6%)
No data	5 (1.8%)	**Reported reasons for ending the study**	
Histologic type		Progression of primary disease	172 (60.8%)
Adenocarcinoma	271 (95.8%)	Deterioration of symptoms	4 (1.4%)
Bronchoalveolar carcinoma	11 (3.9%)	Loss of contact with the patient	7 (2.5%)
Squamous cell carcinoma	1 (0.4%)	Adverse event related to Bevacizumab treatment	13 (4.6%)
Stage		Patient’s decision	17 (6.0%)
III B	52 (18.4%)	Patient’s death	16 (5.7%)
IV	226 (79.9%)	Other	45 (15.9%)
No data	5 (1.8%)	No data	9 (3.2%)

5.1.2. Treatment

Prior to enrolment, 64 patients (22.6%) had undergone surgical intervention, 18 patients (6.4%) had received adjuvant/neoadjuvant chemotherapy, and 18 patients (6.4%) had received radiotherapy (Table 4).

Patients received cisplatin (N=148, 52.3%) or carboplatin (N=124, 43.8%) treatment in accordance with the protocol in approximately half-and-half proportion during the study. No data are available for 11 patients (3.9%). The other components of the combination chemotherapy are shown in Table 4.

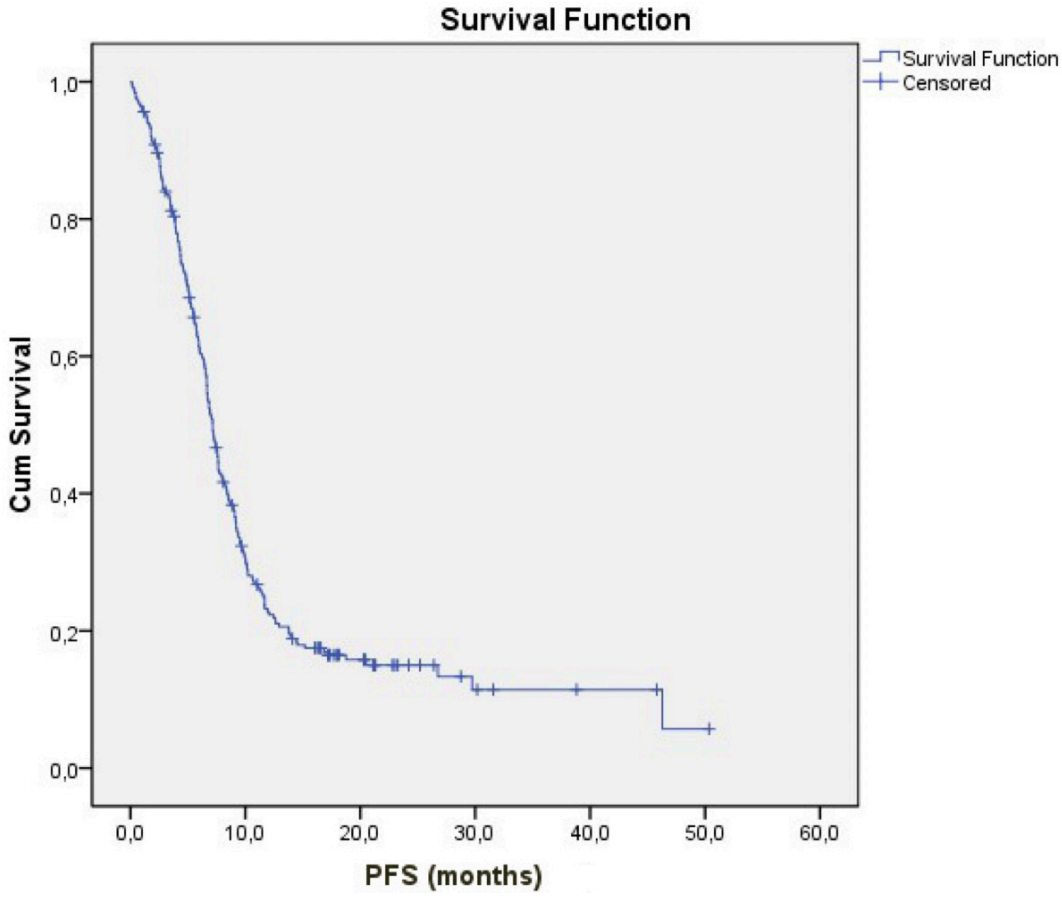
The vast majority of patients (N=262, 92.6%) received bevacizumab in 3-weekly cycles. A treatment of different cycle frequency was applied in two patients (0.7%), and no data were available for 19 patients (6.7%). The median number of bevacizumab treatment cycles in the retrospectively evaluated patient population was 6.

The most common reason for ending the study was documented as progression of the primary disease in more than half of the study subjects (60.8%). Patient's decision, patient's death, adverse event related to bevacizumab therapy, loss to follow-up, and symptom deterioration accounted for ending the study in 6.0%, 5.7%, 4.6%, 2,5% and 1.4% of the cases, respectively. Other reasons behind ending the study occurred in 15.9%; no data were available in 3.2% of cases.

5.1.3. Efficacy analysis

5.1.3.1. Progression-free survival

The PFS in the total study patient population was 7.162 ± 0.282 (CI95%: 6.609-7.715) months (Figure 24). The subgroup-analysis of PFS by gender showed that the survival time with bevacizumab treatment was longer in women (median: 7.589 ± 0.647, CI95%: 6.321-8.858 months) than in men (median: 6.669 ± 0.375, CI95%: 5.934-7.405 months). This difference, however, was not significant (p=0.542).



24. Figure - Progression-free survival in the total population (Kaplan-Meier)

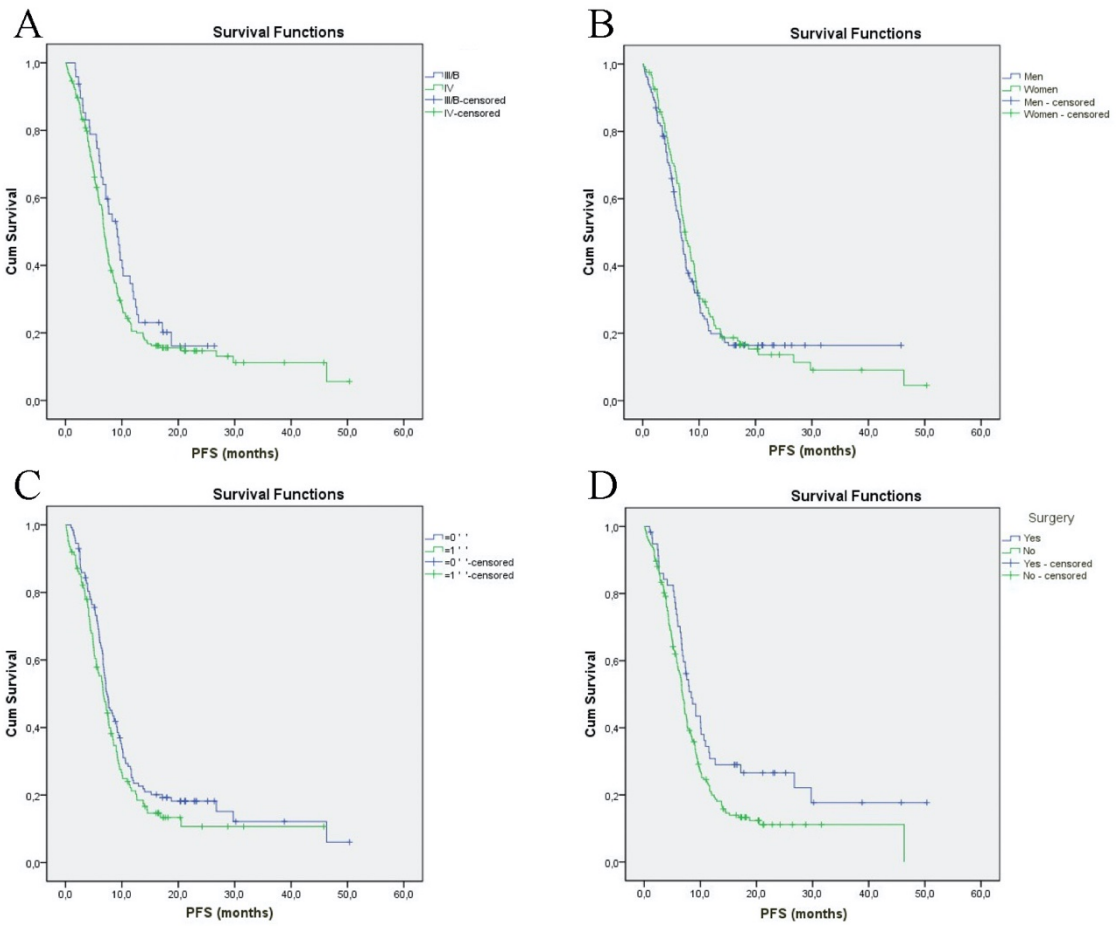
The median PFS was higher in patients with an ECOG status of 0 at enrolment (median: 7.326 ± 0.535, CI95%: 6.278 ± 8.375 months) than in patients with a baseline ECOG status of 1 (median: 6.702 ± 0.597 months, CI95%: 5.531-7.873 months). However, the difference between the two groups was not remarkable (p=0.123).

Similarly, PFS was not significantly influenced by the localization of the tumor (central vs. non-central, p=0.813).

Interestingly, the median PFS in patients who had undergone surgical intervention prior to enrolment (median: 8.411 ± 0.947, CI95%: 6.554-10.267 months) was notably higher (p=0.017) compared with patients with no such prior intervention (median: 6.834 ± 0.265, CI95%: 6.314-7.353 months). In contrast, neither adjuvant/neoadjuvant chemotherapy (p=0.165) nor radiotherapy (p=0.165) applied prior to enrolment had a significant impact on median PFS.

The platinum derivative used had no significant influence on median PFS, either (p=0.199).

Nearly 10% of the patient population with evaluable data were over 70 years of age at the time of enrolment. The median PFS was not significantly different between patients under or above 70 years of age (p=0.541).



25. Figure - Analysis of progression-free survival. A: in different tumor stages; B: According to gender; C: by ECOG PS and D: by history of surgery (Kaplan-Meier). Avalanche study.

Of note, median PFS was significantly higher (p<0.001) in patients receiving bevacizumab maintenance therapy (median: 9.166 ± 0.601, CI95%: 7.988-10.345 months) compared with those who received no maintenance therapy (median: 5.815 ± 0.574, CI95%: 4.690-6.940 months) (Figure 26).

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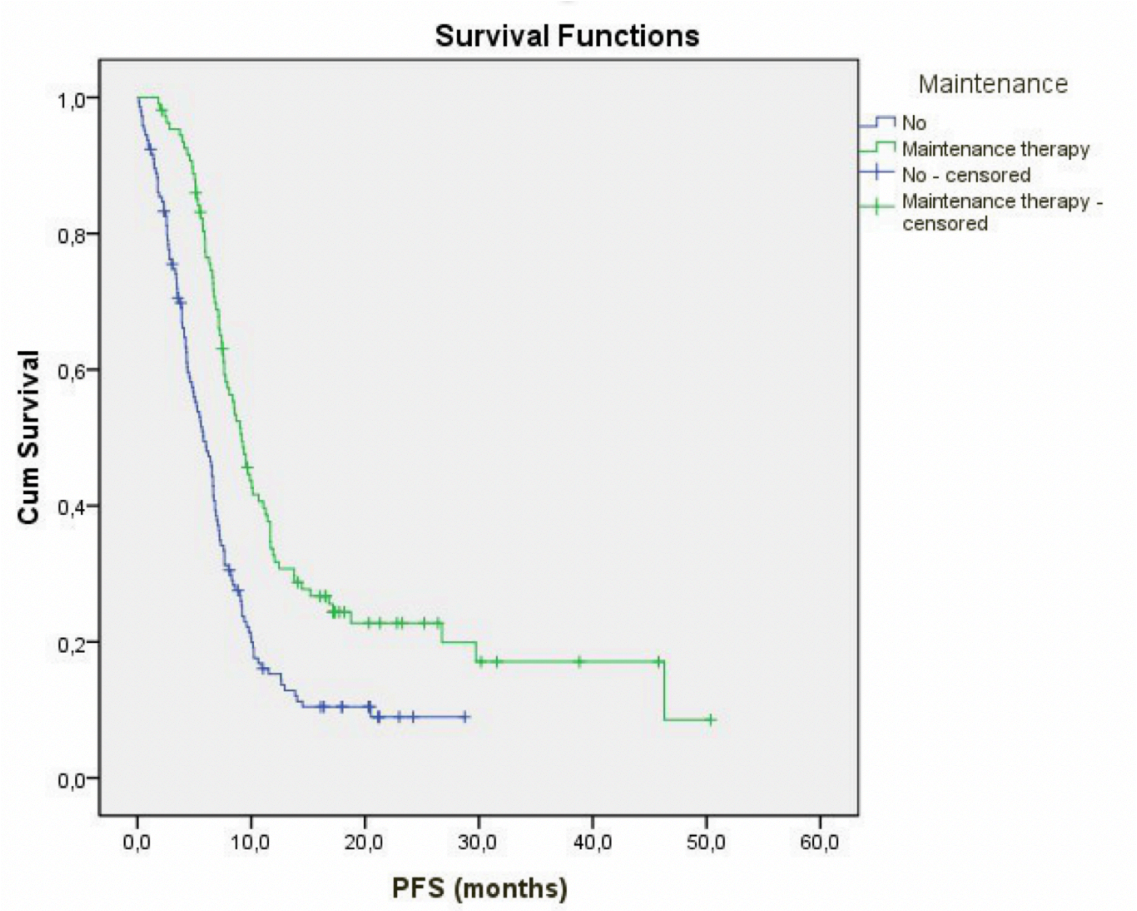

26. Figure - Analysis of progression-free survival by BEV maintenance therapy (Kaplan-Meier)

5.1.3.2. Secondary endpoints

5.1.3.2.1. Tumor response

Disease control was achieved in a remarkable 86.5% with CR in 2.3%, and PR in 44.4% of the cases with evaluable data. PD was recorded in 13.5% of evaluable cases and sufficient data was not available in 32.6% (Table 5).

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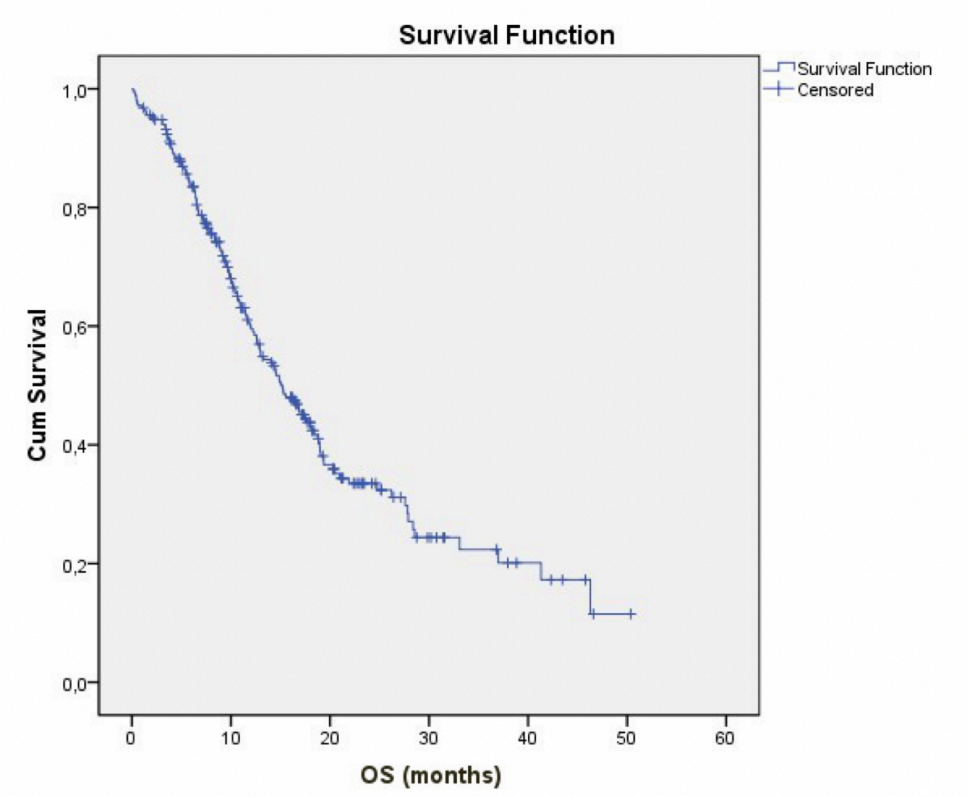
8. Table - Best tumor response reached during the first-line treatment in the Avalanche study

Tumor response	N	Patient population with evaluable data N = 133 (%)	Total patient population N = 216 (%)
Complete remission (CR)	3	2.3%	1.5%
Partial remission (PR)	59	44.4%	29.9%
Stable disease (SD)	53	39.8%	26.9%
Progressive disease (PD)	18	13.5%	9.1%
Not assessable (NA)	83	-	32.6%



27. Figure - Overall survival in enrolled and evaluated patients (Kaplan-Meier) in the Avalanche study

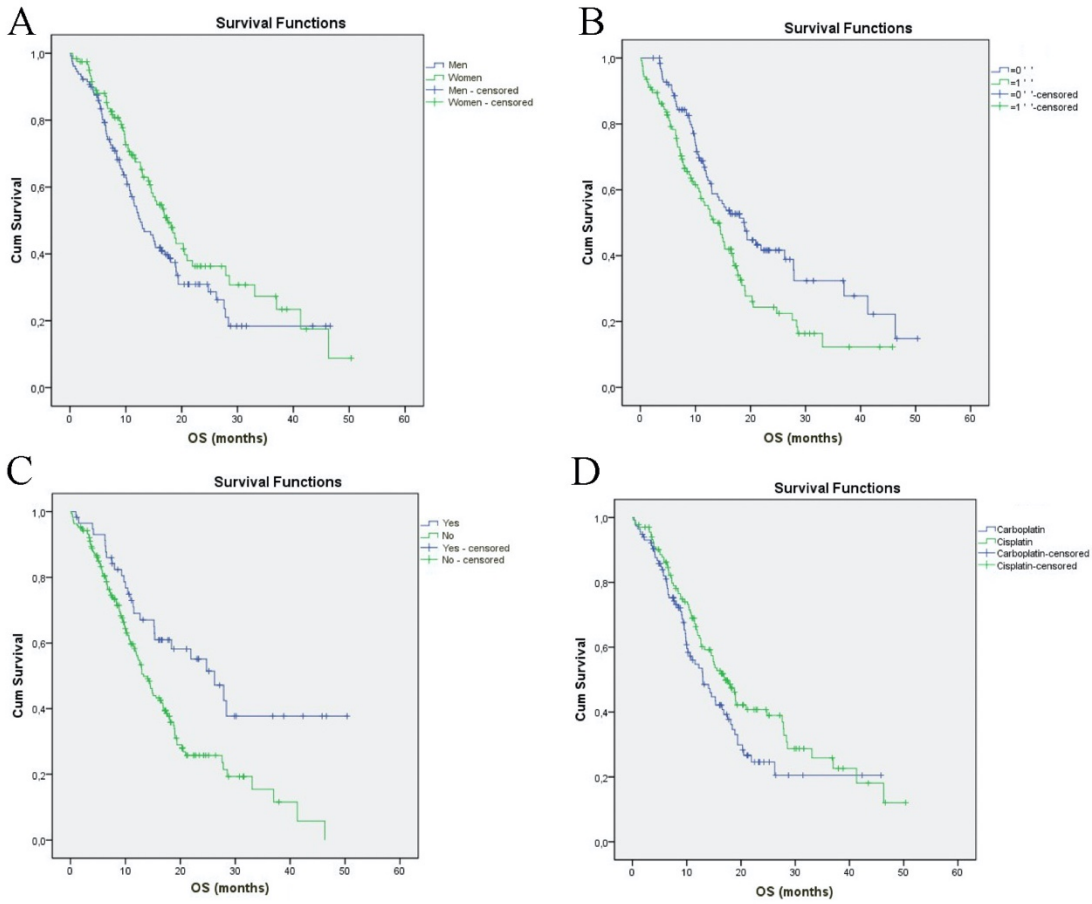
5.1.3.2.2. Overall survival

The median OS in the total study population was 15.179 ± 1.377 months (CI95%: 12.480-17.877) (Figure 27).

As with PFS, we performed subgroup-analysis of OS by gender, ECOG status, prior surgical procedure and chemotherapy.

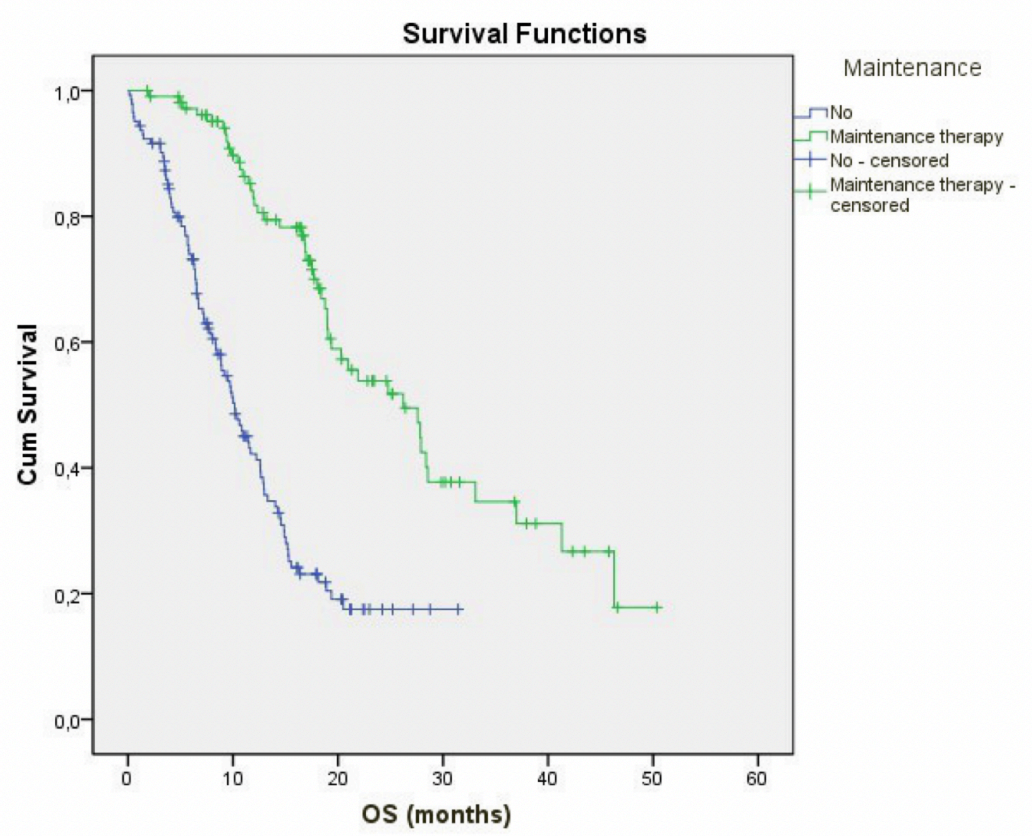
The localization of the tumor had no impact on OS (p=0.992) in the patient population studied.

Surprisingly, we found a tendency towards a higher median OS for patients over 70 years of age (18.398 ± 3.869 months, CI95%: 10.815-25.982 months) compared with patients younger than 70 years (15.014 ± 1.329 months, CI95%: 12.410-17.619 months), although this difference remained not significant (p=0.638).



28. Figure - Analysis of overall survival. A: According to gender; B: by ECOG PS; C: by history of surgery and D: according to the platina derivate used (Kaplan-Meier). Avalanche study.

A remarkably longer ($p<0.001$) OS was observed in patients receiving bevacizumab maintenance therapy (median: 26.218 ± 3.946 months, CI95%: 18.484-33.952 months) than in those without maintenance bevacizumab therapy (median: 10.152 ± 0.975 months, CI95%: 8.240-12.064 months) (Figure 29).



29. Figure - Analysis of overall survival by Avastin® maintenance therapy (Kaplan-Meier)

5.1.3.2.3. Safety and adverse events

As per the protocol, possible adverse events (AE) encountered during the study were recorded in the Case Report Form. Data on AE were recorded from the start of treatment until the end of treatment.

During the study, a total of 157 AEs were reported for 59 patients, 14 of which were serious (sAE) (Table 6).

Of all the adverse events, 63 (40.1%) events resolved without sequelae, the investigators reported improvement for 61 cases (38.9%) and the event resolved with remaining symptoms in 7 cases (4.5%). 2 AEs (1.3%) had not resolved, 14 AEs (8.9%) persisted unchanged from observation until the last follow-up of the patient, 5 AEs (3.2%) led to the death of the patient, and the outcome was unknown for 4 AEs (2.5%).

Of the above-mentioned AEs, 14 were categorized as sAE, which were the following: Anemia (3 cases), pulmonary embolism (3 cases), hemoptysis (2 cases), deep vein thrombosis (2 cases), hypertension (1 case), neutropenia (1 case), thrombocytopenia (1 case), uremia (1 case). 5 of these (two cases of pulmonary embolism, hemoptysis, hypertension and uremia) led to the death of the patient.

During the study period, 16 (5.6%) of the 283 enrolled and evaluable patients died. The investigators reported the cause of death as disease progression in 11 cases (3.8%), while a serious adverse event was behind the death of the patient in 5 cases (1.7%).

In summary, the participating investigators did not encounter and report on any new information on the safety profile of bevacizumab. Indeed, the rate of reported adverse events falls behind the rate expected based on literature data.

9. Table - Adverse events reported in the study (summary)

Adverse event	N (%)	Adverse event	N (%)
Anemia	23 (14.7%)	Dermatitis (forehead, back)	1 (0.7%)
Thrombocytopenia	14 (9%)	Dermatitis (generalized)	1 (0.7%)
Neutropenia	12 (7.7%)	Cholesterol increased	1 (0.7%)
Hypertension	7 (4.5%)	Exsiccosis	1 (0.7%)
Nausea	7 (4.5%)	Ulcer (in the mouth, tongue)	1 (0.7%)
Epistaxis	6 (3.9%)	Gastroesophageal reflux disease	1 (0.7%)
Chest pain	5 (3.2%)	Weakness	1 (0.7%)
Acute bronchitis	4 (2.6%)	Vomiting	1 (0.7%)
Weight loss	4 (2.6%)	Abdominal pain	1 (0.7%)
Bone pain	3 (2%)	Ileus	1 (0.7%)
Diarrhea	3 (2%)	Ischemic cerebral vascular lesions	1 (0.7%)
Pulmonary embolism	3 (2%)	Arthralgia	1 (0.7%)
Hemoptysis	3 (2%)	Swelling of arm	1 (0.7%)
Hyponatremia	3 (2%)	Hand swelling	1 (0.7%)
Deep vein thrombosis	3 (2%)	Leg swelling	1 (0.7%)
Hoarseness	3 (2%)	Laryngotracheitis	1 (0.7%)
Cough	2 (1.3%)	Febrile neutropenia	1 (0.7%)
Fever	2 (1.3%)	Prostration	1 (0.7%)
Respiratory infection	2 (1.3%)	Leukopenia	1 (0.7%)
Obstipation	2 (1.3%)	Breast swelling	1 (0.7%)
Pneumonia	2 (1.3%)	Esophageal ulcer	1 (0.7%)
Pyuria	2 (1.3%)	Duodenal ulcer	1 (0.7%)
Tachycardia	2 (1.3%)	Suffusion without trauma	1 (0.7%)
Throat pain	2 (1.3%)	Dizziness	1 (0.7%)
Lung abscess	1 (0.7%)	Thrombosis (left femoral vein)	1 (0.7%)
Agranulocytosis	1 (0.7%)	Uremia	1 (0.7%)
Acute osteomyelitis (jaw)	1 (0.7%)	Urticaria	1 (0.7%)
Allergic dermatitis	1 (0.7%)	Iron deficiency	1 (0.7%)
Allergic reaction	1 (0.7%)	Bleeding following superficial injury	1 (0.7%)
Hip pain (right-sided)	1 (0.7%)	Clear-cell renal carcinoma	1 (0.7%)
Decubitus	1 (0.7%)	Numbness (of the soles)	1 (0.7%)

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5.2. KRAS mutation as a biomarker for anti-VEGF therapy in NSCLC

5.2.1. Incidence of KRAS mutations in LADC patients treated with bevacizumab and chemotherapy

All patients had advanced LADC and Caucasian background. Patients with tumors harboring an EGFR mutation were excluded. One hundred and seventy patients of the full cohort of 501 cases were identified as KRAS-mutant (33.9%) and 331 (66.1%) as KRAS WT, Table 7 and Table 8). 38.5% (n=95) of the patients treated in the BEV/CHT group were KRAS-mutant (Table 7), whereas in the CHT group (Table 8) this ratio was 29.5% (n=75) (P=0.012). There were no significant differences between the BEV/CHT and CHT groups with respect to age (P=0.193), smoking status (p=0.072), gender (p=0.506) or tumor stage (P=0.610) (data not shown). The only difference was seen is performance status, where there were more ECOG 0 (vs. EVOG 1) patients in the BEV/CHT group than in the CHT alone group (P=0.031; data not shown), which might be due to the BEV selection criteria. In the BEV/CHT sub-cohort, 35 (36.8%), 19 (20%) and 20 (21%) cases were classified as G12C, G12D and G12V mutants, respectively (Table 9). Other rare (i.e. n<3) KRAS exon 2 mutation subtypes (G12A, G12R, G12S, G13C, G13D) were also found in the BEV group. Subtype specific mutations were technically not assessable in 21 cases (Table 9).

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10. Table – Patient characteristics in the BEV/CHT group

	No. of patients (%)		KRAS status		*P* value[a]
			Wild type (%)	Mutant (%)	
All patients	247		152 (61.5%)	95 (38.5%)	
Age (years)[b]		**Median:**	62	58	0.09
		SD*:	9.2	8.2	
		Range:	53	44	
Smoking[c]					
Never-smoker	30 (12%)		24	6	0.008
Ever-smoker	167 (68%)		93	74	
No data (n=50)					
Gender[c]					
Female	106 (43%)		52	54	0.002
Male	141 (57%)		100	41	
ECOG[c]					
0	139 (56%)		87	52	0.056
1	108 (44%)		65	43	
Stage[c]					
III	55 (22 %)		38	17	0.16
IV	192 (78%)		114	78	
Survival[d]					
Median PFS (months)			8.63	7.03	**0.0255**
Median OS (months)			21.57	14.23	**0.0186**

*a) P value is calculated between wild type and all mutant groups, b) Mann-Whitney test is used in case of continuous variable (age) as the data are not normally distributed (Saphiro-Wilk test), c) Fisher's exact test is used between categorical variables, d) survival difference between the wild type and the mutant group was calculated using log rank regression analysis, e) PFS was not determined in the CHT group, *SD: standard deviation*

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11. Table – Patient characteristics in the CHT only group

	No. of patients (%)		**KRAS status**		***P* value[a]**
			Wild type (%)	**Mutant (%)**	
All patients	254		179 (70.5%)	75 (29.5%)	
Age (years)[b]		**Median:**	63	61	0.297
		SD*:	7.8	8.7	
		Range:	46	46	
Smoking[c]					
Never-smoker	21 (8%)		15	6	0.435
Ever-smoker	188 (74%)		135	53	
No data (n=45)					
Gender[c]					
Female	118 (46.5%)		79	39	0.27
Male	136 (53.5%)		100	36	
ECOG					
0	128 (50.5%)		94	34	0.335
1	126 (49.5%)		85	41	
Stage					
III	66 (26%)		44	22	0.351
IV	188 (74%)		135	53	
Survival[d,e]					-
Median OS (months)			11	10	**0.6771**

*a) P value is calculated between wild type and all mutant groups, b) Mann-Whitney test is used in case of continuous variable (age) as the data are not normally distributed (Saphiro-Wilk test), c) Fisher's exact test is used between categorical variables, d) survival difference between the wild type and the mutant group was calculated using log rank regression analysis, e) PFS was not determined in the CHT group, *SD: standard deviation*

In order to study the clinical relevance of KRAS mutations, we performed comparative statistical analyses of KRAS status and clinicopathological variables in both the BEV/CHT (Table 7) and the CHT sub-cohorts (Table 8). As for the BEV/CHT group, ever-smoking and KRAS mutational statuses showed a significant positive association (P=0.008, Table 7). KRAS mutation was also significantly more common in female BEV/CHT patients (vs. males; P=0.002, Table 8). ECOG status and clinical stage did not differ between KRAS-mutant and KRAS WT patients in the BEV/CHT group significantly (P=0.056 and P=0.16, respectively, Table 7). The presence of KRAS mutation did not associate with age in the BEV/CHT group (P=0.09, Table 8). Of note, we did not detect significant associations of KRAS mutational status with age, smoking status, gender, ECOG status, stage or OS in the CHT group (Table 8). While reasons for the differences in the associations between KRAS mutational status and clinicopathological variables in the BEV/CHT vs. the CHT sub-cohorts are not entirely clear, a possible explanation is that they are due to the selection criteria for BEV therapy.

12. Table - Correlation of clinicopathologic features, outcome variables and KRAS codon 12 subtypes in patients with advanced LADC treated with BEV (n = 95)

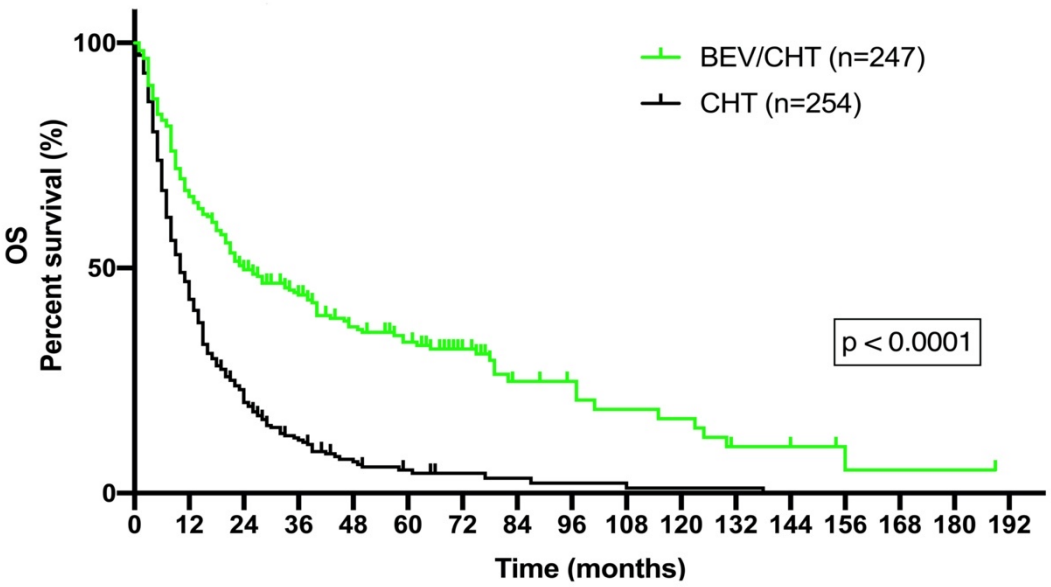
All patients		G12C	G12D	G12V	Rare mutation	*P*
		35	19	20	21	value[a]
Age (years)[b]	**Median:**	61	61	56	56	
	SD*:	7.7	7.1	8.7	7.2	0.579
	Range:	32	30	31	19	
Smoking[c]						
Never-smoker		2	0	2	2	
Ever-smoker		33	15	12	14	0.182
No data (15)						
Gender[c]						
Female		21	6	12	15	0.072
Male		14	13	8	6	
ECOG[c]						
0		17	7	11	17	0.594
1		18	12	9	4	
Stage[c]						
III		12	3	1	1	0.071
IV		23	16	19	20	
Survival[d]						
Median PFS (months)		8,53	3,77	8,17	6.77	0,0057
Median OS (months)		19,20	7,27	14,97	12.97	0.0414

*[a] P value is calculated between KRAS codon 12 subtypes, [b] Kruskal-Wallis test, [c] Chi-square test, [d] log rank regression analysis, *SD: Standard Deviation*

5.2.2. The presence of KRAS mutations has clinical utility in predicting disease outcome in LADC patients receiving concurrent antiangiogenic and chemotherapy

As expected, patients in the BEV/CHT group had significantly longer median OS than those receiving CHT only (P<0.0001, log-rank test; Figure 30). This difference was even more remarkable when only KRAS WT patients were compared (P<0.0001, log-rank test,

Figure 31A). Notably, the addition of BEV to CHT was also associated with significant benefit in OS if KRAS-mutant patients were compared with those in the CHT alone sub-cohort (P=0.0002, log-rank test, Figure 31A).



30. Figure - Comparison of survival outcomes in patients with advanced LADC according to treatment regimen. Advanced LADC patients receiving BEV/CHT showed significantly higher median OS compared to those treated with CHT only (median OSs were 24 vs. 10 months, respectively, P<0.0001, log-rank test).

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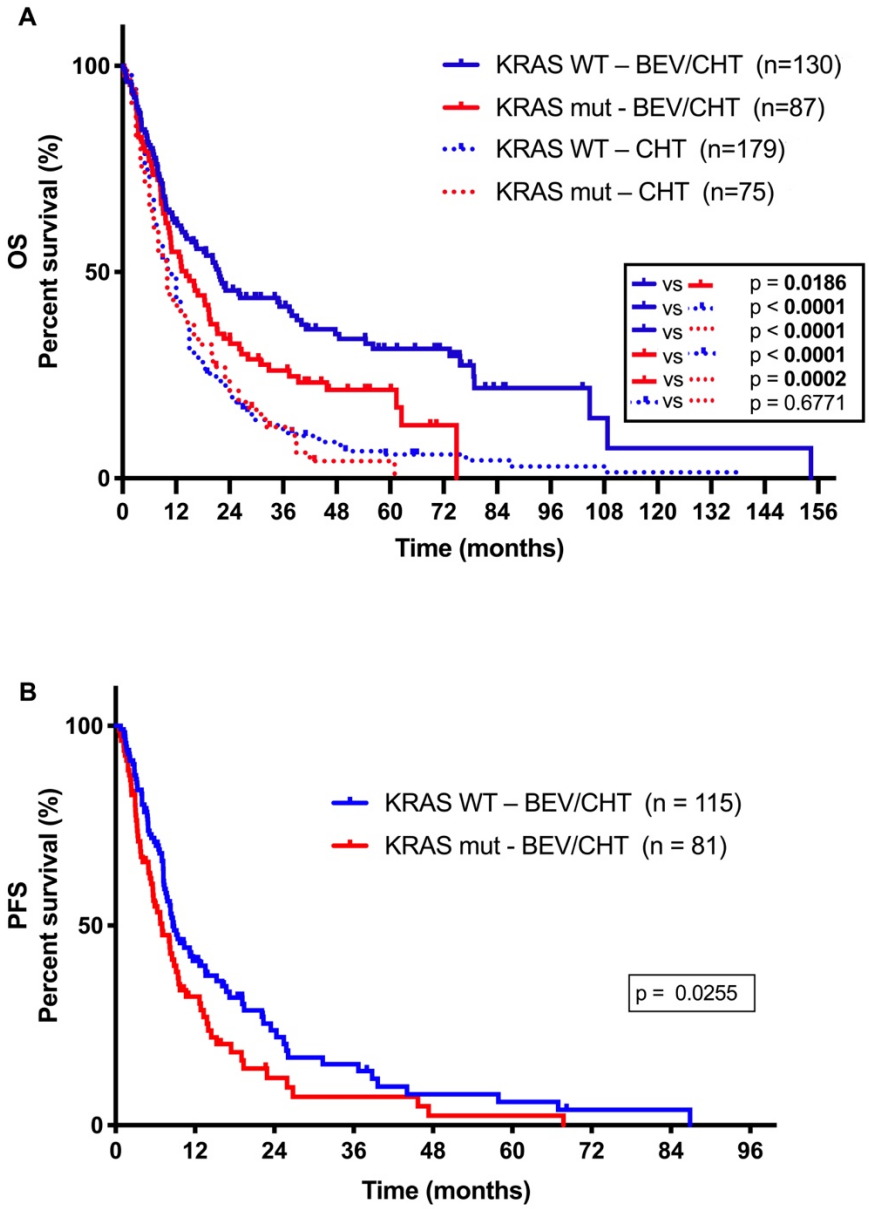

31. Figure - Kaplan-Meier plots for the OS (A) and PFS (B) in LADC patients according to KRAS mutation status. (A) LADC patients with KRAS WT tumors and receiving BEV/CHT had significantly increased median OS (vs. those with KRAS WT tumors and receiving CHT only; median OS 21.57 vs. 14.23 months, respectively, P=0.0186, log-rank test). Median OS was also increased in KRAS-mutant LADC patients receiving BEV/CHT compared to those treated with CHT only (median OSs were 18 vs. 10 months, respectively, P=0.0002, log-rank test). No significant differences in OS have been observed for patients receiving CHT only and with KRAS WT versus KRAS-mutant tumors (median OSs were 11 vs. 10 months, respectively P=0.6771, log-rank test). Of note, in the BEV/CHT group, patients with KRAS WT LADC had a significantly better OS than those with tumors harboring KRAS mutations (median OSs were 39 vs. 18 months, respectively, P=0.0186, log-rank test). (B) Similarly, in the BEV/CHT group, patients with KRAS WT LADC had significantly longer median PFS (vs. those with KRAS-mutant tumors; median PFSs were 8.63 vs. 7.03 months, respectively, P=0.0255, log-rank test).

We next investigated if KRAS mutational status influences the efficacy of CHT with or without BEV in advanced LADC. There was no difference in OS between patients with KRAS-mutant versus KRAS WT tumors in the CHT alone group (P=0.6771, log-rank test, Figure 31A). Importantly, however, in the BEV/CHT group we found that KRAS-mutant LADC patients had significantly shorter median PFS and OS than did KRAS WT patients (P=0.0255 and P=0.0186, respectively, log-rank test; Figures 31B and 31A). In support of this, multivariate Cox regression analyses revealed that KRAS status (mutant vs. WT) at diagnosis influenced OS (HR 0.645, 95% CI 0.458-0.908, P= 0.012) and PFS (HR 0.597, 95% CI 0.402-0.887, P= 0.011) independently from age (continuous; P values were 0.081 and 0.628, respectively), gender (female vs. male; P values were 0.005 and 0.001, respectively), smoking status (never- vs. ever-smoker; P values were 0.907 and 0.835, respectively), ECOG PS (0 vs. 1; P values were 0.193 and 0.177, respectively) and tumor stage (III. vs. IV; P values were 0.048 and 0.617, respectively; Table 13). These analyses also identified more advanced tumor stage as a significant independent negative prognostic factor for OS but not for PFS (P values were 0.048 and 0.617, respectively, Table 13). Gender proved to be an independent prognosticator for both OS and PFS in a multivariate Cox regression model as well (P values were 0.005 and 0.001, respectively, Table 13).

13. Table - Clinicopathological variables and PFS and OS of LADC patients treated with BEV/CHT in the multivariate Cox proportional hazards model

	PFS	**OS**
Age (continuous)		
HR	0.628	0.978
95% CI	0.966-1.021)	(0.955-1.003)
P	0.628	0.081
Gender (female vs. male)		
HR	0.248	0.390
95% CI	(0.125-0.494)	(0.203-0.751)
P	0.001	0.005
Smoking (never- vs. ever-smokers)		
HR	0.944	0.968
95% CI	(0.548-1.626)	(0.562-1.669)
P	0.835	0.907
ECOG PS (0 vs. 1)		
HR	0.765	0.772
95% CI	(0.518-1.129)	(0.523-1.140)
P	0.177	0.193
Stage (III vs. IV)		
HR	0.879	0.603
95% CI	(0.531-1.455)	(0.365-0.996)
P	0.617	0.048
KRAS status (WT vs. mutant)		
HR	0.597	0.645
95% CI	(0.402-0.887)	(0.458-0.908)
P	0.011	0.012

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

5.2.3. Distinct efficacy of BEV/CHT in advanced LADC patients with different subtype-specific KRAS mutations

Next, we looked at the clinicopathological characteristics of KRAS codon 12-mutant LADC patients receiving BEV/CHT and performed a statistical analysis on their associations with amino acid-specific mutational status. We identified 35 (36.8%) G12C, 19 G12D (20%), 20 G12V (21%), 3 G12A (3.2%%), 1 G12S (1%), 1 G12R (1%), 3 G13D (3.1%), and 1 G13C (1%) cases. Significant associations of subtype-specific KRAS mutational status with age, smoking status, gender, ECOG PS or tumor stage were not detected (Table 9). Importantly, patients with KRAS G12D mutant tumors had significantly shorter OS than those presenting with KRAS WT or with other KRAS codon 12 or 13 mutant (G12/13x) tumors (P=0.0223 and P=0.0144, respectively; log-rank test, Figure 32A). In line with the OS data, KRAS G12D mutation conferred a significant disadvantage for PFS when compared with KRAS WT (P<0.0001; log-rank test, Figure 2B) or all the other codon 12 or 13 KRAS (G12/13x) mutations (P=0.0032; log-rank test, Figure 32B). Of note, the OS of G12D KRAS mutant patients in the BEV/CHT group was comparable to that of patients in the CHT alone sub-cohort (Figure 33).

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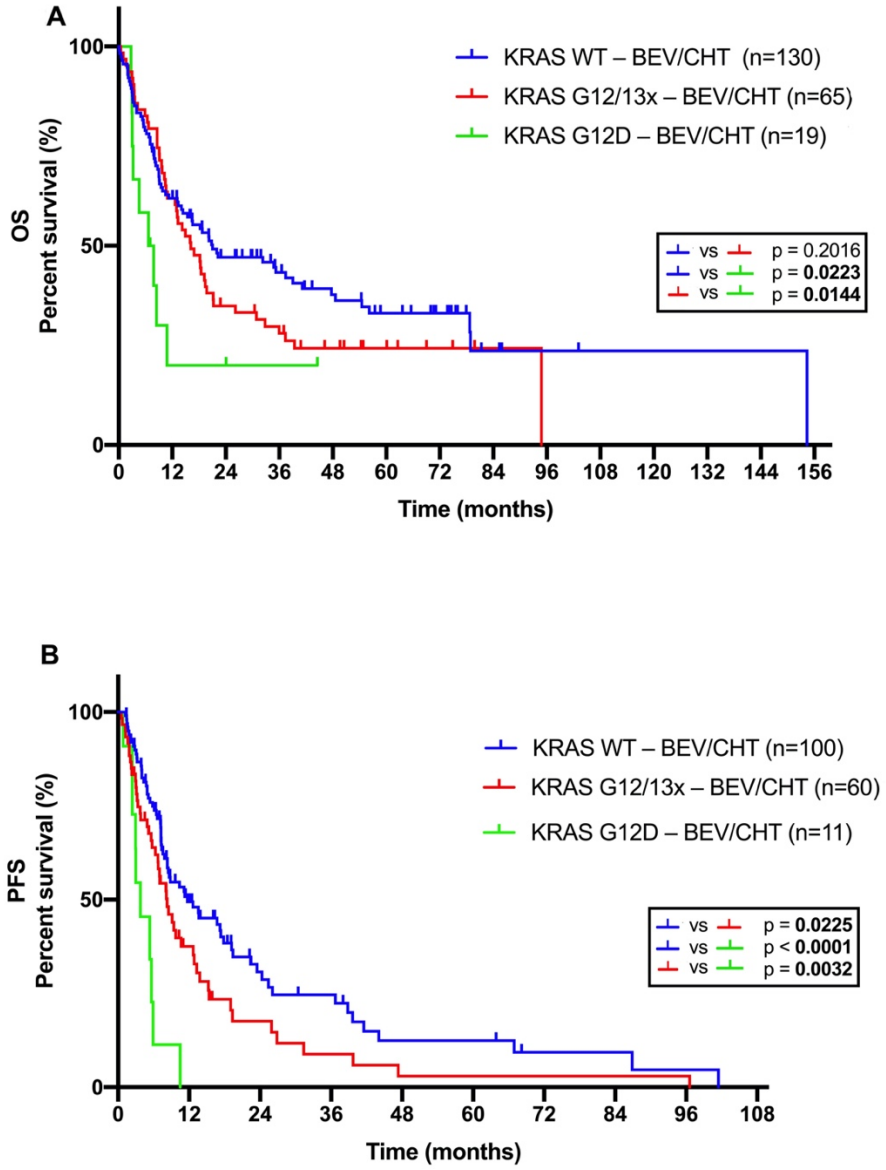

32. Figure - Kaplan-Meier plots for the OS (A) and PFS (B) in LADC patients receiving BEV/CHT according to subtype-specific codon 12 KRAS mutations. (A) KRAS G12D mutation was associated with significantly shorter OS in LADC patients (vs. KRAS G12x and 13x mutations or WT KRAS; median OSs were 7.2, 16.1 and 21 months, respectively, P values were 0.0144 and 0.0223, respectively, log-rank test). (B) LADC patients with tumors harboring KRAS G12D mutations had also significantly shorter median PFS than those with other codon 12 (G12x) and 13 (G13x) KRAS mutant or with KRAS WT tumors (median PFSs were 3.7, 8.27 and 11.7 months, respectively; P values were 0.0032 and <0.0001, respectively, log-rank test).

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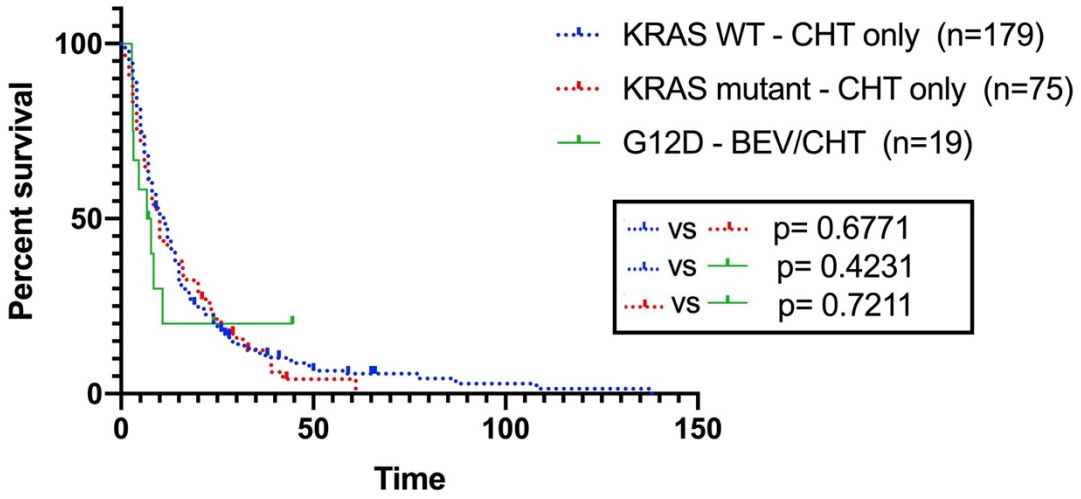

33. Figure - Kaplan-Meier curves for the OS of LADC patients treated with CHT alone and LADC patients with KRAS G12D mutations in the BEV/CHT sub-cohort. Patients with tumors harboring KRAS G12D mutations and treated with BEV/CHT had comparable OS to that of patients with KRAS WT or KRAS mutant tumors in the CHT alone sub-cohort.

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6. Discussion

6.1. The safety and efficacy of bevacizumab in addition to platinum based chemotherapy in patients with advanced NSCLC

Various randomized trials showed superior survival data and acceptable safety results with the use of bevacizumab in NSCLC(205, 206, 238, 239). Most of these trials, however, were not concluded in an unselected, real-world environment. Of note, there are still several questions yet to be answered regarding the drug's safety, efficacy and optimal treatment protocol. The AVALANCHE observational cohort study (OCS) provided an opportunity to examine the safety and efficacy of bevacizumab in combination with chemotherapy in a real-life setting in Hungarian everyday practice.
Generally the results of observational studies cannot be directly compared with those of a randomized study. However, the indicators of effectiveness in the AVALANCHE study (which included a higher variety of patients) are consistent with those of several randomized trials shown in Table 14.

Survival data:
The median PFS and OS in our study were longer than in the AVAiL (206, 207), the E4599 (205) or the ARIES(238) studies. These OS outcomes are also comparable with the results of the phase IV SAiL trial conducted between 2006 and 2008 in Europe. SAiL reported 14.6 months (95% CI, 13.8–15.3) OS, that was shorter than the reported OS in AVALANCHE. The PFS in AVALANCHE was 7.162 ± 0.282 months (CI95%: 6.609-7.715). SAiL trial reported time-to-progression of 7,8 months (95% CI, 7.5-8.1) but not PFS. The SAiL study let the choice of platinum doublet chemotherapy regimen to the investigator’s decision similarly to our study. However, non-platinum doublets and single-agent chemotherapy regimens were also allowed in SAiL study unlike in AVALANCHE. Other differences included that SAiL enrolled a selected patient population that was generally healthier and younger(239).

Treatment response:

ORR outcomes in AVALANCHE were also comparable with the ORR results of the above-mentioned studies. The 46.7% ORR was higher than the 34.6%, 37.8% and the 34.9% of the AVAiL 7.5mg/kg, AVAiL 15mg/kg and the E4599 trials, respectively. The SAiL and ARIES trials showed higher ORR. SAiL reported 3% CR and 48% PR (239) which is also comparable to the 2.3% CR and 44.4% PR rate of AVALANCHE.

Survival analysis by different patient subgroups:

Sandler et al. reported that women had significantly lower OS in the E4599 trial. They, however, also stated that this difference could be the result of imbalances of treatment regiments or baseline prognostic factors between the two groups (205). The AVAiL studies (207) and our AVALANCHE trial, on the other hand, found comparable results between women and men. Women had longer PFS and OS than men in the AVALANCHE, however, only OS was on the boundary of significance (p=0.071). Although, OS was reported higher in both AVAiL studies and the AVALANCHE trial, this survival advantage of women can also be accounted for by the generally longer survival of women with lung cancer that has been previously reported in statistical reports (7, 240).

As for the patients' age, nearly 10% of the patient population with evaluable data were over 70 years of age and no significant difference was found between the two groups regarding PFS. Surprisingly, however, OS was reported to be longer in patients over 70 years of age, although this difference was not significant. Contrary to our findings, the E4599 study found that patients older than 65 years of age had a significantly higher HR for death and suggested that these patients might not benefit from bevacizumab treatment (205). The AVAiL studies reported similar HRs for OS in both groups. One concern in previous studies was that the risk of bleeding could be higher in older patients, however neither the E4599, nor the AVAiL studies nor the SAiL study back up this hypothesis (241).

We observed higher PFS and OS in patients with an ECOG status of 0 at enrolment, although only OS showed a significant difference. This result is not surprising in light of the fact that ECOG performance status is an important prognostic factor in lung cancer

(242-245). Of note, the E4599 and the AVAiL studies did not find a significant difference in the HR for OS between the ECOG 0 and the ECOG 1 group (205, 207).

Johnson et al. assumed that central tumor location might cause pulmonary hemorrhage more often thus decreasing the OS (203). However, this was not supported by subsequent data. Neither SAiL, nor ARIES showed significantly more pulmonary bleeding with centrally located tumors (246, 247). Based on a retrospective analysis of the E4599 study data, Sandler et al. suggested that pulmonary hemorrhage was connected to cavitation of NSCLC instead of central location (248). Further studies did not support this assumption. Our data do not reinforce any of these suggestions. Neither the PFS, nor the OS was significantly longer with central tumors, and cavitated tumors were not assessed separately.

Although previous chemo- or radiotherapy did not influence PFS or OS, we found significantly longer PFS and OS in the patient group that underwent surgery before enrolment in this study. There is no available data to back up this finding. The most probable reason behind it is that the number of cancer sites is lower in these patients. Further assessment would be needed to draw further conclusions.

Platinum based chemotherapy has been shown in multiple studies to result in a small but significant survival benefit when compared to supportive care (249, 250). The most commonly used platinum derivatives are cisplatin and carboplatin. Neither of the above mentioned two drugs were associated with higher PFS, OS or lower toxicity when compared to each other(251-256). Interestingly, patients treated with cisplatin were found to have a longer OS (16.953±1.775 months) than those receiving carboplatin (OS: 12.977 ± 1.692 months). The statistical difference was on the boundary of significance (p=0.06). Santana-Davila et al. showed that oncologists more often administered cisplatin to relatively younger patients with less comorbidities. This could be a reason for the longer OS. However, it has also been shown that morbidity is higher in patients receiving cisplatin and they experience a higher need for health care (252).

Our patients receiving bevacizumab maintenance therapy showed significantly higher PFS and OS, which correlates with previous results published by Reck et al (206). In addition, Dranitsaris et al. found that bevacizumab maintenance therapy contributed to a significant OS benefit (257). In the Phase IIIB AvaALL study, bevacizumab was administered even after disease progression. A significantly higher PFS of 10.1 months

was achieved in this experimental arm compared to the control arm where only supportive care was used after disease progression (258). There are several trials debating whether bevacizumab or bevacizumab with pemetrexed is more effective for maintenance therapy. AVAPERL and POINTBREAK, two phase III trials designed to evaluate bevacizumab maintenance therapy with or without pemetrexed, showed significantly longer PFS, however the difference regarding OS was not significant in either of them (223, 259).

Our rate of reported adverse events falls behind that of expected based on previous trials. Lynch et al. reported that in the ARIES trial 19.7% of patients experienced one or more protocol-specified adverse event (238), which is somewhat lower than the 20.8% of patients reported in AVALANCHE. However, when looking at the serious adverse events, the 10.9% reported in ARIES is appreciably higher than the 0.5% reported in AVALANCHE. Notably, the study protocols can vary in the qualification of serious adverse events. Crinò et al. reported a rate of 38% for serious adverse events, although only 13% was deemed related to bevacizumab by the investigators (239). There is a special interest in similar studies regarding pulmonary bleeding, one of the most common serious adverse event following bevacizumab therapy. AVAiL 7.5mg/kg, AVAiL 15mg/kg, E4599, ARIES and SAiL reported 4%, 5%, 4.7%, 4.1% and 9.5% for the prevalence of any grade pulmonary hemorrhage, respectively. In contrary to this, pulmonary hemorrhage only occurred in 2 patients (0.7%) in AVALANCHE.

In summary, patients in Hungary commonly receive bevacizumab for advanced NSCLC in combination with a range of chemotherapeutics. Despite the less strictly selected patient population and treatment regimens survival outcomes and treatment response rates are comparable with those of the previous large RCT (randomized clinical trials). In our study, both PFS and OS were significantly longer and ORR significantly higher in patients who received bevacizumab maintenance therapy. The adjuvant/neoadjuvant chemotherapy or radiotherapy received prior to enrolment, the localization of the primary tumor, the presence of metastases or the age of the patient had no influence on the efficacy of bevacizumab treatment. On the other hand, previous surgery and cisplatin chemotherapy were associated with better outcomes. We also found low rates of adverse events and acceptable safety profile.

Limitations

The study design did not allow the comparison of PFS and OS assessed in the study, with placebo or any active comparator, and the comparative assessment of the significance of the prognostic factors studied, either. Due to the high censoring rate, the median OS could not be determined after the closure of the study; therefore, a retrospective data collection was required.

The Avalanche study, like most OCSs had limitations such as reporting errors, missing data, potential biases regarding data entry and confoundment. In this study, reporting centers were asked to enroll all eligible patients to reduce selection bias, however, unintended selection bias cannot be excluded. All known strong confounders were collected and analyzed to reduce confounding bias. Clinical reporting errors were reduced by systematic data reviews occurring every 3 months.

A further limitation of the current study was that in 12/40 planned sites, due to their lower patient turnover, we did not identify eligible patients within the recruiting period. Thus, representing the real life setting, not all centers enrolled patients and there were also smaller centers where fewer patients were recruited.

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1. Table - Baseline patient characteristics and effectiveness of Bevacizumab with First-Line Chemotherapy for nsNSCLC in the AVALANCHE OCS, ARIES OCS, the Phase IV SAiL Study, and the Phase III Clinical Trials E4599 and AVAiL

Baseline patient characteristics										Results			
	Age (years)	**Gender**		**ECOG status**			**Stage**			**Chemotherapy Regimen Used**	**Median follow-up** (months)	**Median PFS** (months)	**Median OS** (months)	**ORR**[a] (%)
		Female	Male	0	1	2	IIIB	IV	Recurrent					
AVAiL[b] 7.5mg/kg (n=345 ITT) (n=307 PP)	< 65: 70.95% > 65: 29.05%	35.55%	64.45%	39.73%	60.27%	0%	14.88%	77.02%	8.1%	Gemcitabine + Cisplatin	≥ 7 for PFS ≥ 12,5 for OS	6,5	13,4 (11,1 - 15,1)	34,6
AVAiL 15mg/kg (n=351 ITT) (n=285 PP)	< 65: 69.92% > 65: 30.08%	36.67%	63.33%	40.1%	59.9%	0%	15.9%	76.64%	7.3%	Gemcitabine + Cisplatin	≥ 7 for PFS ≥ 12,5 for OS	6,7	13,6 (11,8 - 15,8)	37,8
E4599[c] (n=434)	< 70: 76% > 70: 24%	48%	52%	47%	53%	0%	14%	86%	0%	Paclitaxel + carboplatin	19	6,2	12,3 (11,3 - 13,7)	34,9
SAIL[d] (n=2212)	58.8 (24-86)*	40%	60%	37%	57%	6%	20%	80%	0%	Investigator`s choice	12,5 (SD, 7.1-12.5)	7,8 (7.5-8.1)	14,6 (13,8 - 15,3)	51,5
ARIES[e] (n=1967)	> 65: 51.5% > 75: 18.8%	46.7%	53.3%	36%	48.7%	9.3%	Locally advanced** 16.6%		Metastatic** 83.4%	Investigator`s choice	12,5 (SD, 0.2-65.5)	6,6 (6.3-6.9)	13 (12,2 - 13,8)	49
AVALANCHE (n=283)	58.16 ± 9.032*	49.5%	50.5%	33%	67%	0%	19.2%	80.8%	0%	Investigator`s choice	n.a.	7.162 ± 0.282 (6.609-7.715)	15.179 (12.480-17.877)	46,7

a: Patients experiencing a complete response or a partial response. b: AVAiL - Avastin in Lung; c: E4599 - Eastern Cooperative Oncology Group 4599; d: Safety of Avastin in Lung, The SAiL study reported time to progression (TTP) outcomes instead of PFS. e: ARIES - Avastin Regimens: Investigation of Effectiveness and Safety; * Age was reported as average in the SAIL and AVALANCHE studies; ** Stage was reported as such in the ARIES study

6.2. KRAS mutation as a biomarker for anti-VEGF therapy in NSCLC

Although KRAS is the most frequently mutated oncogene in NSCLC, our knowledge on the effect of KRAS mutation on response to BEV in lung cancer is very limited. Biomarkers of BEV efficacy including imaging markers and circulating levels of angiogenic cytokines have been tested both in preclinical and clinical studies. For instance, VEGF levels in immunodepleted plasma of cancer patients were found to be significantly reduced following BEV treatment (260). However, VEGF-A, as measured by using an ELISA that recognizes all VEGF-A isoforms, was not predictive in a comprehensive evaluation of four phase III trials of BEV in CRC, NSCLC and renal cancer (261). Interestingly, recent data suggest use of TP53 status as a biomarker for response to BEV in NSCLC (262, 263). Nevertheless, as in other solid tumor types, a reliable biomarker to identify patients with LADC who will benefit from BEV is yet to be discovered. Here we analyzed the KRAS exon 2 mutational status in a large Caucasian patient cohort (n=501) with stage III–IV, EGFR WT LADC treated with platinum-based chemotherapy alone or in combination with BEV.

In the current LADC cohort, 33.9% of the patients had KRAS mutation. The incidence of KRAS mutation was higher in the BEV/CHT-treated group as compared to the CHT group (38.5% vs. 29.5%, respectively, P=0.012). With an incidence of 36.8% G12C was the most frequent subtype in the BEV/CHT group, followed by the G12V (21.1%) and the G12D (20%) subtypes. Other rare mutational subtypes (i.e. G12A, G12R, G12S, G13C, G13D) were identified in 22.1% of the patients. These findings are in line with data previously reported by us and by others in large NSCLC studies (219, 264).

Next, we investigated if KRAS mutational status had an effect on response to BEV. Although KRAS status had no impact on OS of LADC patients receiving CHT alone, in the BEV/CHT group patients carrying KRAS mutation had significantly shorter OS. Multivariate analysis also confirmed the role of KRAS as a negative predictor of response to BEV. In lung cancer, so far only two studies addressed the impact of KRAS mutation on the efficacy of BEV. Results coming from both of these studies are in line with our data. Chaft et al. treated 50 stage IB-IIIA NSCLC patients in the neoadjuvant setting in combination with CHT and evaluated their pathological response (265). None of the 10 KRAS mutant patients responded, in comparison to 11 of 31 KRAS WT cases. Although these authors administered BEV in combination with CHT, based on our current data and

also on previous reports from our group and others, the efficacy of CHT is not affected by KRAS status in NSCLC (218, 219). Thus, the better response rate in the KRAS WT group of the Chaft study can be attributed to BEV and not to CHT (233). In further support of this, Brady et al. studied 93 stage IV NSCLC patients receiving CHT alone or in combination with BEV and observed that while CHT was as effective in KRAS WT patients as in those with KRAS-mutant tumors, BEV improved OS and PFS only in patients with KRAS WT but not with KRAS-mutant tumors (234).

Mechanisms of resistance to antiangiogenic agents such as BEV include hypoxia-mediated mechanisms (266), the downregulation of target receptors and the activation of compensatory angiogenic pathways (267-269), proangiogenic hematopoietic or endothelial progenitor cell release from the bone marrow (270), inadequate intratumoral distribution of antiangiogenic drugs (271), and also a switch from endothelial sprouting to a nonangiogenic vascularization mechanism such as vessel cooption (a frequently occurring vascularization pattern in primary and secondary lung tumors that mediates resistance to anti-angiogenic therapy) (272-275).) It is not completely clear, though, whether and how KRAS mutation can contribute to these resistance mechanisms. Notably, however, tumor spread through air spaces (STAS) (276) and "tumor islands" (277) are closely related morphological features to vessel cooption (278) and were found to be significantly associated with KRAS mutations in NSCLC (276, 277). Moreover, mutant KRAS has been shown to induce the expression of VEGF in transformed fibroblasts or epithelial cells in vitro. KRAS mutation led to increased expression of other angiogenic growth factors such as TGF-beta and alpha (279). Elevated VEGF mRNA levels were detected in tumor cell lines expressing mutant KRAS (92). Genetic disruption of the mutant KRAS allele in human colon carcinoma cells resulted in decreased VEGF secretion (92). Transfection of human pancreatic epithelial cells with KRAS12V induced the expression of VEGF and CXC chemokines through Erk and c-Jun signaling and enhanced endothelial tube formation in co-cultures, which could be inhibited by CXCR2 or VEGF targeting (280). And lastly, doxycycline withdrawal led to tumor regression and endothelial apoptosis in a doxycycline-inducible RAS-driven INK4a deficient murine model of melanoma (226).

In CRC patients receiving BEV, results on associations between KRAS mutational status and outcome have been inconsistent, with a larger number of studies reporting no

associations (227, 229, 230, 281, 282) than those demonstrating significant associations (231, 232). Interestingly, however, in a recent CRC study, Fiala et al. demonstrated that G12V and G12A mutation were predictors of shorter PFS and OS, while patients with tumors harboring other KRAS mutations had similar outcome to those with KRAS WT tumors (231). Notably, another group reported that the presence of KRAS G12D mutation was significantly associated with poorer outcome in CRC patients receiving BEV containing regimens (283). As for NSCLC, Scheffler et al. found recently that patients with KRAS G12D mutation exhibit a high frequency of co-occurring mutations in the angiogenesis-associated PDGF (platelet derived growth factor receptor) / PDGF-receptor pathway (284). In line with this, amongst the three major codon 12 KRAS mutation subtypes (G12C, G12V and G12D) G12D proved to be a predictor of poor outcome in our BEV/CHT sub-cohort. Patients with LADC harboring this mutation had significantly worse PFS and OS than those with tumors harboring other KRAS mutations or WT KRAS.

The biological importance of KRAS mutational subtypes has been demonstrated in a study by Figueras et al. who introduced either codon 12 or codon 13 KRAS mutation into NIH3T3 cells and analyzed the VEGF levels and the activity of VEGF promoter in these transfected sublines. Despite the lower VEGF expression, codon 12 mutant tumors exhibited higher microvessel density, while tumors harboring the codon 13 mutation developed angiogenic sprouts with larger diameters (285).

In our cohort, only two patients carried codon 13 mutation of KRAS, thus we could not evaluate the BEV response in this subgroup. Nevertheless, our study suggests that specific KRAS mutation subtypes can have a major impact on tumor vascularization and, potentially, on response to anti-angiogenic treatment.

Like all retrospective analyses, our study has limitations. First, although we excluded patients with EGFR mutant tumors from our study, we did not analyze KRAS-WT patients for additional oncogenic driver mutations. Second, we did not study KRAS mutant patients for co-occurring mutations in additional tumor-associated pathways (284). Third, because this large retrospective cohort did not include reliable RECIST data (286) for all patients, we did not investigate the correlation of KRAS mutational status with tumor response according to RECIST criteria. Finally, because there is a massive body of literature on the predictive and prognostic role of KRAS mutations in CHT-

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treated LADC patients (216-220, 284, 286-288) and the main aim of the current study was to investigate the relationship between *KRAS* status and the efficacy of BEV, only the OS but not the PFS data were used in our analyzes in the CHT alone sub-cohort.

7. Conclusion

7.1. The safety and efficacy of bevacizumab in addition to platinum based chemotherapy in patients with advanced NSCLC

To conclude the results of the AVALANCHE study, it can be stated that comparable results were seen in the Hungarian clinical practice as in former international studies. Marked improvement was seen in PFS and OS of patients with locally advanced, metastatic, or recurrent non-small cell lung cancer other than predominantly squamous cell histology when BEV was added to standard platinum based CHT. In the meantime, combination therapy with BEV proved to be safe, with acceptable amount of adverse events and low percentage of treatment discontinuation. BEV is a valid option in the treatment of stage IIIB and IV nsNSCLC in the Hungarian clinical setting.

7.2. KRAS mutation as a biomarker for anti-VEGF therapy in NSCLC

In conclusion, when combined with standard first-line chemotherapy, BEV has led to increased OS and thus has been approved in patients with advanced or recurrent nsNSCLC without targetable molecular abnormalities (203, 205, 222, 223, 289, 290). However, although serious efforts have been made to identify patients responsive to BEV, there is as yet no validated predictive biomarker in this field.

Here, we present novel evidence for use of BEV in stage III-IV LADC patients with KRAS-mutant tumors -and especially with KRAS G12D-mutant tumors -, demonstrating inferior activity of this drug compared to that in LADC patients with non–KRAS-mutant tumors. Our data may not only help to improve the efficacy of BEV, but through better patient selection, could also help to decrease the unnecessary use of this expensive agent in subgroups of KRAS-mutant human LADC patients. Unnecessary and ineffective use of BEV can potentially even harm patients through adverse events while achieving no PFS, OS or quality of life benefit and also putting a financial strain of health care.

Subtype specific *KRAS* mutational status can be an easily accessible marker, since its already part of the routine molecular testing in NSCLC. Using subtype specific *KRAS* mutation status as a biomarker could help clinicians to administer individualized therapy. To validate this potential biomarker prospective studies are needed.

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8. Summary

Although angiogenesis has long been regarded as essential to tumor progression, anti-angiogenics have provided only modest clinical results so far. The current thesis is based on two different studies on the clinical efficacy of bevacizumab (BEV), an antiangiogenic inhibitor, in lung adenocarcinoma (LADC) patients.

In the first study (AVALANCHE; NCT03170284), advanced LADC patients received BEV with any platinum-doublet for up to 6 cycles. Primary endpoint was progression-free survival (PFS), secondary endpoints included overall survival (OS), tumor control rate and safety. Longer PFS and OS were observed in patients who received BEV therapy (median OS, 26.2 versus 10.2 months ($p<0.001$); median PFS, 9.2 versus 5.8 months ($p<0.001$)). Response rate: complete remission / partial response / stable disease / progressive disease /not reported were: 1.5/29.9/26.9/9.1/32.6% of all patients. These clinical outcomes were consistent with pivotal studies.

In the second study, our aim was to investigate the prognostic and predictive role of KRAS (Kirsten rat sarcoma 2 viral oncogene homolog) mutations in human LADC patients treated with BEV. To this aim, the association between KRAS status and clinicopathological variables was retrospectively analyzed in 501 stage IIIB-IV LADC patients receiving first-line platinum-based chemotherapy (CHT) with or without BEV. Comparing the 247 BEV/CHT and the 254 CHT patients, we only found difference in OS between patients with KRAS-mutant versus KRAS wild-type tumors in the BEV/CHT group: patients with KRAS-mutant tumors demonstrated significantly shorter PFS ($p = 0.0255$) and OS ($p = 0.0186$) in response to BEV/CHT compared to KRAS wild-type patients. KRAS mutation was an independent predictor of shorter PFS (hazard ratio (HR), 0.597; $p = 0.011$) and OS (HR, 0.645; $p = 0.012$) in the BEV/CHT group. G12D KRAS-mutant patients receiving BEV/CHT showed significantly shorter PFS (3.7 months versus 8.27 months in the G12/13x group; $p = 0.0032$) and OS (7.2 months versus 16.1 months in the G12/13x group; $p = 0.0144$). BEV treatment was associated with significantly longer PFS and OS, however KRAS-mutant advanced LADC patients receiving BEV/CHT treatment exhibited inferior PFS and OS compared to those with KRAS wild-type LADC. G12D mutations may define a subset of KRAS-mutant LADC patients unsuitable for antiangiogenic therapy with BEV.

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9. Összefoglalás

Annak ellenére, hogy régóta ismert az angiogenezis nélkülözhetetlen szerepe a tumor növekedésében, az érképződést gátló kezelések eddig csak szerény klinikai eredményeket hoztak tüdőrákok esetén. Jelen tézis két különböző kutatás eredményei alapján készült, amik a bevacizumab (BEV), egy angiogenezis inhibitor, klinikai hatékonyságát vizsgálták tüdő adenocarcinomás (LADC) betegeknél.

Az első klinikai vizsgálatban (AVALANCHE; NCT03170284) előrehaladott LADC betegek kaptak 6 ciklus BEV-ot a standard platina alapú kezelés mellett. A vizsgálat elsődleges végpontja a progressziómentes túlélés (PFS), míg másodlagos végpontjai az össztúlélés (OS), a terápiára adott klinikai válasz és a biztonság voltak. Hosszabb PFS és OS volt megfigyelhető azokban a betegekben, akik BEV terápiában részesültek (medián OS, 26.2 vs 10.2 hónap ($p<0.001$); medián PFS, 9.2 versus 5.8 hónap ($p<0.001$)). A terápiás válasz tekintetében a teljes remisszió / részleges válasz / stabil betegség / progresszív betegség / nem jelentett: 1.5/29.9/26.9/9.1/32.6% volt. Ezek a klinikai eredmények összemérhetőek voltak a korábbi meghatározó vizsgálatok eredményeivel.

A második vizsgálatban az volt a célunk, hogy megfigyeljük a KRAS (Kirsten rat sarcoma 2 viral oncogene homolog) mutáció prognosztikai és prediktív értékét előrehaladott LADC betegekben, akik BEV terápiában részesülnek. Ennek elérése érdekében retrospektívan elemeztük 501 IIIB-IV. stádiumú LADC beteg klinikopathológiai paramétereit, akik platina alapú kemoterápiás (CHT) kezelésben részesültek BEV-el vagy nélküle. Amikor összehasonlítottuk a 247 BEV/CHT terápiában részesülő beteget a 254 csak CHT beteggel, azt találtuk, hogy csak a BEV/CHT csoportban okozott túlélési különbséget a KRAS mutáció: a KRAS mutáns betegek PFS-e ($p = 0.0255$) és OS-e ($p = 0.0186$) is szignifikánsan alacsonyabb volt, mint a KRAS wild-type betegeké. A KRAS mutáció önálló prediktív faktornak bizonyult a rövidebb PFS (hazard ratio (HR), 0.597; $p = 0.011$) és OS (HR, 0.645; $p = 0.012$) tekintetében is a BEV/CHT csoportban. A G12D KRAS mutációt hordozó betegek szignifikánsan rövidebb PFS-t (3.7 hónap vs 8.27 hónap a G12/13x csoportban; $p = 0.0032$) és OS-t (7.2 hónap vs 16.1 hónap a G12/13x csoportban; $p = 0.0144$) mutattak BEV/CHT terápia esetén, mint a bármely más KRAS mutációt hordozók. A G12D KRAS mutáció a LADC betegek egy olyan alcsoportját jelezheti, akiknél az angiogenezis gátló terápia BEV-el hatástalan.

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11. Bibliography of the candidate's publications

11.1. Publications related to the thesis

Ghimessy AK, Gellert A, Schlegl E, Hegedus B, Raso E, Barbai T, Timar J, Ostoros G, Megyesfalvi Z, Gieszer B, Moldvay J, Renyi-Vamos F, Lohinai Z, Hoda MA, Klikovits T, Klepetko W, Laszlo V, Dome B.

KRAS Mutations Predict Response and Outcome in Advanced Lung Adenocarcinoma Patients Receiving First-Line Bevacizumab and Platinum-Based Chemotherapy.

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The efficacy and safety of bevacizumab in addition to platinum-based chemotherapy for the first-line treatment of patients with advanced nonsquamous non-small-cell lung cancer: Final results of AVALANCHE, an observational cohort study.

Oncol Lett. 2019 Feb;17(2):1750-1760. *contributed equally

11.2. Publications not related to the thesis

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The efficacy and safety of bevacizumab in addition to platinum-based chemotherapy for the first-line treatment of patients with advanced nonsquamous non-small-cell lung cancer: Final results of AVALANCHE, an observational cohort study

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Abstract. The previous results of former clinical studies confirmed that first-line bevacizumab (BEV) in combination with chemotherapy improves clinical outcomes in patients with advanced non-squamous non-small cell lung cancer. The AVALANCHE study (ClinicalTrials.gov Identifier NCT03170284) was undertaken to assess the clinical outcomes of first-line BEV combined with standard platinum-based regimens in the Hungarian clinical practice. This observational study was conducted in 28 Hungarian sites, with patients enrolled between July 2008 and April 2011. Patients with untreated locally advanced, metastatic or recurrent lung adenocarcinoma received BEV (7.5 mg/kg, q3w) with any platinum-doublet for up to 6 cycles, and then non-progressors proceeded to receive BEV until disease progression or unacceptable toxicity. The primary endpoint was time-to-progression, and secondary endpoints included overall survival (OS), tumour control rate and safety. Patients were also analysed as two cohorts (non-progressors vs. progressors) based on whether or not they received BEV maintenance therapy following completion of first-line chemotherapy plus BEV. The study enrolled 283 patients (median age: 58.2 (18-78) years; males: 50.5%; stage: III/B: 18.4%, IV: 79.9%; adenocarcinoma/other: 95.8/4.2%; ECOG PS 0/1/2/≥3: 30.8/59.7/2.6/1.4%). Centrally located tumours were reported in 21.6%. Cisplatin/carboplatin-based regimens: 53.8/46.2%. A total of 43% of patients received BEV maintenance therapy. The median number of BEV cycles was 6. Median progression-free survival (PFS) was 7.2 months and OS was 15.2 months for the entire cohort. Longer PFS and OS were observed in patients who received BEV maintenance therapy [median OS, 26.2 vs. 10.2 months (P<0.001); median PFS, 9.2 vs. 5.8 months (P<0.001)]. Contrary to the results of previous OCS no significant difference was recorded in the different age groups or gender. Best tumour response: Complete remission/partial remission/stable disease/progressive disease/not reported were: 1.5/29.9/26.9/9.1/32.6% of all patients. In conclusion, clinical outcomes obtained in this real-life population were consistent with pivotal studies. BEV maintenance treatment was associated with a significantly longer PFS and OS.

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Abbreviations: AE, adverse event; ALK, anaplastic lymphoma kinase; BEV, bevacizumab; CI, confidence interval; CR, complete response; CRF, case report form; ECOG, Eastern Cooperative Oncology group; EGFR, epidermal growth factor receptor; EMA, European medicines agency; NA, not applicable; NSCLC, non-small cell lung cancer; nsNSCLC, non-squamous non-small cell lung cancer; OCS, observational cohort study; OS, overall survival; PD, progressive disease; PFS, progression-free survival; PR, partial response; RCT, randomized clinical trial; SAE, serious adverse event; SD, stable disease; SD, standard deviation; TNM, Internationally accepted classification of malignant tumours; TTP, time-to-progression; VEGF, vascular endothelial growth factor; WHO, World Health Organization

Key words: bevacizumab, non-small cell lung cancer, first-line, observational study

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Introduction

Lung cancer is the second most common malignant tumour. However it causes more deaths than breast, prostate and colon cancer combined (1). Hungary has the highest mortality rates of lung cancer in the world regarding both men and women. Hungary, unlike other developed countries, records a growing number of new cases. While the incidence hasn't increased over the last few years in men, it continuously does in women (2).

Survival rates remain poor in non-small cell lung cancer (NSCLC) with 49% 5-year survival rate with early (stage IA) NSCLC and 1% 5-year survival rate in stage IV. One reason for such poor survival is that more than 50% of patients are diagnosed with advanced disease (3).

Although many advances have been made in the treatment of unresectable (stage IIIB), metastatic (stage IV) or recurrent NSCLC, such as the introduction of targeted therapy for specific oncogenic drivers (EGFR, ALK mutations etc.), platinum-based chemotherapy (with or without radiotherapy) still remains the first choice in most cases.

Targeted therapies showed superior survival data, demonstrated improved response rates and are associated with less toxicity. Druggable mutations for EGFR and ALK mutation, however, only occur in 25 and 5%, respectively (4,5). Vascular endothelial growth factor (VEGF) is a key factor to endothelial cell growth and one of the most important regulators of angiogenesis. Increased expression of VEGF can be demonstrated in most solid tumours including NSCLC (6). In many cases, VEGF overexpression is associated with an increased risk of relapse and metastasis (7-10). According to preclinical studies, anti-VEGF monoclonal antibodies are capable of inhibiting the growth of human tumour xenografts both in monotherapy and in combination with chemotherapy (11-14). Bevacizumab (BEV) (Avastin®; Genentech/Roche, San Francisco, CA, USA) is a humanized monoclonal antibody that acts by binding and neutralizing the VEGF-A isoform, thus preventing VEGF ligand-receptor binding. It has demonstrated its efficacy in colorectal (15,16), ovarian (17), breast (18,19) and renal cancer (20,21). This was the first antivascular drug to be licensed for the treatment of NSCLC.

According to a phase II study (22), BEV treatment in combination with chemotherapy in NSCLC was more effective than chemotherapy alone. The combination was also well tolerated, however, the incidence of lung haemorrhage increased. In a post hoc multivariate analysis, squamous cell histology was identified as an independent risk factor for bleeding (23). Consequently, patients with squamous cell histology were excluded from most of the clinical trials of BEV in NCSLC.

Subsequent to the above Phase II study, the Eastern Cooperative Oncology Group (ECOG) E4599 trial was initiated (24). This study, which was the first published Phase III randomized trial of an antiangiogenesis agent in combination with chemotherapy in patients with advanced NSCLC, randomized chemotherapy-naive patients with predominantly non-squamous cell histology were included. In the BEV treatment arm, following completion of chemotherapy, single-agent BEV was continued until disease progression. Results showed that the addition of BEV was associated with a significant improvement in the median overall survival (OS) compared with chemotherapy alone. Progression-free survival (PFS) was also significantly improved.

A second Phase III trial (Avastin® in Lung; AVAiL), evaluating BEV in combination with cisplatin and gemcitabine (25) (another commonly used and efficacious regimen in NSCLC) was originally initiated with a primary end point of OS. However, after the positive OS results of E4599, the study design was amended so as to change the primary end point from OS to PFS. Patients were randomly assigned to receive cisplatin 80 mg/m^2 and gemcitabine 1250 mg/m^2 for up to six cycles plus low-dose BEV (7.5 mg/kg), high-dose BEV (15 mg/kg) or placebo every 3 weeks until disease progression. PFS was significantly prolonged with BEV. Interestingly, according to the final efficacy analysis, OS was >13 months in all treatment groups, which was the longest OS reported for advanced non-squamous NSCLC in a clinical trial setting, although it did not yield a statistically significant prolongation with either BEV dose (26).

As a result of the above trials, BEV in combination with platinum-based chemotherapy was approved for the first-line treatment of patients with advanced NSCLC by the European Medicines Agency (EMA) in August 2007.

Although BEV was approved with platinum-based chemotherapy in NSCLC in 2007, so far no Hungarian data have been available. The AVALANCHE study (ClinicalTrials.gov, identifier: NCT03170284) was undertaken to assess the clinical outcomes of first-line BEV combined with standard platinum-based regimens in Hungarian clinical practice.

Patients and methods

Study design. AVALANCHE (ClinicalTrials.gov, identifier: NCT03170284) was a multi-centre single-arm observational study designed to assess the efficacy and safety of BEV therapy in patients with advanced, unresectable, metastatic or recurrent nsNSCLC (other than predominantly squamous cell histology) in the routine oncology practice in Hungary. Further objective of the study was to assess and identify possible treatment-related prognostic factors.

Patients. This study was originally projected to enrol 150 patients from 40 Hungarian study centres. Fortunately, however, due to the high number of patients recruited by some centres, nearly 300 patients were enrolled.

Patients with histology or cytology proven unresectable advanced, metastatic or recurrent (stage IIIB/IV) NSCLC other than predominantly squamous cell histology were included in the present study. There were 143 male (50.5%) and 135 female (47.7%) patients and no data on gender was available in 5 patients (1.8%) (Table I).

The exclusion criteria were the following: i) hypersensitivity to the active substance or to any of the excipients of Avastin®; ii) hypersensitivity to products derived from Chinese hamster ovary (CHO) cells or to other recombinant human or humanized antibodies; iii) pregnancy and iv) presence of untreated central nervous system metastases. The present study was done in accordance with the Declaration of Helsinki, Good Clinical Practice International Conference on Harmonisation Tripartite Guidelines, laws and regulations of the participating institutes' country. The present study was approved by the Hungarian Ethics Committee and Health Authority. All patients provided written informed consent.

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Table I. Patient demographics and treatment.

Characteristics	No. of patients, n (%)
Evaluable patient population	283 (99.6)
Patient population evaluable in terms of PFS	252 (88.7)
Patient population evaluable in terms of OS	250 (88)
Age (years)	
Mean	58.16±9.032
Men	58.30±8.986
Women	58.02±9.113
Gender	
Male	143 (50.5)
Female	135 (47.7)
No data	5 (1.8)
Histologic type	
Adenocarcinoma	271 (95.8)
Bronchoalveolar carcinoma	11 (3.9)
Squamous cell carcinoma	1 (0.4)
Stage	
III B	52 (18.4)
IV	226 (79.9)
No data	5 (1.8)
Previous treatment	
Previous surgery	64 (22.6)
Adjuvant/neoadjuvant chemotherapy	18 (6.4)
Radiotherapy	18 (6.4)
Chemotherapeutic agent during study	
Paclitaxel	132 (46.6)
Gemcitabine	111 (39.2)
Docetaxel	18 (6.4)
Vinorelbine	2 (0.7)
Other	7 (2.5)
No data	13 (4.6)
Reported reasons for ending the study	
Progression of primary disease	172 (60.8)
Deterioration of symptoms	4 (1.4)
Loss of contact with the patient	7 (2.5)
Adverse event associated with BEV treatment	13 (4.6)
Patient's decision	17 (6.0)
Mortality	16 (5.7)
Other	45 (15.9)
No data	9 (3.2)

PFS, progression-free survival; OS, overall survival; BEV, bevacizumab.

Treatment. Eligible patients received first-line BEV with cisplatin or carboplatin in accordance to the approved and reimbursed BEV indication in Hungary (BEV 7.5 mg/kg, every 3 weeks with any platinum-doublet for up to 6 cycles) then non-progressors proceeded to receive BEV until disease progression or unacceptable toxicity. The maintenance therapy regimen was 7,5 mg/kg every 3 weeks until PD or intolerable toxicity. The third component of the combination chemotherapy was one of the following: paclitaxel, gemcitabine, docetaxel or vinorelbine. Based on the therapeutic protocol, patients were followed up until the first progression of their primary disease, or death, or withdrawal of consent, or loss of contact with the patient, or closure of the study, whichever occurred first.

Progression-free and OS. Investigators seemed to be frequently using PFS and time-to-progression (TTP) interchangeably in clinical trials in the early 2000s (27). The protocol of our study defined TTP as the time elapsed from the date of enrolment until the first documented progression or the death of the patient from any cause which is in accordance with the current definition of PFS. To avoid confusion, PFS will be used hereinafter for the denomination of the primary endpoint of the study. Progression was determined by the investigator at the routine clinical practice follow-up examinations. PFS was calculated from the start of BEV treatment.

Secondary endpoints included best tumour response (complete remission (CR), partial remission (PR), stable disease (SD), progressive disease (PD)), OS (based on retrospective analysis) and indicators of safety (serious and non-serious adverse events). Objective response rate (ORR) was calculated from patients experiencing complete or partial remission.

Basic demographic data, basic vital parameters, primary disease-related historical data, ECOG performance status, data related to BEV treatment, results of the staging assessments as well as the patient's comorbidities and concomitant treatments were recorded in an electronic case-report form.

Following the closure of the study, data for the assessment of the PFS were available for 252 patients. As per the amended protocol, the secondary endpoint (OS) was retrospectively analysed based on data from 250 patients.

During the treatment period regular monitoring visits were conducted to ensure high-quality data collection. Data related to BEV treatment, blood pressure, body weight, concomitant treatments and adverse events were registered.

The following data were recorded at the end-of-treatment visit: End date of BEV treatment, reason for ending treatment, ECOG status, best tumour response observed during treatment, concomitant treatments administered during BEV treatment and adverse events observed during BEV treatment.

Statistical analysis. Continuous variables were compared with Student's t-tests if the sample distribution was normal or with Mann-Whitney U test if the sample distribution was asymmetric. Categorical data were compared using Fisher's exact probability and χ^2 tests. PFS (primary study endpoint) and OS in the total population were analysed using Kaplan-Meier curves. Both PFS and OS were assessed separately in subgroups according to gender, age, ECOG status, the platinum derivate used, the use of maintenance therapy and weather prior surgical intervention was done. Log-rank test was used for comparison between the above mentioned groups.

PFS was defined as the time elapsed from the start of BEV treatment until the first documented progression or the death

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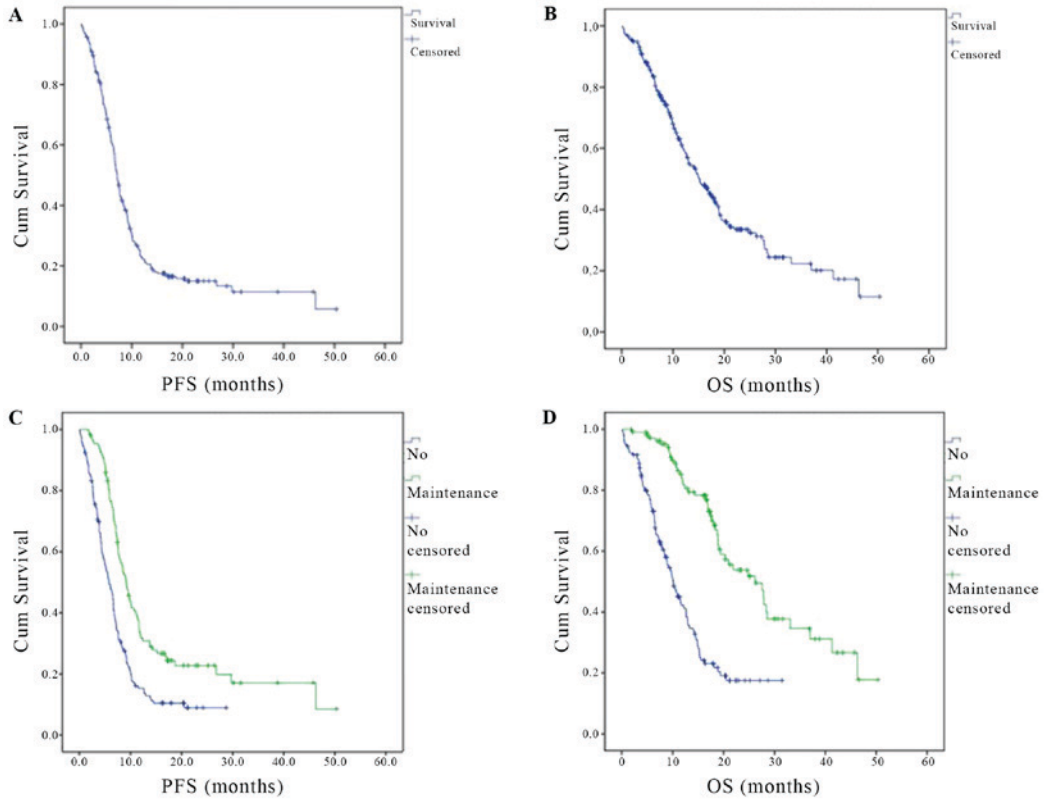

Figure 1. Kaplan-Meier plots of (A) PFS in the total population. (B) OS in enrolled and evaluated patients. (C) Analysis of PFS by Bevacizumab maintenance therapy. (D) Analysis of OS by Bevacizumab maintenance therapy. OS, overall survival; PFS, progression-free survival.

of the patient from any cause. For study subjects who had not shown progression and had not died by the closure of the study, the data were censored at the date of the last contact.

OS was defined as the time elapsed from the date of enrolment until the death of the patient from any cause. Regarding subjects who had not died by the closure of the study, the OS data were analysed retrospectively after the end of the study in the knowledge of their dates of death. Otherwise, data were censored at the date of the last contact.

P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were conducted using Statistica 8.0 (StatSoft, Inc., Tulsa, OK, USA) software program.

Results

Baseline characteristics of the patients. A total of 284 patients with corresponding diagnosis were identified at the Hungarian study sites, and were subsequently enrolled into the study between 17th June 2008 and 3rd May 2011, out of which data of 283 patients were evaluable. From among the 41 study centres originally involved, no patients were enrolled at 16 sites, thus in fact 25 centres participated actively. The highest number of patients enrolled at one centre was 36, whereas the smallest was 1. One patient did not comply with all the inclusion and exclusion criteria: The patient's histological diagnosis was squamous cell carcinoma; therefore evaluable patient population was 283. Central localization of the primary tumour was reported in 61 patients (21.6%) and cavitated tumour in 4 patients (1.4%) in the total patient population.

The study population had to be reduced to 252 in case of PFS and 250 regarding OS. In case of PFS 31 patients and in case of OS 33 patients had to be excluded from the data assessment due to missing or incomplete information. These information could not be recovered retrospectively.

The demographic characteristics of the enrolled and evaluable patients are summarized in Table I.

Treatment. Prior to enrolment, 64 patients (22.6%) had undergone surgical intervention, 18 patients (6.4%) had received adjuvant/neoadjuvant chemotherapy, and 18 patients (6.4%) had received radiotherapy (Table I).

Patients received cisplatin (N=148, 52.3%) or carboplatin (N=124, 43.8%) treatment in accordance with the protocol in approximately half-and-half proportion during the study. No data are available for 11 patients (3.9%). The other components of the combination chemotherapy are shown in Table I.

The vast majority of patients (N=262, 92.6%) received BEV in 3-weekly cycles. A treatment of different cycle frequency was applied in two patients (0.7%), and no data were available for 19 patients (6.7%). The median number of BEV treatment cycles in the retrospectively evaluated patient population was 6.

The most common reason for ending the study was documented as progression of the primary disease in more than half of the study subjects (60.8%). Patient's decision, patient's death, adverse event related to BEV therapy, loss to follow-up, and symptom deterioration accounted for ending the study in 6.0, 5.7, 4.6, 2,5 and 1.4% of the cases, respectively. Other reasons behind ending the study occurred in 15.9%; no data were available in 3.2% of cases.

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Table II. Best tumor response reached during the first-line treatment.

Response	N	Patient population with evaluable data (n=133), (%)	Total patient population (n=216), (%)
Complete remission	3	2.3	1.5
Partial remission	59	44.4	29.9
Stable disease	53	39.8	26.9
Progressive disease	18	13.5	9.1
Not assessable	83	-	32.6

Efficacy analysis

PFS. The PFS in the total study patient population was 7.162±0.282 ($CI_{95\%}$: 6.609-7.715) months (Fig. 1A). The subgroup-analysis of PFS by gender showed that the survival time with BEV treatment was longer in women (median: 7.589±0.647, $CI_{95\%}$: 6.321-8.858 months) than in men (median: 6.669±0.375, $CI_{95\%}$: 5.934-7.405 months). This difference, however, was not significant (P=0.542).

The median PFS was higher in patients with an ECOG status of 0 at enrolment (median: 7.326±0.535, $CI_{95\%}$: 6.278±8.375 months) than in patients with a baseline ECOG status of 1 (median: 6.702±0.597 months, $CI_{95\%}$: 5.531-7.873 months). However, the difference between the two groups was not remarkable (P=0.123).

Similarly, PFS was not significantly influenced by the localization of the tumour (central vs. non-central, P=0.813).

Interestingly, the median PFS in patients who had undergone surgical intervention prior to enrolment (median: 8.411±0.947, $CI_{95\%}$: 6.554-10.267 months) was notably higher (P=0.017) compared with patients with no such prior intervention (median: 6.834±0.265, $CI_{95\%}$: 6.314-7.353 months). In contrast, neither adjuvant/neoadjuvant chemotherapy (P=0.165) nor radiotherapy (P=0.165) applied prior to enrolment had a significant impact on median PFS.

The platinum derivative used had no significant influence on median PFS, either (P=0.199).

Nearly 10% of the patient population with evaluable data were over 70 years of age at the time of enrolment. The median PFS was not significantly different between patients under or above 70 years of age (P=0.541).

Of note, median PFS was significantly higher (P<0.001) in patients receiving BEV maintenance therapy (median: 9.166±0.601, $CI_{95\%}$: 7.988-10.345 months) compared with those who received no maintenance therapy (median: 5.815±0.574, $CI_{95\%}$: 4.690-6.940 months) (Fig. 1C).

Secondary endpoints

Tumour response. Disease control was achieved in a remarkable 86.5% with CR in 2.3%, and PR in 44.4% of the cases with evaluable data. PD was recorded in 13.5% of evaluable cases and sufficient data was not available in 32.6% (Table II).

OS. The median OS in the total study population was 15.179±1.377 months ($CI_{95\%}$: 12.480-17.877) (Fig. 1).

As with PFS, we performed subgroup-analysis of OS by gender, ECOG status, prior surgical procedure and chemotherapy. Results can be seen on Table III.

The localization of the tumour had no impact on OS (P=0.992) in the patient population studied.

Surprisingly, we found a tendency towards a higher median OS for patients over 70 years of age (18.398±3.869 months, $CI_{95\%}$: 10.815-25.982 months) compared with patients younger than 70 years (15.014±1.329 months, $CI_{95\%}$: 12.410-17.619 months), although this difference remained not significant (P=0.638).

A remarkably longer (P<0.001) OS was observed in patients receiving BEV maintenance therapy (median: 26.218±3.946 months, $CI_{95\%}$: 18.484-33.952 months) than in those without maintenance BEV therapy (median: 10.152±0.975 months, $CI_{95\%}$: 8.240-12.064 months) (Fig. 1D).

Safety and adverse events. As per the protocol, possible adverse events (AE) encountered during the study were recorded in the Case Report Form. Data on AE were recorded from the start of treatment until the end of treatment.

During the study, a total of 157 AEs were reported for 59 patients, 14 of which were serious (sAE) (Table IV).

Of all the adverse events, 63 (40.1%) events resolved without sequelae, the investigators reported improvement for 61 cases (38.9%) and the event resolved with remaining symptoms in 7 cases (4.5%). 2 AEs (1.3%) had not resolved, 14 AEs (8.9%) persisted unchanged from observation until the last follow-up of the patient, 5 AEs (3.2%) led to the death of the patient, and the outcome was unknown for 4 AEs (2.5%).

Of the above-mentioned AEs, 14 were categorized as sAE, which were the following: Anaemia (3 cases), pulmonary embolism (3 cases), haemoptysis (2 cases), deep vein thrombosis (2 cases), hypertension (1 case), neutropenia (1 case), thrombocytopenia (1 case), uraemia (1 case). 5 of these (two cases of pulmonary embolism, haemoptysis, hypertension and uraemia) led to the death of the patient.

During the study period, 16 (5.6%) of the 283 enrolled and evaluable patients died. The investigators reported the cause of death as disease progression in 11 cases (3.8%), while a serious adverse event was behind the death of the patient in 5 cases (1.7%).

In summary, the participating investigators did not encounter and report on any new information on the safety profile of BEV. Indeed, the rate of reported adverse events falls behind the rate expected based on literature data.

Discussion

Various randomised trials showed superior survival data and acceptable safety results with the use of BEV in

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Table III. Subgroup analysis of OS.

	Gender		ECOG status		Prior surgery		Prior chemo-/radiotherapy		Platinum derivative used	
Variable	Male	Female	ECOG 0	ECOG 1	Yes	No	Chemotherapy	Radiotherapy	Cisplatin	Carboplatin
Median OS	12.583	17.511	18.891	13.306	26.218	13.306	Not significant P=0.237	Not significant P=0.237	16.953	12.977
CI 95%	9.544-15.622	14.320-20.703	14.869-22.914	10.385-16.227	18.721-33.714	11.373-15.239			13.475-20.431	9.661-16.294
P-value	0.071		0.004		0.001				0.006	

OS, overall survival; CI, confidence interval; ECOG, Eastern Cooperative Oncology Group.

NSCLC (24,25,28,29). Most of these trials, however, were not concluded in an unselected, real-world environment. Of note, there are still several questions yet to be answered regarding the drug's safety, efficacy and optimal treatment protocol. The AVALANCHE observational cohort study (OCS) provided an opportunity to examine the safety and efficacy of BEV in combination with chemotherapy in a real-life setting in Hungarian everyday practice.

Generally the results of observational studies cannot be directly compared with those of a randomized study. However, the indicators of effectiveness in the AVALANCHE study (which included a higher variety of patients) are consistent with those of several randomized trials shown in Table V.

The median PFS and OS in our study were longer than in the AVAiL (25,26), the E4599 (24) or the ARIES (28) studies. These OS outcomes are also comparable with the results of the phase IV SAiL trial conducted between 2006 and 2008 in Europe. SAiL reported 14.6 months (95% CI, 13.8-15.3) OS, that was shorter than the reported OS in AVALANCHE. The PFS in AVALANCHE was 7.162±0.282 months ($CI_{95\%}$: 6.609-7.715). SAiL trial reported TTP of 7,8 months (95% CI, 7.5-8.1) but not PFS. The SAiL study let the choice of platinum doublet chemotherapy regimen to the investigator's decision similarly to our study. However, non-platinum doublets and single-agent chemotherapy regimens were also allowed in SAiL study unlike in AVALANCHE. Other differences included that SAiL enrolled a selected patient population that was generally healthier and younger (29).

ORR outcomes in AVALANCHE were also comparable with the ORR results of the above-mentioned studies. The 46.7% ORR was higher than the 34.6%, 37.8% and the 34.9% of the AVAiL 7.5 mg/kg, AVAiL 15 mg/kg and the E4599 trials, respectively. The SAiL and ARIES trials showed higher ORR. SAiL reported 3% CR and 48% PR (29) which is also comparable to the 2.3% CR and 44.4% PR rate of AVALANCHE.

Sandler *et al* (24) reported that women had significantly lower OS in the E4599 trial. They, however, also stated that this difference could be the result of imbalances of treatment regiments or baseline prognostic factors between the two groups (24). The AVAiL studies (26) and our AVALANCHE trial, on the other hand, found comparable results between women and men. Women had longer PFS and OS than men in the AVALANCHE, however, only OS was on the boundary of significance (P=0.071). Although, OS was reported higher in both AVAiL studies and the AVALANCHE trial, this survival advantage of women can also be accounted for by the generally longer survival of women with lung cancer that has been previously reported in statistical reports (1,30).

As for the patients' age, nearly 10% of the patient population with evaluable data were over 70 years of age and no significant difference was found between the two groups regarding PFS. Surprisingly, however, OS was reported to be longer in patients over 70 years of age, although this difference was not significant. Contrary to our findings, the E4599 study found that patients older than 65 years of age had a significantly higher HR for death and suggested that these patients might not benefit from BEV treatment (24). The AVAiL studies reported similar HRs for OS in both groups. One concern in previous studies was that the risk of bleeding could be higher in older patients, however neither

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Table IV. Summary of the adverse events reported in the present study.

Adverse event	n (%)
Anemia	23 (14.7)
Thrombocytopenia	14 (9)
Neutropenia	12 (7.7)
Hypertension	7 (4.5)
Nausea	7 (4.5)
Epistaxis	6 (3.9)
Chest pain	5 (3.2)
Acute bronchitis	4 (2.6)
Weight loss	4 (2.6)
Bone pain	3 (2)
Diarrhea	3 (2)
Pulmonary embolism	3 (2)
Hemoptysis	3 (2)
Hyponatremia	3 (2)
Deep vein thrombosis	3 (2)
Hoarseness	3 (2)
Cough	2 (1.3)
Fever	2 (1.3)
Respiratory infection	2 (1.3)
Obstipation	2 (1.3)
Pneumonia	2 (1.3)
Pyuria	2 (1.3)
Tachycardia	2 (1.3)
Throat pain	2 (1.3)
Lung abscess	1 (0.7)
Agranulocytosis	1 (0.7)
Acute osteomyelitis (jaw)	1 (0.7)
Allergic dermatitis	1 (0.7)
Allergic reaction	1 (0.7))
Hip pain (right-sided)	1 (0.7)
Decubitus	1 (0.7)
Dermatitis (forehead, back)	1 (0.7)
Dermatitis (generalized)	1 (0.7)
Cholesterol increased	1 (0.7)
Exsiccosis	1 (0.7)
Ulcer (in the mouth, tongue)	1 (0.7)
Gastroesophageal reflux disease	1 (0.7)
Weakness	1 (0.7)
Vomiting	1 (0.7)
Abdominal pain	1 (0.7)
Ileus	1 (0.7)
Ischemic cerebral vascular lesions	1 (0.7)
Arthralgia	1 (0.7)
Swelling of arm	1 (0.7)
Hand swelling	1 (0.7)
Leg swelling	1 (0.7)
Laryngotracheitis	1 (0.7)
Febrile neutropenia	1 (0.7)
Prostration	1 (0.7)
Leukopenia	1 (0.7)
Breast swelling	1 (0.7)
Esophageal ulcer	1 (0.7)
Duodenal ulcer	1 (0.7)
Suffusion without trauma	1 (0.7)
Dizziness	1 (0.7)
Thrombosis (left femoral vein)	1 (0.7)
Uremia	1 (0.7)
Urticaria	1 (0.7)
Iron deficiency	1 (0.7)
Bleeding following superficial injury	1 (0.7)
Clear-cell renal carcinoma	1 (0.7)
Numbness (of the soles)	1 (0.7)

the E4599, nor the AVAiL studies nor the SAiL study back up this hypothesis (31).

We observed higher PFS and OS in patients with an ECOG status of 0 at enrolment, although only OS showed a significant difference. This result is not surprising in light of the fact that ECOG performance status is an important prognostic factor in lung cancer (32-35). Of note, the E4599 and the AVAiL studies did not find a significant difference in the HR for OS between the ECOG 0 and the ECOG 1 group (24,26).

Johnson *et al* (22) assumed that central tumour location might cause pulmonary haemorrhage more often thus decreasing the OS. However, this was not supported by subsequent data. Neither SAiL, nor ARIES showed significantly more pulmonary bleeding with centrally located tumours (36,37). Based on a retrospective analysis of the E4599 study data, Sandler *et al* (38) suggested that pulmonary haemorrhage was connected to cavitation of NSCLC instead of central location. Further studies did not support this assumption. Our data do not reinforce any of these suggestions. Neither the PFS, nor the OS was significantly longer with central tumours, and cavitated tumours were not assessed separately.

Although previous chemo- or radiotherapy did not influence PFS or OS, we found significantly longer PFS and OS in the patient group that underwent surgery before enrolment in this study. There is no available data to back up this finding. The most probable reason behind it is that the number of cancer sites is lower in these patients. Further assessment would be needed to draw further conclusions.

Platinum based chemotherapy has been shown in multiple studies to result in a small but significant survival benefit when compared to supportive care (39,40). The most commonly used platinum derivatives are cisplatin and carboplatin. Neither of the above mentioned two drugs were associated with higher PFS, OS or lower toxicity when compared to each other (41-46). Interestingly, patients treated with cisplatin were found to have a longer OS (16.953±1.775 months) than those receiving carboplatin (OS: 12.977±1.692 months). The statistical difference was on the boundary of significance (P=0.06). Santana-Davila *et al* (42) showed that oncologists more often administered cisplatin to relatively younger patients with less comorbidities. This could be a reason for the longer OS. However, it has also been

Table V. Baseline patient characteristics and effectiveness of Bevacizumab with First-Line Chemotherapy for nsNSCLC in the AVALANCHE OCS, ARIES OCS, the Phase IV SAiL Study, and the Phase III Clinical Trials E4599 and AVAiL.

	Baseline patient characteristics									Results				
		Gender (%)		ECOG status (%)			Stage (%)							
Trial	Age (years)	Female	Male	0	1	2	IIIB	IV	Recurrent	Chemotherapy regimen used	Median follow-up (months)	Median PFS (months)	Median OS (months)	ORR[c] (%)
AVAiL 7.5 mg/kg (n=345 ITT) (n=307 PP)	<65: 70.95% >65: 29.05%	35.55	64.45	39.73	60.27	0	14.88	77.02	8.1	Gemcitabine + Cisplatin	≥7 for PFS, ≥12.5 for OS	6.5	13.4 (11.1-15.1)	34.6
AVAiL 15 mg/kg (n=351 ITT) (n=285 PP)	<65: 69.92% >65: 30.08%	36.67	63.33	40.1	59.9	0	15.9	76.64	7.3	Gemcitabine + Cisplatin	≥7 for PFS, ≥12.5 for OS	6.7	13.6 (11.8-15.8)	37.8
E4599 (n=434)	<70: 76%; >70: 24%	48	52	47	53	0	14	86	0	Paclitaxel + carboplatin	19	6.2	12.3 (11.3-13.7)	34.9
SAIL (n=2,212)	58.8 (24-86)[a]	40	60	37	57	6	20	80	0	Investigator's choice	12.5 (SD: 7.1-12.5)	7.8 (7.5-8.1)	14.6 (13.8-15.3)	51.5
ARIES (n=1,967)	>65: 51.5%; >75: 18.8%	46.7	53.3	36	48.7	9.3	Locally advanced =16.6[b]		Metastatic =83.4[b]	Investigator's choice	12.5 (SD: 0.2-65.5)	6.6 (6.3-6.9)	13 (12.2-13.8)	49
AVALANCHE (n=283)	58.16±9.032[b]	49.5	50.5	33	67	0	19.2	80.8	0	Investigator's choice	n.a.	7.162±0.282 (6.609-7.715)	15.179 (12.480-17.877)	46.7

[a]Age was reported as the average in the SAIL and AVALANCHE studies. [b]Stage was reported as such in the ARIES study. ORR, objective response rate [c](patients who experienced a complete or partial response); AVAiL, Avastin in lung; E4599, eastern cooperative oncology group (ECOG) 4599; SAIL, safety of avastin in lung [The SAiL study reported time to progression outcomes instead of PFS]; ARIES, avastin regimens: investigation of effectiveness and safety; PFS, progression-free survival; OS, overall survival; n.a., not available; ITT, intention-to-treat; PP, per protocol; SD, standard deviation.

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shown that morbidity is higher in patients receiving cisplatin and they experience a higher need for health care (42).

Our patients receiving BEV maintenance therapy showed significantly higher PFS and OS, which correlates with previous results published by Reck *et al* (25). In addition, Dranitsaris *et al* (47) found that BEV maintenance therapy contributed to a significant OS benefit. In the Phase IIIB AvaALL study, BEV was administered even after disease progression. A significantly higher PFS of 10.1 months was achieved in this experimental arm compared to the control arm where only supportive care was used after disease progression (48). There are several trials debating whether BEV or BEV with pemetrexed is more effective for maintenance therapy. AVAPERL and POINTBREAK, two phase III trials designed to evaluate BEV maintenance therapy with or without pemetrexed, showed significantly longer PFS, however the difference regarding OS was not significant in either of them (49,50).

Our rate of reported adverse events falls behind that of expected based on previous trials. Lynch *et al* (28) reported that in the ARIES trial 19.7% of patients experienced one or more protocol-specified adverse event, which is somewhat lower than the 20.8% of patients reported in AVALANCHE. However, when looking at the serious adverse events, the 10.9% reported in ARIES is appreciably higher than the 0.5% reported in AVALANCHE. Notably, the study protocols can vary in the qualification of serious adverse events. Crinò *et al* (29) reported a rate of 38% for serious adverse events, although only 13% was deemed related to BEV by the investigators. There is a special interest in similar studies regarding pulmonary bleeding, one of the most common serious adverse event following BEV therapy. AVAiL 7.5 mg/kg, AVAiL 15 mg/kg, E4599, ARIES and SAiL reported 4, 5, 4.7, 4.1 and 9.5% for the prevalence of any grade pulmonary haemorrhage, respectively. In contrary to this, pulmonary haemorrhage only occurred in 2 patients (0.7%) in AVALANCHE.

In summary, patients in Hungary commonly receive BEV for advanced NSCLC in combination with a range of chemotherapeutics. Despite the less strictly selected patient population and treatment regimens survival outcomes and treatment response rates are comparable with those of the previous large RCT (randomised clinical trials). In our study, both PFS and OS were significantly longer and ORR significantly higher in patients who received BEV maintenance therapy. The adjuvant/neoadjuvant chemotherapy or radiotherapy received prior to enrolment, the localization of the primary tumour, the presence of metastases or the age of the patient had no influence on the efficacy of BEV treatment. On the other hand, previous surgery and cisplatin chemotherapy were associated with better outcomes. We also found low rates of adverse events and acceptable safety profile.

The study design did not allow the comparison of PFS and OS assessed in the study, with placebo or any active comparator, and the comparative assessment of the significance of the prognostic factors studied, either. Due to the high censoring rate, the median OS could not be determined after the closure of the study; therefore, a retrospective data collection was required.

The Avalanche study, like most OCSs had limitations such as reporting errors, missing data, potential biases regarding data entry and confoundment. In this study, reporting centres were asked to enrol all eligible patients to reduce selection bias, however, unintended selection bias cannot be excluded. All known strong confounders were collected and analysed to reduce confounding bias. Clinical reporting errors were reduced by systematic data reviews occurring every 3 months.

A further limitation of the current study was that in 12/40 planned sites, due to their lower patient turnover, we did not identify eligible patients within the recruiting period. Thus, representing the real life setting, not all centres enrolled patients and there were also smaller centres where fewer patients were recruited.

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Availability of data and materials

The datasets generated and analysed during the present study are not publicly available due to patient confidentiality but are available from the corresponding author on reasonable request and with permission of F Hoffmann-La Roche AG.

Authors' contributions

ET analysed and interpreted the data and contributed to the study design. ÁKG analysed and interpreted the collected data and wrote the manuscript. EJ, ZS, GL, PD, ZV, LH and EC enrolled the patients to the present study and collected the data. VS enrolled the patients and designed the study.

Ethics approval and consent to participate

The present study was approved by the Hungarian Ethics Committee and Health Authority. All patients provided written informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of any associated data.

Competing interests

The BEV used in the present study was obtained from Genentech/Roche (South San Francisco, CA, USA). The present study was also sponsored by F Hoffmann-La Roche. The funding body contributed to data collection and analysis; however, the sponsor did not influence the content of the report and did not contribute to the writing of this manuscript. The authors declare that they have no competing interests.

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KRAS Mutations Predict Response and Outcome in Advanced Lung Adenocarcinoma Patients Receiving First-Line Bevacizumab and Platinum-Based Chemotherapy

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Abstract: Bevacizumab, combined with platinum-based chemotherapy, has been widely used in the treatment of advanced-stage lung adenocarcinoma (LADC). Although KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutation is the most common genetic alteration in human LADC and its role in promoting angiogenesis has been well established, its prognostic and predictive role in the above setting remains unclear. The association between KRAS exon 2 mutational status and clinicopathological variables including progression-free survival and overall survival (PFS and OS, respectively) was retrospectively analyzed in 501 Caucasian stage IIIB-IV LADC patients receiving first-line platinum-based chemotherapy (CHT) with or without bevacizumab (BEV). EGFR (epidermal growth factor receptor)-mutant cases were excluded. Of 247 BEV/CHT and 254 CHT patients, 95 (38.5%) and 75 (29.5%) had mutations in KRAS, respectively. KRAS mutation was associated with smoking ($p = 0.008$) and female gender ($p = 0.002$) in the BEV/CHT group. We found no difference in OS between patients with KRAS-mutant versus KRAS wild-type tumors in the CHT-alone group ($p = 0.6771$). Notably, patients with KRAS-mutant tumors demonstrated significantly shorter PFS ($p = 0.0255$) and OS ($p = 0.0186$) in response to BEV/CHT compared to KRAS

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wild-type patients. KRAS mutation was an independent predictor of shorter PFS (hazard ratio, 0.597; $p = 0.011$) and OS (hazard ratio, 0.645; $p = 0.012$) in the BEV/CHT group. G12D KRAS-mutant patients receiving BEV/CHT showed significantly shorter PFS (3.7 months versus 8.27 months in the G12/13x group; $p = 0.0032$) and OS (7.2 months versus 16.1 months in the G12/13x group; $p = 0.0144$). In this single-center, retrospective study, KRAS-mutant LADC patients receiving BEV/CHT treatment exhibited inferior PFS and OS compared to those with KRAS wild-type advanced LADC. G12D mutations may define a subset of KRAS-mutant LADC patients unsuitable for antiangiogenic therapy with BEV.

Keywords: bevacizumab; non-small-cell lung cancer; advanced-stage lung adenocarcinoma; platinum-based chemotherapy; KRAS mutation

1. Introduction

The KRAS (V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) protein, encoded by the KRAS proto-oncogene, is a small GTPase (guanosine triphosphatase) that plays a key role in regulating various cell functions [1]. Alterations of the *KRAS* gene are typically missense mutations that can lead to the oncogenic conversion of KRAS resulting in the constitutive activation of its effector pathways and thus cancer development and progression [2]. KRAS is frequently mutated in pancreatic and colorectal cancer (CRC), and in lung adenocarcinoma (LADC). With an incidence of up to 30%, KRAS mutation is the most common driver mutation in LADC. The most prevalent G12C and G12V KRAS mutation subtypes are associated with smoking, while the G12D subtype has been observed in those who have never smoked [3,4]. Several other rare mutations of KRAS codon 12, 13, and 61 have also been reported [3].

The prognostic and predictive power of the KRAS mutation in non-small-cell lung cancer (NSCLC) patients remains controversial. It was first reported in the late 1980s that KRAS mutation is associated with poorer survival [5,6], and since then several groups confirmed these findings [7,8]. However, most of these studies were rather heterogeneous in terms of histology, tumor stage, and methodologies of KRAS mutation detection. Although two different meta-analyses concluded that KRAS mutation is a negative prognosticator in LADC [9,10], the most comprehensive study of more than 1500 NSCLC patients (including 300 KRAS-mutant cases) from four trials of adjuvant chemotherapy (CHT) reported that KRAS mutation had no clear prognostic or predictive relevance with regards to response to CHT [11].

Previously, our group performed a mutation subtype-specific analysis of 505 stage III–IV LADC patients treated with platinum-based CHT and found that there were no significant differences in progression-free survival (PFS) and overall survival (OS) among patients with wild-type (WT), codon 12, and codon 13 KRAS mutations. Importantly, however, G12V KRAS-mutant patients tended to have a higher response rate and a modestly longer median PFS [12].

The importance of subtype-specific KRAS mutation analysis was further highlighted in the preclinical study of Garassino et al. These authors investigated the role of different KRAS mutation subtypes (G12C, G12V, and G12D) in the in vitro chemosensitivity of human NSCLC cells and found that the expression of G12C was associated with a reduced response to cisplatin and an increased sensitivity to taxol and pemetrexed. In the same study, G12D mutation led to resistance to taxol and sensitivity to sorafenib, whereas the G12V mutation sensitized the cells to cisplatin [13].

Increased expression and the negative prognostic role of vascular endothelial growth factor (VEGF, the key angiogenic cytokine) have been reported in most solid tumors including NSCLC [14] [15]. Several phase 2 and 3 clinical trials demonstrated that the addition of bevacizumab (BEV, a humanized monoclonal antibody against the VEGF-A isoform) to CHT improves the PFS and OS of NSCLC patients [16–20]. Accordingly, BEV in combination with platinum-based CHT was approved for the first-line treatment of patients with advanced-stage NSCLC by the FDA (U.S. Food and Drug

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Administration) and the EMA (European Medicines Agency) in 2006 and 2007, respectively. The efficacy of BEV in a real-life setting in Hungary was shown in the Avalanche study [21].

Although the RAS/RAF/MEK/ERK signaling pathway has been implicated in the regulation of VEGF expression and angiogenesis [22], only a few studies have investigated the effect of KRAS mutation on the efficacy of BEV therapy. Most studies focused on CRC, where the addition of BEV to CHT prolonged survival regardless of KRAS mutational status [23–26]. Two different groups, however, demonstrated that G12V, G12A [27], and G12D [28] KRAS mutations are associated with poor outcome in metastatic CRC patients receiving BEV. As for nonsquamous NSCLC, in a phase 2 trial evaluating the addition of neoadjuvant BEV to CHT, Chaft et al. found that no patient (0 out of 10) with KRAS mutation showed a pathological response to neoadjuvant BEV/CHT, in comparison to 11 of 31 KRAS WT patients [29]. In another small study of stage IV NSCLC, BEV therapy was associated with improved OS and PFS in KRAS WT ($n = 26$), but not in KRAS-mutant ($n = 16$) patients [30]. Here, we report the results of the first study, to our knowledge, of amino acid substitution-specific KRAS mutational status analysis in a large cohort of BEV/CHT-treated stage III–IV Caucasian patients.

2. Results

2.1. Incidence of KRAS Mutations in LADC Patients Treated with Bevacizumab and Chemotherapy

All patients had advanced LADC and Caucasian background. Patients with tumors harboring an EGFR mutation were excluded. One hundred and seventy patients of the full cohort of 501 cases were identified as KRAS-mutant (33.9%) and 331 (66.1%) as KRAS WT (see Table 1A,B). While 38.5% ($n = 95$) of the patients treated in the BEV/CHT group were KRAS-mutant (Table 1A), in the CHT group (Table 1B) this ratio was 29.5% ($n = 75$) ($p = 0.012$). There were no significant differences between the BEV/CHT and CHT groups with respect to age ($p = 0.193$), smoking status ($p = 0.072$), gender ($p = 0.506$), and tumor stage ($p = 0.610$) (data not shown). The only difference was seen in performance status (PS): there were more ECOG (Eastern Cooperative Oncology Group) 0 (vs. EVOG 1) patients in the BEV/CHT group than in the CHT-alone group ($p = 0.031$; data not shown), which might be due to the BEV selection criteria. In the BEV/CHT subcohort, 35 (36.8%), 19 (20%), and 20 (21%) cases were classified as G12C, G12D, and G12V mutants, respectively (Table S1). Other rare (i.e., $n < 3$) KRAS exon 2 mutation subtypes (G12A, G12R, G12S, G13C and G13D) were also found in the BEV group. Subtype-specific mutations were technically not assessable in 21 cases (Table S1).

Table 1. Patient characteristics in the bevacizumab/chemotherapy (BEV/CHT) and chemotherapy (CHT) groups.

	No. of Patients (%)	**KRAS Status**		***p*-value** [a]
		Wild type (%)	Mutant (%)	
A. BEV/CHT				
All patients	247	152 (61.5%)	95 (38.5%)	
Age (years) [b]	Median:	62	58	0.09
	SD*:	9.2	8.2	
	Range:	53	44	
Smoking [c]				
Never-smoker	30 (12%)	24	6	0.008
Ever-smoker	167 (68%)	93	74	
No data ($n = 50$)				
Gender [c]				
Female	106 (43%)	52	54	0.002

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Male	141 (57%)	100	41	
ECOG [c]				
0	139 (56%)	87	52	0.056
1	108 (44%)	65	43	
Stage [c]				
III	55 (22%)	38	17	0.16
IV	192 (78%)	114	78	
Survival [d]				
Median PFS (months)		8.63	7.03	**0.0255**
Median OS (months)		21.57	14.23	**0.0186**

B. CHT					
All patients	254		179 (70.5%)	75 (29.5%)	
Age (years) [b]		Median:	63	61	0.297
		SD *:	7.8	8.7	
		Range:	46	46	
Smoking [c]					
Never-smoker	21 (8%)		15	6	0.435
Ever-smoker	188 (74%)		135	53	
No data (n = 45)					
Gender [c]					
Female	118 (46.5%)		79	39	0.27
Male	136 (53.5%)		100	36	
ECOG					
0	128 (50.5%)		94	34	0.335
1	126 (49.5%)		85	41	
Stage					
III	66 (26%)		44	22	0.351
IV	188 (74%)		135	53	
Survival [d,e]					-
Median OS (months)			11	10	**0.6771**

[a] p value is calculated between wild type and all mutant groups, [b] Mann–Whitney test is used in case of continuous variable (age) as the data are not normally distributed (Shapiro–Wilk test), [c] Fisher's exact test is used between categorical variables, [d] survival difference between the wild type and the mutant group was calculated using log rank regression analysis, [e] PFS was not determined in the CHT group, * SD: standard deviation, BEV/CHT: bevacizumab/chemotherapy, KRAS: V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog, ECOG: Eastern Cooperative Oncology Group, PFS: progression-free survival, OS: overall survival.

In order to study the clinical relevance of KRAS mutations, we performed comparative statistical analyses of KRAS status and clinicopathological variables in both the BEV/CHT (Table 1A) and the CHT subcohorts (Table 1B). As for the BEV/CHT group, ever-smoking and KRAS mutational statuses showed a significant positive association (p = 0.008; see Table 1A). KRAS mutation was also significantly more common in female BEV/CHT patients (vs. males; p = 0.002; see Table 1A). ECOG status and clinical stage did not differ significantly between KRAS-mutant and KRAS WT patients in the BEV/CHT group (p = 0.056 and p = 0.16, respectively; see Table 1A). The presence of KRAS mutation was not associated with age in the BEV/CHT group (p = 0.09; see Table 1A). Of note, we did not detect significant associations of KRAS mutational status with age, smoking status, gender, ECOG status, stage, or OS in the CHT group (Table 1B). While the reasons for the differences in the associations between KRAS mutational status and clinicopathological variables in the BEV/CHT vs.

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the CHT subcohorts are not entirely clear, a possible explanation is that they are due to the selection criteria for BEV therapy.

2.2. The Presence of KRAS Mutations has Clinical Utility in Predicting Disease Outcome in LADC Patients Receiving Concurrent Antiangiogenic and Chemotherapy

As expected, patients in the BEV/CHT group had significantly longer median OS than those receiving CHT only ($p < 0.0001$, log-rank test; Figure S2). This difference was even more remarkable when only KRAS WT patients were compared ($p < 0.0001$, log-rank test; see Figure 1A). Notably, the addition of BEV to CHT was also associated with a significant benefit in OS if KRAS-mutant patients were compared with those in the CHT-alone subcohort ($p = 0.0002$, log-rank test; see Figure 1A).

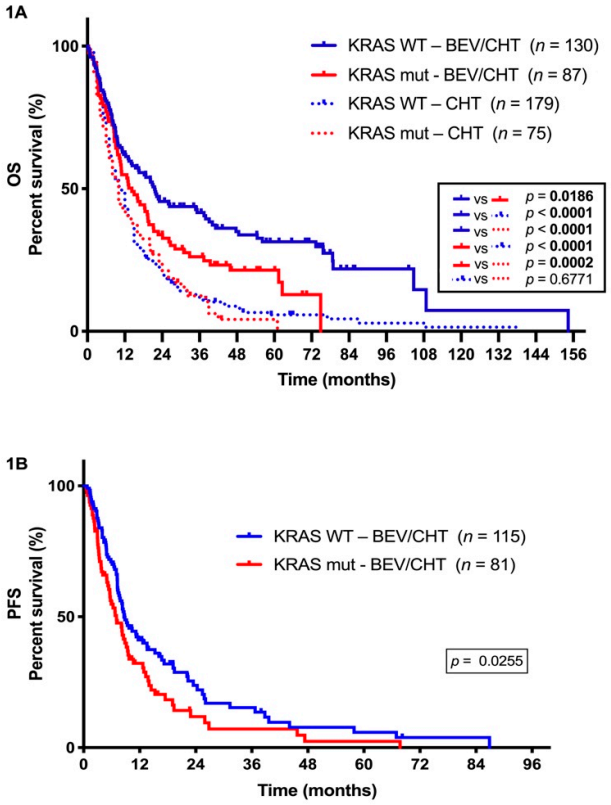

Figure 1. Kaplan–Meier plots for the overall survival (OS) (**A**) and progression-free survival (PFS) (**B**) in lung adenocarcinoma (LADC) patients according to V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) mutation status. (**A**) LADC patients with KRAS wild-type (WT) tumors and receiving bevacizumab/chemotherapy (BEV/CHT) had significantly increased median OS (vs. those with KRAS WT tumors and receiving CHT only; median OS 21.57 vs. 14.23 months, respectively, $p = 0.0186$, log-rank test). Median OS was also increased in KRAS-mutant LADC patients receiving BEV/CHT compared to those treated with CHT only (median OSs were 18 vs. 10 months, respectively, $p = 0.0002$, log-rank test). No significant differences in OS have been observed for patients receiving CHT only and with KRAS WT versus KRAS-mutant tumors (median OSs were 11 vs. 10 months, respectively $p = 0.6771$, log-rank test). Of note, in the BEV/CHT group, patients with KRAS WT LADC had a significantly better OS than those with tumors harboring KRAS mutations (median OSs were 39 vs. 18 months, respectively, $p = 0.0186$, log-rank test). (**B**) Similarly, in the BEV/CHT group, patients with KRAS WT LADC had significantly longer median PFS (vs. those with KRAS-mutant tumors; median PFSs were 8.63 vs. 7.03 months, respectively, $p = 0.0255$, log-rank test).

We next investigated whether the KRAS mutational status influences the efficacy of CHT with or without BEV in advanced LADC. There was no difference in OS between patients with KRAS-

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mutant versus KRAS WT tumors in the CHT-alone group (p = 0.6771, log-rank test; see Figure 1A). Importantly, however, in the BEV/CHT group we found that KRAS-mutant LADC patients had a significantly shorter median PFS and OS than did KRAS WT patients (p = 0.0255 and p = 0.0186, respectively, log-rank test; see Figures 1A,B). In support of this, multivariate Cox regression analyses revealed that KRAS status (mutant vs. WT) at diagnosis influenced OS (hazard ratio (HR) 0.645, 95% confidence interval (CI) 0.458–0.908, p = 0.012) and PFS (HR 0.597, 95% CI 0.402–0.887, p = 0.011) independently from age (continuous; P values were 0.081 and 0.628, respectively), gender (female vs. male; p values were 0.005 and 0.001, respectively), smoking status (never vs. ever; p values were 0.907 and 0.835, respectively), ECOG PS (0 vs. 1; P values were 0.193 and 0.177, respectively) and tumor stage (III. vs. IV; p values were 0.048 and 0.617, respectively; see Table 2). These analyses also identified more advanced tumor stage as a significant independent negative prognostic factor for OS, but not for PFS (p values were 0.048 and 0.617, respectively; see Table 2). Gender proved to be an independent prognosticator for both OS and PFS in a multivariate Cox regression model as well (p values were 0.005 and 0.001, respectively; see Table 2).

Table 2. Clinicopathological variables and progression-free survival (PFS) and overall survival (OS) of lung adenocarcinoma (LADC) patients treated with bevacizumab/chemotherapy (BEV/CHT) in the multivariate Cox proportional hazards model.

Clinicopathological Variables	PFS	OS
Age (continuous)		
HR	0.628	0.978
95% CI	0.966–1.021)	(0.955–1.003)
p	0.628	0.081
Gender (female vs. male)		
HR	0.248	0.390
95% CI	(0.125–0.494)	(0.203–0.751)
p	0.001	0.005
Smoking (never- vs. ever-smokers)		
HR	0.944	0.968
95% CI	(0.548–1.626)	(0.562–1.669)
p	0.835	0.907
ECOG PS (0 vs. 1)		
HR	0.765	0.772
95% CI	(0.518–1.129)	(0.523–1.140)
p	0.177	0.193
Stage (III vs. IV)		
HR	0.879	0.603
95% CI	(0.531–1.455)	(0.365–0.996)
p	0.617	0.048
KRAS status (WT vs. mutant)		
HR	0.597	0.645
95% CI	(0.402–0.887)	(0.458–0.908)
p	0.011	0.012

HR, hazard ratio; CI, confidence interval; ECOG PS, Eastern Cooperative Oncology Group performance status.

2.3. Distinct Efficacy of BEV/CHT in Advanced LADC Patients with Different Subtype-Specific KRAS Mutations

Next, we looked at the clinicopathological characteristics of KRAS codon 12-mutant LADC patients receiving BEV/CHT and performed a statistical analysis on their associations with amino acid-specific mutational status. We identified 35 (36.8%) G12C, 19 G12D (20%), 20 G12V (21%), three G12A (3.2%%), one G12S (1%), one G12R (1%), three G13D (3.1%), and one G13C (1%) cases. Significant associations of subtype-specific KRAS mutational status with age, smoking status, gender, ECOG PS, or tumor stage were not detected (Table S1). Importantly, patients with KRAS G12D

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mutant tumors had a significantly shorter OS than those presenting with KRAS WT or with other KRAS codon 12 or 13 mutant (G12/13x) tumors ($p = 0.0223$ and $p = 0.0144$, respectively; log-rank test; see Figure 2A). In line with the OS data, KRAS G12D mutation conferred a significant disadvantage for PFS compared with KRAS WT ($p < 0.0001$; log-rank test; see Figure 2B) or all the other codon 12 or 13 KRAS (G12/13x) mutations ($p = 0.0032$; log-rank test; see Figure 2B). Of note, the OS of G12D KRAS-mutant patients in the BEV/CHT group was comparable to that of patients in the CHT-alone subcohort (Figure S3).

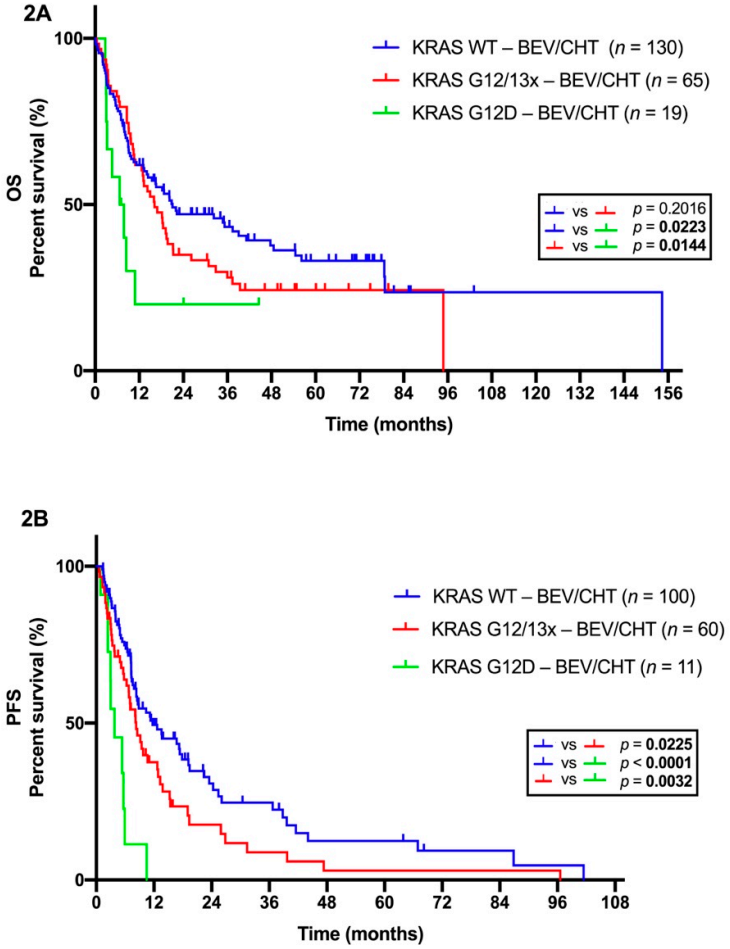

Figure 2. Kaplan–Meier plots for the OS (**A**) and PFS (**B**) in LADC patients receiving BEV/CHT according to subtype-specific codon 12 KRAS mutations. (**A**) KRAS G12D mutation was associated with significantly shorter OS in LADC patients (vs. KRAS G12x and 13x mutations or WT KRAS; median OSs were 7.2, 16.1, and 21 months, respectively, *p* values were 0.0144 and 0.0223, respectively, log-rank test). (**B**) LADC patients with tumors harboring KRAS G12D mutations had also significantly shorter median PFS than those with other codon 12 (G12x) and 13 (G13x) KRAS-mutant or with KRAS WT tumors (median PFSs were 3.7, 8.27, and 11.7 months, respectively; *p* values were 0.0032 and <0.0001, respectively; log-rank test).

3. Discussion

Although KRAS is the most frequently mutated oncogene in NSCLC, our knowledge on the effect of KRAS mutation on the response to BEV in lung cancer is very limited. Biomarkers of BEV efficacy including imaging markers and circulating levels of angiogenic cytokines have been tested in both preclinical and clinical studies. For instance, VEGF levels in immunodepleted plasma of cancer patients were found to be significantly reduced following BEV treatment [31]. However, VEGF-A, as measured using an Enzyme-linked immunosorbent assay that recognizes all VEGF-A

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isoforms, was not predictive in a comprehensive evaluation of four phase III trials of BEV in CRC, NSCLC, and renal cancer [32]. Interestingly, recent data suggest use of TP53 (tumor protein 53) status as a biomarker for the response to BEV in NSCLC [33,34]. Nevertheless, as in other solid tumor types, a reliable biomarker to identify patients with LADC who will benefit from BEV is yet to be discovered. Here we analyzed the KRAS exon 2 mutational status in a large Caucasian patient cohort (n = 501) with stage III–IV, EGFR WT LADC treated with platinum-based chemotherapy alone or in combination with BEV.

In the current LADC cohort, 33.9% of the patients had a KRAS mutation. The incidence of KRAS mutations was higher in the BEV/CHT-treated group as compared to the CHT group (38.5% vs. 29.5%, respectively, p = 0.012). With an incidence of 36.8%, G12C was the most frequent subtype in the BEV/CHT group, followed by the G12V (21.1%) and G12D (20%) subtypes. Other rare mutational subtypes (i.e., G12A, G12R, G12S, G13C, and G13D) were identified in 22.1% of the patients. These findings are in line with data previously reported by us and others in large NSCLC studies [12,35].

Next, we investigated whether the KRAS mutational status had an effect on the response to BEV. Although KRAS status had no impact on the OS of LADC patients receiving CHT alone, in the BEV/CHT group patients with a KRAS mutation had a significantly shorter OS. Multivariate analysis also confirmed the role of KRAS as a negative predictor of response to BEV. In lung cancer, so far only two studies have addressed the impact of KRAS mutation on the efficacy of BEV. The results from both of these studies are in line with our data. Chaft et al. treated 50 stage IB–IIIA NSCLC patients in the neoadjuvant setting in combination with CHT and evaluated their pathological response [36]. None of the 10 KRAS-mutant patients responded, in comparison to 11 of 31 KRAS WT cases. Although these authors administered BEV in combination with CHT, based on our current data and also on previous reports from our group and others, the efficacy of CHT is not affected by KRAS status in NSCLC [11,12]. Thus, the better response rate in the KRAS WT group of the Chaft study can be attributed to BEV and not to CHT [29]. In further support of this, Brady et al. studied 93 stage IV NSCLC patients receiving CHT alone or in combination with BEV and observed that, while CHT was as effective in KRAS WT patients as in those with KRAS-mutant tumors, BEV improved OS and PFS in patients with KRAS WT, but not those with KRAS-mutant tumors [30].

Mechanisms of resistance to antiangiogenic agents such as BEV include hypoxia-mediated mechanisms [37], the downregulation of target receptors and the activation of compensatory angiogenic pathways [38–40], proangiogenic hematopoietic or endothelial progenitor cell release from the bone marrow [41], inadequate intratumoral distribution of antiangiogenic drugs [42], and a switch from endothelial sprouting to a nonangiogenic vascularization mechanism such as vessel cooption (a frequently occurring vascularization pattern in primary and secondary lung tumors that mediates resistance to anti-angiogenic therapy) [43–46].) It is not completely clear, though, whether and how KRAS mutation can contribute to these resistance mechanisms. Notably, however, tumor spread through air spaces (STAS) [47] and "tumor islands" [48] are closely related morphological features to vessel cooption [49] and were found to be significantly associated with KRAS mutations in NSCLC [47,48]. Moreover, mutant KRAS has been shown to induce the expression of VEGF in transformed fibroblasts or epithelial cells in vitro. KRAS mutation led to the increased expression of other angiogenic growth factors such as TGF (transforming growth factor)-beta and alpha [50]. Elevated VEGF mRNA levels were detected in tumor cell lines expressing mutant KRAS [51]. Genetic disruption of the mutant KRAS allele in human colon carcinoma cells resulted in decreased VEGF secretion [51]. The transfection of human pancreatic epithelial cells with KRAS12V induced the expression of VEGF and CXC (C-X-C motif) chemokines through Erk and c-Jun signaling and enhanced endothelial tube formation in co-cultures, which could be inhibited by CXC receptor 2 or VEGF targeting [52]. Lastly, doxycycline withdrawal led to tumor regression and endothelial apoptosis in a doxycycline-inducible RAS (rat sarcoma)-driven INK4a (inhibitor of cyclin-dependent kinase 4a) deficient murine model of melanoma [22].

In CRC patients receiving BEV, results on associations between KRAS mutational status and outcome have been inconsistent, with a larger number of studies reporting no associations [23,25,26,53,54] than those demonstrating significant associations [27,28]. Interestingly, however, in a

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recent CRC study, Fiala et al. demonstrated that G12V and G12A mutation were predictors of shorter PFS and OS, while patients with tumors harboring other KRAS mutations had a similar outcome to those with KRAS WT tumors [27]. Notably, another group reported that the presence of a KRAS G12D mutation was significantly associated with poorer outcome in CRC patients receiving BEV-containing regimens [55]. As for NSCLC, Scheffler et al. recently found that patients with KRAS G12D mutation exhibit a high frequency of co-occurring mutations in the angiogenesis-associated PDGF (platelet-derived growth factor receptor)/PDGF-receptor pathway [56]. In line with this, among the three major codon 12 KRAS mutation subtypes (G12C, G12V, and G12D) G12D proved to be a predictor of poor outcome in our BEV/CHT subcohort. Patients with LADC harboring this mutation had significantly worse PFS and OS than those with tumors harboring other KRAS mutations or WT KRAS.

The biological importance of KRAS mutational subtypes has been demonstrated in a study by Figueras et al., who introduced either a codon 12 or a codon 13 KRAS mutation into NIH3T3 cells and analyzed the VEGF levels and the activity of VEGF promoter in these transfected sublines. Despite the lower VEGF expression, codon 12 mutant tumors exhibited a higher microvessel density, while tumors harboring the codon 13 mutation developed angiogenic sprouts with larger diameters [57].

In our cohort, only two patients carried a codon 13 mutation of KRAS, so we could not evaluate the BEV response in this subgroup. Nevertheless, our study suggests that specific KRAS mutation subtypes can have a major impact on tumor vascularization and, potentially, on the response to anti-angiogenic treatment.

Like all retrospective analyses, our study has limitations. First, although we excluded patients with EGFR-mutant tumors from our study, we did not analyze KRAS-WT patients for additional oncogenic driver mutations. Second, we did not study KRAS-mutant patients for co-occurring mutations in additional tumor-associated pathways [56]. Third, because this large retrospective cohort did not include reliable RECIST (response evaluation criteria in solid tumors) data [58] for all patients, we did not investigate the correlation between KRAS mutational status and tumor response according to RECIST criteria. Finally, because there is a massive body of literature on the predictive and prognostic role of KRAS mutations in CHT-treated LADC patients [9–13,56,58–60] and the main aim of the current study was to investigate the relationship between KRAS status and the efficacy of BEV, only the OS but not the PFS data were used in our analyses in the CHT-alone subcohort.

4. Materials and Methods

4.1. Study Population

In this single-center, retrospective study, 501 consecutive patients with advanced lung adenocarcinoma (LADC) were included and underwent first-line platinum-based (cisplatin or carboplatin) doublet chemotherapy (CHT) with or without BEV at the National Korányi Institute of Pulmonology, Budapest between 2007 and 2016 (Table 1, Figure S1). The addition of BEV to CHT was individually decided by the treating physician in line with the proof-of-concept BEV clinical trials [16,18] and with the EMA summary of BEV characteristics. According to our inclusion criteria, cytologically or histologically verified unresectable stage IIIB or IV LADC patients were included. Patients with uncontrollable hypertension, hypertensive encephalopathy, arterial or grade 4 venous thromboembolism, nephrotic syndrome (grade 4 proteinuria), pulmonary bleeding, gastrointestinal perforation, need for major surgery, or hypersensitivity to BEV were considered not eligible for BEV therapy (Figure S1).

In the BEV/CHT group (n = 247), platinum was given together with paclitaxel (84.7%) or gemcitabine (15.3%). In order to rule out the potential confounding effect of different treatment regimens, patients receiving other nonplatinum partners, such as pemetrexed or docetaxel, were excluded from the CHT group (n = 254). Additionally, all patients receiving tyrosine–kinase inhibitors in any further line of treatment were excluded. According to the therapy guidelines of the host institute, only ECOG PS 0 or 1 LADC patients were included in this study, since higher PS

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contradicted the use of cytotoxic chemotherapy. Smoking status, TNM stage, and molecular tumor characteristics (EGFR and KRAS mutational status) were defined at the time of diagnosis. For the calculation of PFS and OS, the date of the first CHT was used. Patients with known EGFR mutations were excluded. Clinical follow-up closed on 1 August 2017. The median follow-up was 21 months in the BEV/CHT group, and 10 months in the CHT group. The study and all treatments were conducted in accordance with the current National Comprehensive Cancer Network guidelines, based on the ethical standards prescribed by the Helsinki Declaration of the World Medical Association and with the approval of the national level ethics committee that included a waiver for this retrospective study (52614-4/2013/EKU). Due to the retrospective study design and the anonymity of the patient records, informed consent was not recommended.

4.2. Molecular Diagnosis

All mutational analyses were performed at the time of diagnosis at the 2nd Department of Pathology of the Semmelweis University, as previously described [12,61,62]. DNA isolation was performed from formalin-fixed, paraffin-embedded (FFPE) tissue blocks, cytological specimens of primary tumors, or lymphatic or organ metastases (including pleural effusion).

KRAS exon 2 mutations were identified by microcapillary-based restriction fragment length analysis, as previously described [12,61]. Briefly, a tumor-rich microscopic area on H&E staining had been determined by pathologists prior to macro dissection from FFPE tissue or cytological smears. DNA was extracted using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA) according to the instructions of the manufacturer. The microfluid-based restriction fragment detection system was characterized by 5% mutant tumor cell content sensitivity. The density ratio of the mutated band to the WT one was calculated and samples containing >5% of the non-WT band were considered mutation-positive due to the sensitivity threshold. The base-pair substitutions in the mutant samples were verified and determined by sequencing on the ABI 3130 Genetic Analyzer System (Life Technologies, Carlsbad, CA, USA) with the BigDye® Terminator v1.1 Kit.

4.3. Statistical Methods

Categorical parameters, such as gender (male vs. female), smoking status (never- vs. ever-smoker), ECOG PS (0 vs. 1), and KRAS mutation status (KRAS-mutant vs. WT) were statistically analyzed by Chi-square test or Fisher's exact test. Age, as a continuous variable, was analyzed in the different KRAS mutational groups by Mann–Whitney U test as the data were not normally distributed in each group (as per the Shapiro–Wilk normality test). Kaplan–Meier survival curves and two-sided log-rank tests were used for univariate survival analyses. The median follow-up time was calculated by using the reverse Kaplan–Meier approach. The Cox proportional hazards model was used for uni- and multivariate survival analyses to detect the impact of both continuous and categorical factors and to calculate the hazard ratios (HR) and corresponding 95% confidence intervals (CI). For multivariate survival analyses, the Cox regression model was adjusted for age (as a continuous variable), gender (female versus male), smoking (never- vs. ever-smoker), ECOG PS (0 versus 1) and stage (IIIB versus IV). In order to establish potential predictive factors, interaction terms were calculated between KRAS status and other variables (age, sex, smoking status, ECOG PS, and stage) in the adjusted multivariate Cox regression model. p values are always two-sided and considered statistically significant below 0.05. Metric data are always shown as median or mean and corresponding range or, in the case of OS and PFS, as median and corresponding 95% CI. All statistical analyses were performed using the PASW Statistics 18.0 package (Predictive Analytics Software, SPSS Inc., Chicago, IL, USA). Graphs were created with GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

5. Conclusions

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In conclusion, when combined with standard first-line chemotherapy, BEV has led to increased OS and thus has been approved in patients with advanced or recurrent nonsquamous NSCLC without targetable molecular abnormalities [16,17,19,20,63,64]. However, although serious efforts have been made to identify patients responsive to BEV, there is as yet no validated predictive biomarker in this field. Here, we present novel evidence for use of BEV in stage III–IV LADC patients with KRAS-mutant tumors- and especially with KRAS G12D-mutant tumors- demonstrating inferior activity of this drug compared to that in LADC patients with non-KRAS-mutant tumors. Our data may not only help to improve the efficacy of BEV, but, through better patient selection, could also help to decrease the unnecessary use of this expensive agent in subgroups of KRAS-mutant human LADC patients.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1. Figure S1. Consort diagram for advanced LADC cases. Consort diagram to demonstrate the selection of stage IIIB/IV LADC cases for BEV/CHT or CHT alone in this study. Where patients were excluded, the reasons for exclusion are indicated. Figure S2. Comparison of survival outcomes in patients with advanced LADC according to treatment regimen. Advanced LADC patients receiving BEV/CHT showed significantly higher median OS compared to those treated with CHT only (median OSs were 24 vs. 10 months, respectively, $p < 0.0001$, log-rank test). Figure S3. Kaplan–Meier curves for the OS of LADC patients treated with CHTalone and LADC patients with KRAS G12D mutations in the BEV/CHT subcohort. Patients with tumors harboring KRAS G12D mutations and treated with BEV/CHT had comparable OS to that of patients with KRAS-WT or KRAS-mutant tumors in the CHT-alone subcohort. Table S1. Correlation of clinicopathologic features, outcome variables and KRAS codon 12 subtypes in patients with advanced LADC treated with BEV ($n = 95$).

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