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THE CLINICAL RELEVANCE OF BIOMARKERS FOR AGGRESSION ASSESSMENT AND PROGNOSIS IN NON-SMALL CELL LUNG CANCER

Doctoral thesis

Plzeň, 2011 MUDr. Markéta Pražáková

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KLINICKÝ VÝZNAM BIOMARKERŮ PRO POSOUZENÍ AGRESIVITY A PROGNOZU NEMALOBUNĚČNÉHO KARCINOMU PLIC

Disertační práce

Plzeň, 2011 MUDr. Markéta Pražáková

Děkuji mému školiteli prof. MUDr. O. Topolčanovi CSc. za jeho užitečné rady a oborné vedení a všem kolegům za jejich ochotnou spolupráci a pomoc s laboratorními i klinickými problémy. Zvláštní poděkování patří mé rodině a přátelům za jejich trpělivou podporu.

I would like to thank my supervisor prof. MUDr. O. Topolčan CSc. For his valuable advice and skilled leadership and all colleagues for thein willing help and cooperation in laboratory and clinical tasks. Special thanks belong to my family and friends for their patient support.

MOTTO:

I AM AMONG THOSE WHO THINK THAT SCIENCE HAS GREAT BEAUTY. A SCIENTIST IN HIS LABORATORY IS NOT ONLY A TECHNICIAN: HE IS ALSO A CHILD PLACED BEFORE NATURAL PHENOMENA WHICH IMPRESS HIM LIKE A FAIRY TALE.

MARIE CURIE

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1. INTRODUCTION

Lung cancer is the most frequently diagnosed cancer in the world and the most common cause of cancer mortality worldwide. Despite improvements in diagnostic and therapeutic procedures, high case fatality persists. Early diagnosis of lung cancer is crucial for improving clinical outcome and prognosis, but the early stages of lung cancer often produce no symptoms. For improving lung cancer management and survival, there is a great need to develop screening and early diagnosis strategies that are sensitive, specific, and non-invasive; tools predicting prognosis to optimize treatment and avoid overtreatment and tools identifying potential therapeutic targets. Serum biomarkers offer a simple, non-invasive, cheap, and reliable tool for more efficient lung cancer management. Although several well-known tumor markers have shown considerable diagnostic and prognostic potential or have proved to be useful for the monitoring of systemic treatment and post-operative follow up care, they are not ideal for the detection of lung cancer due to their low specificity and/or sensitivity ranging between 20 and 80%. The identification of novel biomarkers with high specificity and sensitivity, or which can increase specificity and sensitivity of these traditional markers, is essential for more effective lung cancer diagnosis and remains an important goal of clinical research on tumor markers. The serum of lung cancer patients will most likely reveal many more proteins that may be used as biomarkers. The proteins that are secreted from malignant cells into the extracellular microenvironment and whose serum levels correlate with cancer cell proliferation and/or protein overexpression and increase in the relatively early stages of cancer development can be considered as potential serum biomarkers of cancer and new molecular targets for therapeutic intervention. Biomarkers of special interest are those that play critical role in tumor progression process, including pro-angiogenic cytokines such as VEGF, metaloproteinases and their inhibitors, growth factors such as IGF, and others. The value of many candidate biomarkers in the management of NSCLC patients remain unconfirmed and controversial, therefore some of these novel biomarkers, with the most promising profiles, are subject of this work.

This thesis has arisen thanks to long-time collaboration of our Immunoanalytic laboratory and Surgery, Oncology, and Lung departments of University Hospital in Pilsen. The study was sponsored by research project VZ MSM 0021620819 and grant project IGA MZCR 9343- 3.

The thesis is divided into two major sections – theoretical and experimental part.

The theoretical part describes the compendium of epidemiology, etiology and pathogenesis, the new classification, clinical presentation, screening and prognosis of lung cancer. Last chapters of the theoretical part describe tumor markers, their historical background, current clinical applications, and detail characteristics of the most studied serum tumor markers of lung cancer.

The experimental part includes characteristics of a group of patients with NSCLC and a control group of patients with benign lung diseases in a chapter **"Patients and Methods"**. There are described biomarkers measurement techniques and statistical analysis methods.

Results of our study are shown in a chapter **"Results"** documented in tables and figures.

In chapter **"Disscusion"** our results are commented and compared with results of other authors presented in books and articles and we discuss the clinical utility of biomarkers in management of patients with NSCLC.

2. THE THEORETICAL PART

2.1 LUNG CANCER EPIDEMIOLOGY

2.1.1 Introduction

At the beginning of the 20th century lung cancer was a very rare disease, but rates have increased so dramatically that lung cancer can be considered one of the major epidemics of the 20th century [1]. This is largely due to the carcinogenic effects of cigarette smoke. Currently, lung cancer is the most frequently diagnosed cancer in the world and the most common cause of cancer mortality worldwide. Overall, 13% of all new cases of cancer diagnosed every year are lung cancer diagnoses [2].

As we move into the 21st century, the burden will shift from the developed to the less-developed countries. Other epidemiological changes of lung cancer include the narrowing of a difference between men and women affected by the disease, predominance of an adenocarcinoma histological subtypes as well as more never-smokers afflicted with the disease [3-5].

2.1.2 Lung cancer incidence and mortality

Lung cancer has been the most common cancer diagnosed every year since 1985. From this time the estimated numbers of lung cancer cases worldwide has increased by 51% (+44% in men and +76% in women) [6].

Because the prognosis for lung cancer is still very poor, mortality rates closely follow incidence rates. An estimated 1,35 million people worldwide were diagnosed with lung cancer in 2002 (about 71 % were males) and 1,18 million died of lung cancer (72% were males). Lung cancer deaths caused almost 18% of total cancer mortality and around 2% of all mortality worldwide during 2002 (9^{th} cause of death). Lung cancer age-standardized incidence rates were around twice as high in more developed countries compared with less developed countries [6-8] (Table 1,2).

There are some key differences in the epidemiology of lung cancer between more developed and less developed countries. In more developed countries, incidence and mortality rates are generally declining among males and are starting to plateau for females, reflecting previous trends in smoking prevalence. In contrast, there are some populations in less developed countries where increasing lung cancer rates are predicted to continue, due to endemic use of tobacco. A higher proportion of lung cancer cases are attributable to non smoking causes within less developed countries, particularly among women [8].

Table 1.

Age-standardized lung cancer incidence and mortality rates (per 100 000 population) in males for selected countries.

	Males			
	Incidence		Mortality	
	Cases	ASR	Deaths	ASR
World (2002)	965 241	39,5	848 132	34,9
More developed countries (2002)	481 950	61,0	423 507	53,2
Less developed countries (2002)	481 029	28,7	422 681	25,5
United States (2002)	118 873	69,2	94 640	54,8

Rates age-standardized to the WHO World Standard Population.
Data source: GLOBOCAN 2002, International Agency for Research on Cancer (IARC).

	Males			
	Incidence		Mortality	
	Cases	ASR	Deaths	ASR
European Union (27)	***206 161***	***72,1***	***181 854***	***62,6***
Hungary	6 231	119,3	5 780	110,0
Poland	18 376	103,0	16 346	92,1
Belgium	5 890	93,0	6 082	93,8
Lithuania	1 437	91,9	1 168	74,0
Greece	6 316	88,7	5 027	69,0
Italy	34 163	84,7	26 095	63,0
Latvia	909	82,6	874	78,7
Romania	8 792	81,0	7 282	66,9
Estonia	518	80,3	570	88,2
Czech Republic	**4 338**	**78,9**	**4 250**	**77,3**
Slovenia	824	75,7	761	69,0
France	25 405	75,5	20 711	60,0
Slovakia	1 658	71,7	1 484	64,8
Luxembourg	168	69,8	153	62,6
Spain	17 117	68,3	17 345	67,2
Bulgaria	2 966	67,3	2 639	58,3
Cyprus	246	66,1	226	60,3
Denmark	2 088	65,0	1 875	57,9
The Netherlands	5 764	63,4	6 101	67,0
Germany	32 409	61,2	28 887	53,8
Ireland	1 140	60,2	927	48,9
United Kingdom	21 036	57,1	18 945	50,7
Austria	2 483	54,0	2 390	51,3
Norway	1 394	53,8	1 297	48,4
Switzerland	2 269	52,7	1 888	43,4
Finland	1 433	45,8	1 379	43,5
Portugal	2 675	44,5	2 660	43,3
Malta	96	43,9	110	50,6
Iceland	57	40,6	57	40,1
Sweden	1 683	28,6	1 787	29,7

Estimated incidence and mortality from Lung cancer in males, 2006
Age Standardised Rate (European) per 100 000
European Cancer Observatory (ECO)
http ://eu-cancer.iarc.fr//

Table 2.

Age-standardized lung cancer incidence and mortality rates (per 100 000 population) in females for selected countries.

	Females			
	Incidence		Mortality	
	Cases	ASR	Deaths	ASR
World (2002)	386 891	13,5	330 786	11,5
More developed countries (2002)	194 731	18,9	161 472	15,2
Less developed countries (2002)	191 192	10,4	168 481	9,2
United States (2002)	86 024	40,1	65 792	30,0

Rates age-standardized to the WHO World Standard Population.
Data source: GLOBOCAN 2002, International Agency for Research on Cancer (IARC).

	Females			
	Incidence		Mortality	
	Cases	ASR	Deaths	ASR
European Union (27)	***73 972***	***21,3***	***66 302***	***18,0***
Denmark	1 784	48,7	1 628	41,6
Iceland	68	45,6	53	35,1
Hungary	2 978	42,4	2 530	34,6
United Kingdom	15 631	34,6	14 153	29,7
Ireland	766	34,1	599	26,2
Norway	976	33,7	794	26,1
The Netherlands	3 203	32,5	3 160	30,6
Poland	6 793	28,7	5 375	21,8
Switzerland	1 291	26,2	924	18,1
Sweden	1 499	23,8	1 603	23,5
Belgium	1 637	22,9	1 594	20,7
Czech Republic	**1 670**	**22,9**	**1 399**	**19,1**
Slovenia	318	22,9	293	20,2
Austria	1 252	22,3	1 091	18,2
Germany	12 527	20,8	11 630	18,0
Luxembourg	46	16,3	50	17,0
Italy	7 662	15,6	7 343	14,0
Romania	2 153	15,4	1 711	12,1
France	6 004	15,0	5 842	13,7
Finland	617	14,7	548	13,0
Spain	3 786	13,8	2 605	8,9
Estonia	144	13,2	128	11,2
Greece	1 073	12,7	1 000	11,4
Portugal	886	11,7	636	7,9
Slovakia	379	11,6	387	11,6
Bulgaria	649	11,5	531	9,2
Latvia	188	10,2	179	9,1
Lithuania	267	10,0	227	8,3
Cyprus	42	9,5	41	9,4
Malta	18	6,5	19	7,4

Estimated incidence and mortality from Lung cancer in females, 2006
Age Standardised Rate (European) per 100 000
European Cancer Observatory (ECO)
http ://eu-cancer.iarc.fr//

2.1.2.1 Incidence by sex and age

Lung cancer worldwide has a higher incidence among males than any other type of cancer, followed by prostate cancer (more common in developed countries) and stomach cancer (particularly in developing countries). Among females, lung cancer is the fourth most diagnosed cancer, behind breast cancer, cervical cancer (mostly in developing countries), and colorectal cancer (more in developed countries) [6;9] (Figure 1).

Lung cancer is rarely diagnosed in people younger than 44 years, but incidence rises steeply thereafter peaking in people aged 75-84 years. Most cases (80%) occur in people over the age of 55 for both sexes [7].

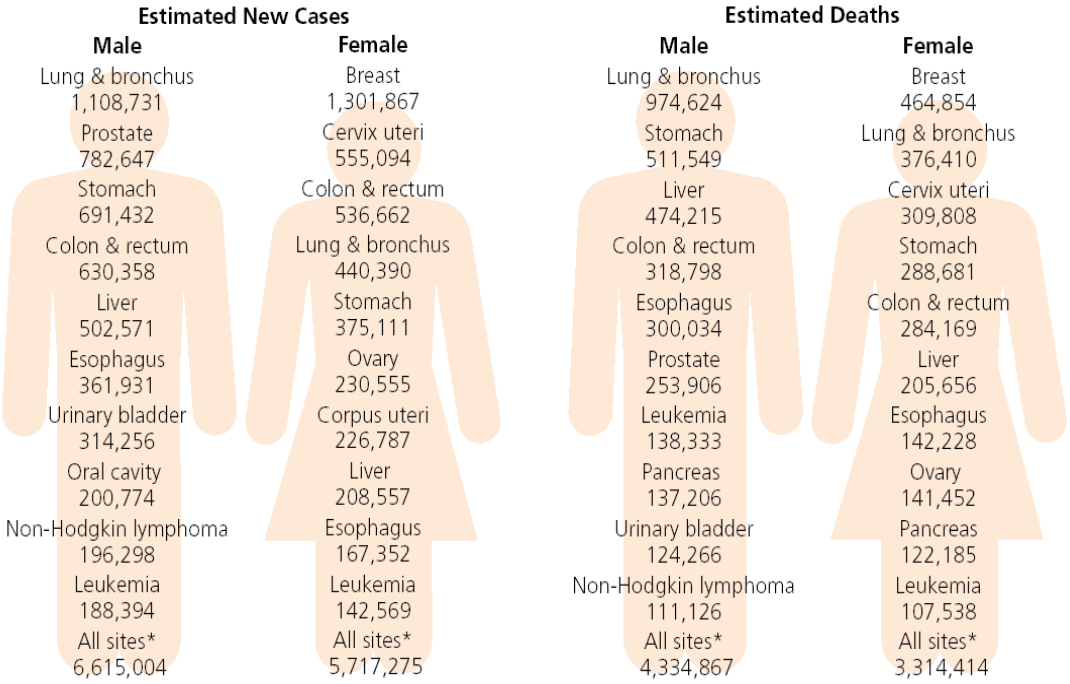


Figure 1. Expected numbers of new cancer cases and deaths worldwide in 2007 for men, women [10].

*Excludes nonmelanoma skin cancer.

2.1.2.2 Geographical differences

Geographic patterns of lung cancer are very much a reflection of past exposure to tobacco smoking and vary hugely between different regions of the world [6]

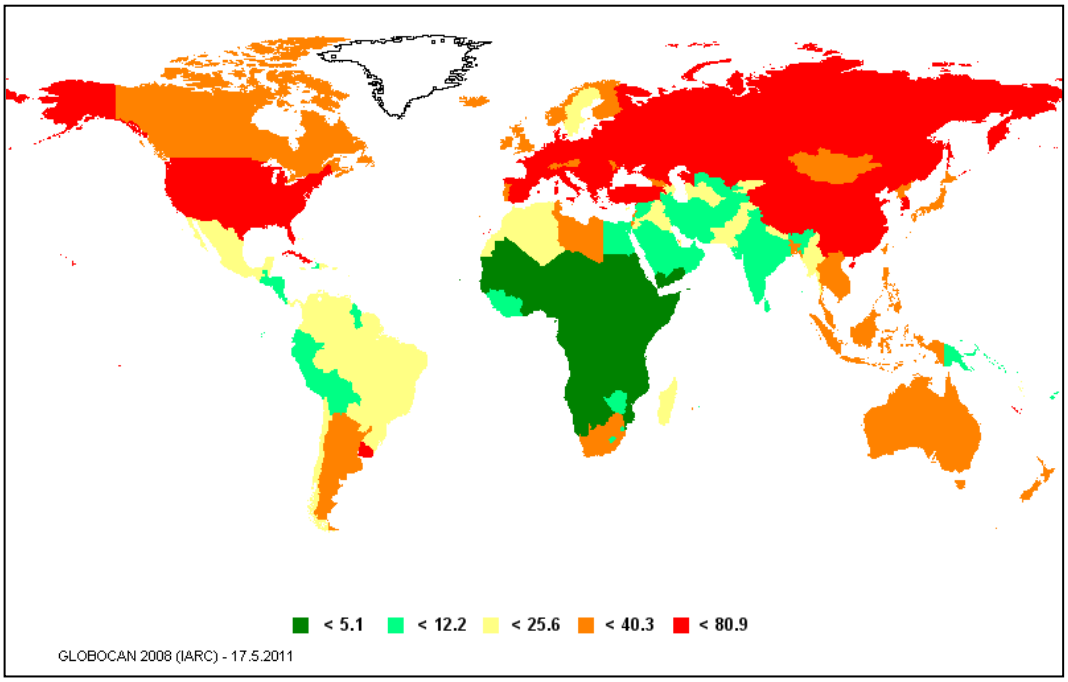


Figure 2. The international variation in age-standardized lung cancer incidence rates for males in 2008.

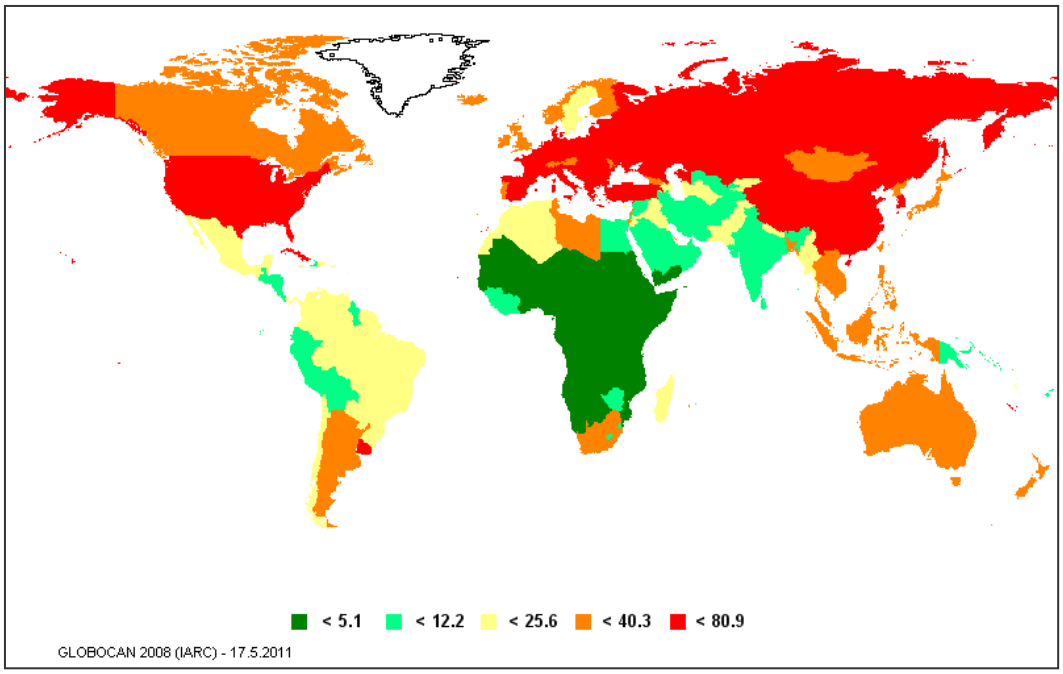


Figure 3. The international variation in age-standardized lung cancer incidence rates for females in 2008.

The highest incidence rates of lung cancer in men are found in Europe (especially Central and Eastern Europe) and Northern America (Canada, the USA) and Russia. Within Europe countries with the highest male rates are Hungary and Poland and the lowest in Sweden, Iceland and Malta (Figure 2). In women, the geographic pattern of lung cancer is somewhat different (Figure 3). For women the highest incidence rates are found in Northern America, North-Western Europe (U.K., Denmark, Iceland) and China. Women in the USA have the world's highest lung cancer incidence rates followed by Canada. Lung cancer rates in Chinese women are higher than the rates among women in many European countries, including Germany and France, despite their lower prevalence of smoking. This is thought to reflect indoor air pollution from unventilated coal-fueled stoves and from cooking fumes.

The lowest lung cancer incidence rates in both men and women are found in African and South Central Asian countries [7;9;10] (Figure 4).

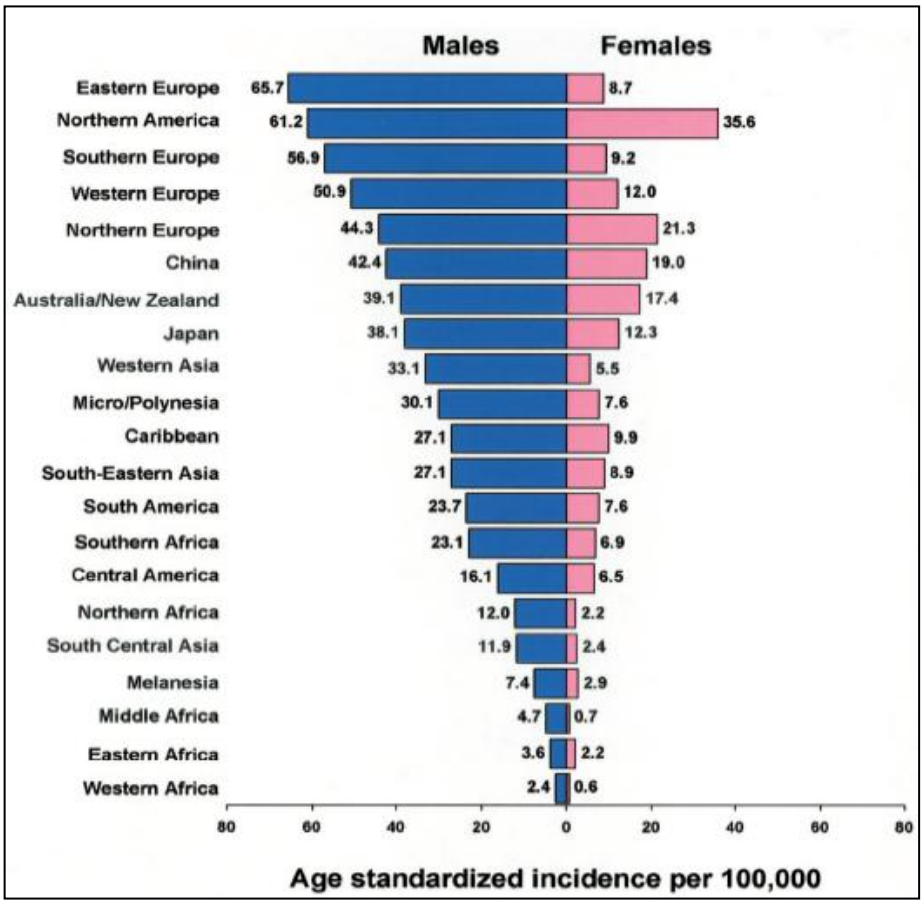


Figure 4.Age-standardized lung cancer incidence rates for males and for females [6].

2.1.2.3 Incidence by histological types

Lung cancer histologies include squamous cell carcinoma, adenocarcinoma, large cell carcinoma, small cell carcinoma and a variety of other less frequent types. The predominant form of lung cancer has been squamous cell carcinoma among males and adenocarcinoma among females, although adenocarcinoma surpassed squamous cell carcinoma in frequency among males in several countries in recent years (North America, China, Japan). In Europe the most common type of lung cancer is still squamous cell carcinoma despite of an increase in the incidence of adenocarcinoma [9;11].

2.1.2.4 Lung cancer mortality

Lung cancer is a leading cause of cancer-related deaths worldwide among men, followed by stomach cancer and liver cancer. Among females, lung cancer is in the second position, behind breast cancer [6;10] (Figure 1). However, in some countries lung cancer has overtaken breast cancer as the leading cause of cancer death among females, in the USA since 1987 and more recently in some European countries including the UK, Sweden and Denmark [8]. Similarly to incidence, the estimated age-standardized mortality rates (MR) for lung cancer during 2002 in more developed countries were about twice that of less developed countries [7] (Table 1,2).

2.1.2.5 Lung cancer incidence and mortality trends

Lung cancer incidence and mortality trends closely reflect patterns in smoking prevalence with a latency period of 20 to 30 years, due to the characteristically long latency period between a time when a person starts to smoke and a time when they are diagnosed with or die of lung cancer [3].

Lung cancer incidence and mortality rates peaked among males in many developed countries (North America, North-Western Europe, Australia) during the 1980s and have since been declining, but they continue to rise in Southern

and Eastern Europe (Spain), China and Japan. Among females lung cancer rates continue to increase or have recently begun to plato in response to the 20 years later peak in smoking prevalence than men. The decrease in lung cancer rates among men is due to reduction in tobacco use during the past 50 years [11;12].

In less developed countries, where smoking is still increasing, incidence and mortality due to lung cancer will increase dramatically in the next decades [3;4;13]. It is supposed that by the year 2025, 85% of the world's smokers will live in less developed countries [8].

The link between lung cancer trends and smoking behavior is demonstrated by changes in the distribution of the histologic subtypes of lung cancer over time. Among males, rates of squamous and small cell carcinomas have decreased, in contrast to stable or increasing rates of adenocarcinoma. Among females, rates of all 3 types have been rising and most rapidly for adenocarcinoma. The increasing incidence of adenocarcinoma has been linked to filtered/low-tar cigarettes that enhance delivery of smoke to peripheral regions of the lungs where adenocarcinomas tend to be form. [8;11].

2.1.3 Epidemiology situation in the Czech Republic (2006)

In 2006 there was a total of 6 188 cases of lung cancer. This type of tumor occurs prevalently in men (90,3 cases per 100 000 men) in comparison with the incidence in women (31,6 per 100 000 women). While the age-standardised incidence as well as mortality in men decreases in long terms, in women both these standardised indicators steadily slowly increase (Figure 5). The levels of incidence and mortality for lung carcinoma is still more than 3,5 times higher in men than in women. The convergent trend of the incidence and mortality rates in men and women will probably continue in the following years and the differences between men and women will probably diminish.

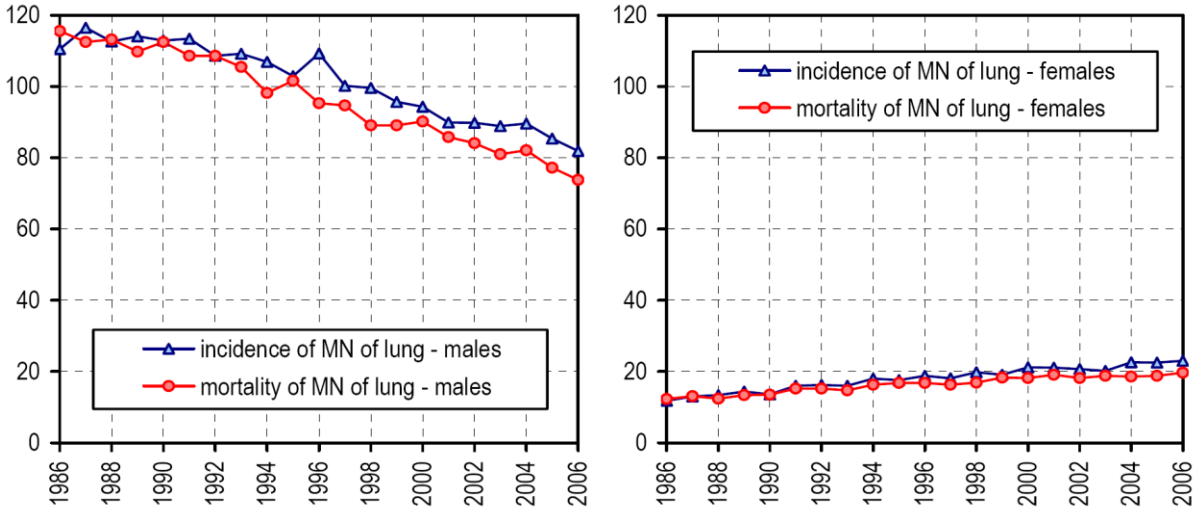


Figure 5. Evolution of age-standardised incidence and mortality for lung cancer in males and females in the Czech Republic 2006 [14].

In Czech men, lung cancer is now the third most common type of cancer after prostate cancer and colorectal cancer, responsible for 16 % of all new male cancer cases. For women, it is the fourth most common type of cancer after breast cancer, colorectal cancer and cancer of the uterus, accounting for 6% of all new female cases.

Lung cancer continued to be the most common cause of cancer death in men with 4065 deaths estimated in 2006 (26.5% of all cancer deaths). Although less common than in men, it is the second cause of death from cancer in women after breast cancer (1451, 11,6 % of total deaths) [14].

2.2 ETIOLOGY AND PATHOGENESIS OF LUNG CANCER

Carcinomas of the lung arise by a stepwise accumulation of genetic abnormalities that transform respiratory epithelium to neoplastic tissue. Interaction of environmental factors with the genome of the respiratory epithelium results in carcinogenesis in genetic susceptible patients. Unlike many other cancers the major environmental insults that inflict genetic damage are known. The well known lung carcinogen is tobacco smoke [2;15].

2.2.1 Tobacco smoking

The smoke inhaled by smokers of cigarettes and other tobacco products contains numerous carcinogens, as well as agents that cause inflammation. A lot of studies have indicated that smoking tobacco is the main cause of lung cancer, with a latency time between the start of smoking and lung cancer of 15 – 50 years [13]. An increased risk of lung cancer in smokers has been demonstrated in epidemiologial studies conducted during 1950s in the United States and United Kingdom [16]. The first publication was in the British Medical Journal in 1950, which confirmed suspicions that lung cancer was associated with cigarette smoking [4].The association between smoking and lung cancer is not solely based on epidemiological studies. Lung tumours of smokers frequently contain a typical molecular fingerprint in the form of G:C –T:A mutations in the TP53 gene which are probably caused by benzopyrene, one of the many carcinogens in tobacco smoke [16].

The geographic variation and time trends in lung cancer incidence and mortality for both sexes are strongly related to smoking behavior (see chapter 2.2.5). Worldwide, 85% of lung cancer in men and 47% of lung cancer in women is estimated as being the consequence of tobacco smoking [16]. Over the past several decades, because of the high increase in tobacco use by women, there has been a corresponding dramatic increase in lung cancer among women. Some studies suggests that women are more susceptible to tobacco-induced

carcinogenesis than men and may show higher risk than men for lung cancer development from smoking [17].

Of the one billion smokers in the world, fewer than 20% will develop lung cancer.

Relevant factors modifing lung cancer risk include [16;18;19]:

- The number of cigarettes and duration of smoking habit ("pack years" = number of cigarette packs smoked per day × number of years as smoker, 1 pack has 20 cigaretes). Duration of smoking is the strongest determinant of risk.
- The age of iniciation of smoking. Early age of starting smoking is an important lung cancer risk later in life.
- The way of smoking (inhalation). Deeply inhalation of cigarette smoke is an important risk.
- The effect of stopping smoking. The risk sharply decreases in ex-smokers after approximately 5 years since stopping. The risk after 20 or more years approaches that of never-smokers.
- Contact with cocarcinogens (industrial carcinogens, asbestos, etc.)

Tobacco smoking increases the risk of all histological lung cancer types, but appears to be strongest for squamous cell carcinoma, followed by small cell and adenocarcinoma [13].

2.2.1.1 Lung cancer in never-smokers

Approximately 10% of lung cancers occur in individuals with no prior history of tobacco smoking. In non-smokers , exposure to secondhand smoke or to other lung carcinogens, such as radon, asbestos, heavy metals, air pollution, and inherited genetic susceptibility, may be contributory [20]. Recent studies suggest that high-dose vitamin E supplementation [21], indoor air pollution from solid fuel use, and cannabis smoking are similarly associated with increased risk for lung cancer [20]. Each cannabis cigarette is equivalent to 20 tobacco cigarettes in risk of lung cancer [22].

Women are almost three times more likely than men to have non-smoking-associated lung cancer [23].

Lung cancers unrelated and related to smoking have strikingly different molecular characteristics. Among the frequently detected molecular alterations, epidermal growth factor receptor (EGFR) mutations are more common in nonsmokers, whereas K-ras mutations, p53 transversion mutations, and p16 promoter hypermethylation are more frequent in tumors of smokers [24]. The distinct biology of lung cancer in never smokers is apparent in differential (better) responses to epidermal growth factor receptor- tyrosine kinase (EGFR-TK) inhibitors (Gefitinib, Erlotinib), and an increased prevalence of adenocarcinoma histology in never smokers [25].

These data suggest that tumors of never-smokers differ from tumors of smokers with respect to etiology, biology, and treatment response. It is not unimaginable that in the near future, never-smokers with lung cancer may be viewed and treated differently from smokers [26;27].

Others lung cancer risk factors include:

2.2.2 Secondhand smoke exposure

The causal association that has been established between secondhand tobacco smoking and lung cancer can explain 1.6% of lung cancers. There is a 20% to 30% increased risk for lung cancer associated with living with a smoker [28]. A recent European study reported that frequent exposure to environmental tobacco smoke during childhood (for daily exposure for many hours) was associated with lung cancer in adulthood [29].

2.2.3 Occupational and environmental exposure

A variety of occupational and environmental exposures to carcinogens have been implicated as potencial risk factors for the development of lung cancer. Cigarette smoking potentiates the effects of many these carcinogens.These include exposure to asbestos and silica fibers, radon and its decay products, heavy metals such as nickel, cobalt, cadmium, chromium, organic compounds such as dichloromethyl ether and polycyclic aromatic hydrocarbons (PAHs), arsenic and beryllium compounds, ionizing radiation, diesel fumes and air pollution [30] :

- Asbestos, is a well known carcinogen that increases the risk of lung cancer in people exposed to airborne fibers, especially in individuals who smoke. Asbestos workers (in textile mills, insulation) have a five times greater risk of developing cancer, and those who smoke have a 50 to 90 times greater risk. It is estimated that about 3-4% of lung cancers are caused by asbestos. Lung cancer typically develop 30 to 35 years after asbestos exposure [30;31].
- Radon gas, is a ubiquitous radioactive gas that results from the radioactive decay of uranium and has been linked to increased lung cancer in miners exposed to relatively high concentrations. The alpha-particles emitted by decay products of radon induce DNA damage in respiratory epithelial cells and can mediate inactivation of the p16 tumor suppressor gene via methylation mechanismus [30]. Low-level indoor radon exposure (e.g., in homes in areas of high radon level in soil) has been also associated with increased lung cancer risk [32;33].
- Ionizing radiation induces DNA damage, and exposure to high-energy ionizing radiation such as plutonium, uranium, radon as well as low-energy ionizing radiation such as X-rays and gamma rays (radiation treatments) increases lung cancer risk [30].
- Air pollution. Available data suggest that 1-2% of lung cancer are directly attributable to pollutans in environmental air, such as metals from smelting and refining industries, PAHs and particulate carcinogens from combustion of fossil fuels , as well as diesel exhaust [30].
- Indoor air pollution have been associated with increased risk of lung cancer, particularly in developing countries. In addition to radon exposure, the use of coal for cooking and heating has been linked to lung cancer in several studies [34;35]. Exposure to cooking oil vapors during high-temperature cooking might have played a role in high lung cancer rates among women in China and Hong Kong [13].

2.2.4 Previous chronic lung diseases

such as chronic obstructive pulmonary disease, idiopatic pulmonary fibrosis, and tuberculosis also are associated with increased lung cancer risk [36].

2.2.5 Diet

The data pertaining to the impact of vitamins and micronutriens, particulary vitamin C, E, carotenoids, retinols and folate, and lung cancer risk are inconclusive. Despite several negative reports [21], most studies suggest that dietary intake of vitamin C, E, folate, and carotenoids (specifically beta-karoten) have a protective effect regarding lung cancer [37]. In contrast, cured meat, deep-fried cooking, and chili have been associated with an increased lung cancer risk [38].

2.2.6 Genetic predisposition

Occasional familliar clustering of lung cancer has suggested a genetic predisposition, as has the variable risk even among heavy smokers. To date, the genes conferring susceptibility to this disease remain elusive [2;30].

Any inherited susceptibility to lung cancer is likely to be mediated through biological differences in the bioactivation or degradation of carcinogens or cellular response to damage (e.g., DNA repair, cell-cycle control). The only direct evidence of a genetic predisposition is provided by the increased risk of lung cancer associated with inherited cancer syndromes caused by germline mutations in Tp53, retinoblastoma (RB), and other genes inherited in an autosomal dominant or recessive manner. A threefold increase in lung cancer risk was found in patients/smokers with Li-Fraumeni syndrome compared to smokers without p53 germline mutations [39]. Other study observed a threefold increase in lung cancer risk among siblings of patients with lung cancer [40]. The present data suggest that a large proportion of lung cancers before age 50 years appears to be heritable and probably due to a high-penetrant recessive gene or genes that predispose to tobacco carcinogens [41;42].

A locus on chromosome 6q23–25 was recently reported as conferring lung cancer susceptibility among families with multiple members affected by lung cancer [43]. A germline EGFR-T790M mutation has been reported to be associated with familial NSCLC, suggesting that this mutation could predispose people to lung cancer [44].

It is considered that susceptibility to lung cancer in each individual is determined by the combination of multiple genetic polymorphisms. A number of studies, focusing on polymorphisms affecting expression and function of enzymes regulating metabolism of tobacco carcinogens, DNA repair, or inflammation, have shown that dozens of genes are associated with cancer risks [30] (Table 3). For example, cytochrome P450 1A1 (CYP1A1) plays a major role in the bioactivation of a number of tobacco procarcinogens derived from cigarette smoke. The role of CYP1A1 in lung carcinogenesis might be more important at low levels of exposure to carcinogens. Much interest has focused on a polymorphism in exon 7 of the CYP1A1 gene, the present data suggest that the CYP1A1 exon 7 polymorphism may confer an increased risk of lung cancer, particularly of SCC, and especially in never-smokers and in female smokers [45].

Table 3. Summarizes the genes implicated to date in lung cancer predisposition [13].

	Gene	Mechanism relevant to cancer
Carcinogen metabolism	*CYP1A1*	Bioactivation of tobacco procarcinogens
	CYP2D6	
	CYP2E1	
	CYP2C9	
	CYP2A6	
	CYP2C19	
	NAT1	Activation and inactivation of tobacco-derived aromatic amines
	NAT2	
	GSTM1	Detoxification of PAH carcinogens
	GSTM4	
	GSTM3	
	GSTT1	
	GSTTP1	
	SULT1A1	Bioactivation of aromatic amines
	mEH	Bioactivation of PAHs
	MPO	Activation of benzo(a)pyrene
	NQO1	Activation of nitrosamines
Methylation	*MTHFR*	Changes in DNA methylation (transcriptional activation/ silencing)
	DNMT3B	
Nucleotide excision repair	*XPA*	Repair of tobacco-related DNA adducts
	XPD	
	XPG	
	XPC	
Homologous recombination	*XRCC3*	Repair of DNA strand breaks generated by reactive oxygen species in tobacco smoke
	DNA ligase I	
	Poly(ADP)-ribose	
Base excision repair	*OGG1*	Repair of DNA damage due to reactive oxygen species in tobacco smoke
	XRCC1	
	APE/ref1	
Free-radical system	*hGPX1*	Detoxification of tobacco smoke-related free radicals
	NE	
	MNSOD	
	MMP-1	
	ADH3	
Apoptosis	*TP53*	Mediation of cellular re-sponses to genotoxic insults by tobacco carcinogens
	TP73	
	TP21	
Proto-oncogene	*HRAS-VNTR*	Control of cell growth and differentiation
	L-MYC	
Other genes	*AGT*	Repair of DNA adducts induced by the tobacco-specific nitrosamine NNK
	RAGE	Regulation of invasive process extension and cell migration in tumor cells
	DRD2	Role in smoking status and addiction

2.2.7 Molecular and genetic pathogenesis of lung cancer

Lung cancer develops from normal respiratory epithelial cells through a multistep process involving successive accumulation of genetic and epigenetic abnormalities, that transform respiratory epithelium to neoplastic tissue. Interaction of environmental factors, such as tobacco smoke, with the genome of the respiratory epithelium results in carcinogenesis in genetic susceptible patients [13;15]. Factors that are unrelated to smoking — including genetic, hormonal, and viral (e.g., human papillomavirus) factors — have been suggested [27].

Tissue injury (e.g., from tobacco smoke, reflected in the discolored smoking-related lungs) initially occurs in the form of genetic and epigenetic changes (e.g., mutations in oncogenes and tumor supressor genes, loss of heterozygosity (LOH), promoter methylation, chromosomal instability) and global transcriptome changes (e.g., inflammation and apoptosis pathways). These changes can persist long term and eventually lead to aberrant pathway activation and cellular function (e.g., dysregulated proliferation and apoptosis) to produce premalignant changes, including dysplasia and clonal patches. Additional changes can result in angiogenesis, invasion and early-stage cancer, and advanced cancer and metastasis. Many molecular changes in earliest-stage cancer also occur in advanced disease (Fig. 6)[46].

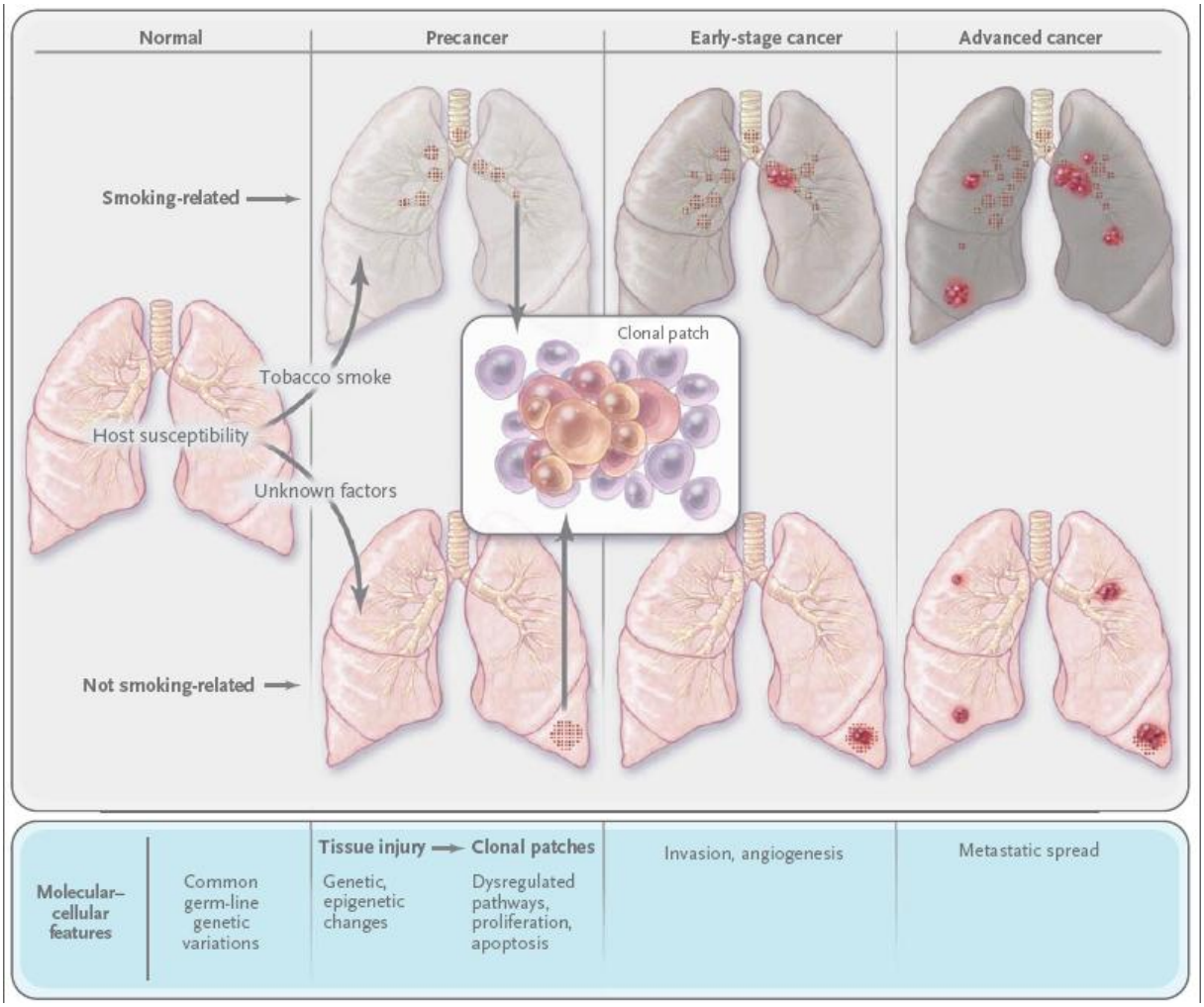


Figure 6. Molecular evolution of lung cancer [46].

The dominant oncogenes that are frequently involved in lung cancer include MYC (formerly c-MYC), K-RAS, EGFR, and ERBB2 (formerly HER2/neu). The commonly delated or inactivated tumor suppressor genes include Tp53, RB, p16INK4a, and multiple loci on chromosome 3p. There are numerous candidate tumor suppressor genes, such as FHIT, RASSF1A, and SEMA3B [47]. Although certain genetic changes are known to be early (inactivation of chromosome 3p suppessor genes) or late (activation of K-RAS), the temporal sequence is not yet well defined. Certain genetic changes such as LOH on chromosome 3p can be found in benign bronchial epithelium of patients with lung cancer, as well as in the respiratory epithelium of smokers without lung cancer, suggesting that large areas of respiratory mucosa are mutagenized after exposure of

carcinogens („field effect“). The cells that accumulate additional mutations ultimately develop into cancer [2].

The profile of molecular and genetic alterations considerably differs between SCLC and NSCLC, as well as among the subtypes of NSCLC (Table 4). Genetic alterations of both the Rb and Tp53 genes are most likely to be important and early events in the development of SCLC, whereas alterations of the EGFR signaling pathway play significant and important roles in NSCLC carcinogenesis. Inactivating mutations (mostly frequent mutations) of the Tp53 gene are found in approximately 50% of NSCLC and more than 70% of SCLC. Rb gene alterations and RB protein loss are found in virtually all SCLC, but rarely in NSCLC. Tp53 alterations are later events in adenocarcinoma, while they occur early in squamous cell carcinoma carcinogenesis. Recent studies demonstrated activating mutations of the EGFR gene play a significantly important role in adenocarcinoma carcinogenesis [48].

K-RAS mutation are found in 30-40 % of adeno, but are extremely rare in other forms of NSCLC or in SCLC [16]. Most K-RAS mutations in adenocarcinoma are smoking-related G -T transversions (substitutions of a purine for a pyrimidine). K-RAS mutations appear to be an early event (e.g., detectable in the preinvasive lesions of atypical adenomatous hyperplasia and bronchoalveolar carcinoma) that precedes smoking-related lung adenocarcinoma. The generally mark a poor prognosis [46].

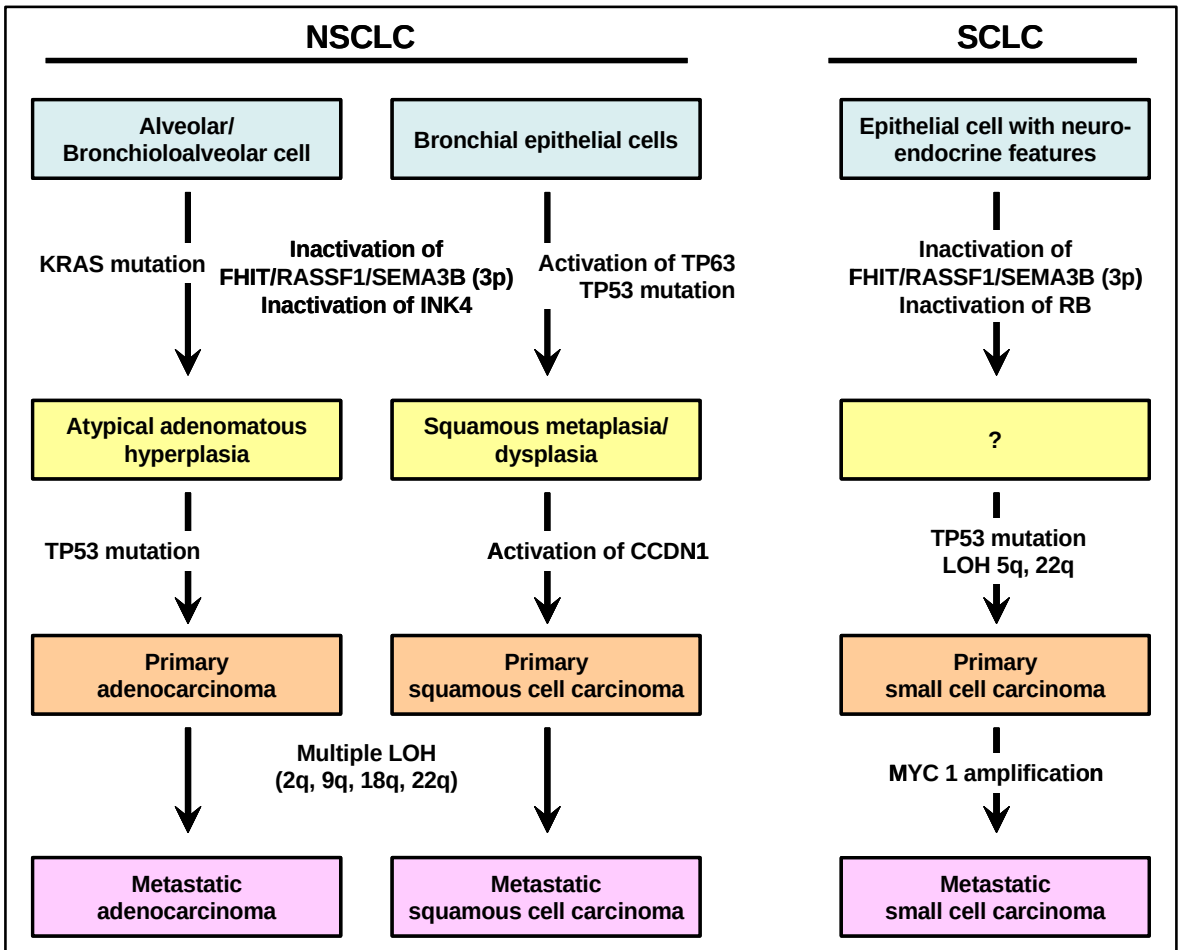
HER2 mutations occur in NSCLC, mainly adenocarcinomas, with frequency less than 5% [49].

Lung cancers unrelated and related to smoking have strikingly different molecular profiles. Smoking is associated strongly with alterations of genes Tp53, K-ras, and PIK3CA, but weakly with EGFR gene mutations, which are more common in nonsmokers [48].

Table 4. Major differences in the genetic and molecular abnormalities in SCLC and NSCLC [15].

Characteristic	SCLC (%)	NSCLC (%)
Point mutations		
K ras	NA	30-50
P53	90	60
Rb	80-100	20-40
c-met	12.5	7
Increased gene copy number		
EGFR	NA	22-32
MYC (formerly c-MYC)	18-30	8-22
Protein overexpression		
BCL2	75-95	10-35
EGFR	NA	60-70
ERBB2 (formerly Her2/neu)	0-13	20-40
GRP	50-75	NA
CCND1	NA	43
MYC (formerly c-Myc)	10-45	10
c-kit	60-90	<10
c-met	80-90	90-100
VEGF	80	75

*EGFR = epidermal growth factor receptor; GRP = gastrin-releasing peptide; NA= not applicable; VEGF = vascular endothelial growth factor.



Genetic models for the development non-small cell lung carcinomas (NSCLC) and of small cell lung carcinoma (SCLC). From J. Yokota and T. Kohno.

Molecular differences between different lung cancer types are being used for the development of more rational targeted therapy. Large-scale molecular genetic studies have led to the discovery of several potential molecular targets for therapeutic design, such as vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR). Various drugs targeted against these molecular changes have been developed and are being tested for clinical use in lung cancer therapy (Table 5 and Figure 7). The promise of these drugs is that they are specific for particular— often aberrant—molecules that are altered in cancer cells but not in normal cells; thus, they have a higher therapeutic ratio for cancer cells compared with normal cells. Some of these drugs, such as the monoclonal anti-VEGF antibody bevacizumab (Avastin), have shown a significant impact on patient survival. In addition, the recent discovery of tyrosine kinase (TK) domain mutations in the EGFR of non-small cell lung cancers (NSCLCs), and the finding that such tumors are particularly sensitive to EGFR TK inhibitor (TKI) therapy, indicate the possibility of molecular typing of tumors to aid in therapy selection [49;50].

Table 5. Genetic alterations found in lung cancer and drugs or therapeutics targeting these alterations [49].

Gene	Type of Alteration	Drug or Therapeutics Targeting Abnormalities
EGFR	Mutation and amplification	Tyrosine kinase inhibitors (gefitinib, elrotinib)
		Chimeric IgG monoclonal antibody (cetuximab)
HER2	Mutation and amplification	Pan-ERBB tyrosine kinase inhibitor (CI-1033)
		Humanized monoclonal antibody (trastuzumab)
c-KIT	Overexpressed	Tyrosine kinase inhibitor (imatinib)
SRC	Constitutively activated	Src inhibitor (dasatinib)
BRAF	Mutation	Raf kinase inhibitor (sorafenib)
RAS	Mutation	Farnesyl transferase inhibitors (tipifarnib, lonafarnib)
MEK	Constitutively activated	Inhibitors of MEK (CI-1040, PD325901)
PI3K/AKT/mTOR	Constitutively activated	PI3K inhibitor (LY294002)
		mTOR inhibitor (rapamycin) and its derivatives (CCI-779, RAD001, AP23576)
BCL2	Overexpressed	Antisense oligonucleotide (oblimersen sodium)
		Inhibitor of BCL2 (ABT-737)
p53	Mutation and deletion	*p53* adenoviral vector (Advexin)
FUS1	Loss of protein expression	*FUS1* nanoparticles (DOTAP:Chol-*FUS1*)
VEGF	Overexpressed	Humanized monoclonal antibody (bevacizumab)
		VEGFR-2 and EGFR inhibitor (ZD6474)
Telomerase	Overexpressed	Telomerase template antagonist (GRN163L)

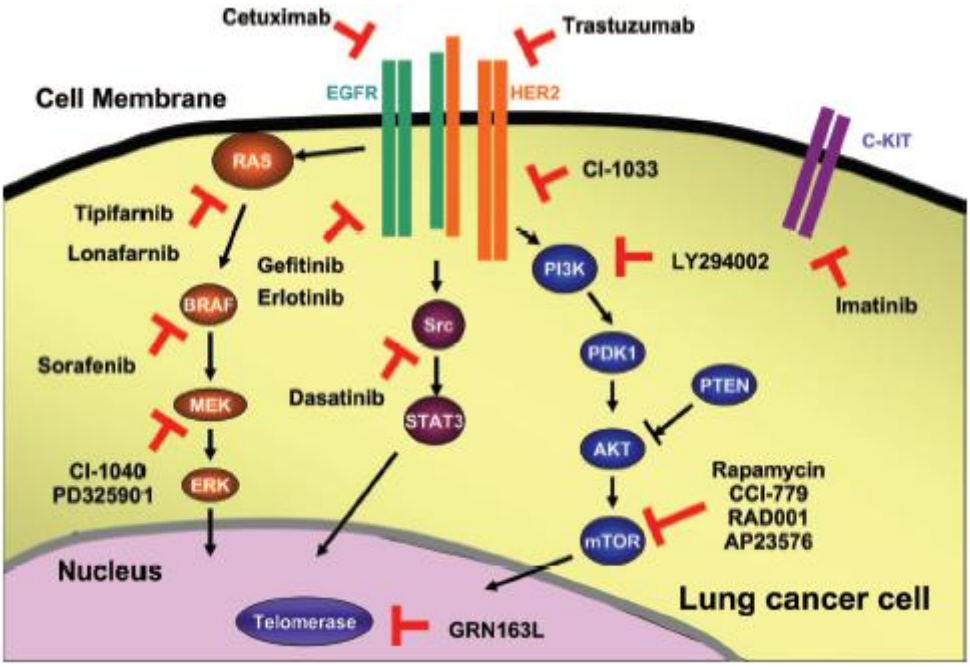


Figure 7. Major growth transduction pathways involved in lung cancer pathogenesis and drugs targeting altered molecules in the pathways [49].

2.3 CLASSIFICATION OF LUNG CANCER

2.3.1 WHO histological classification

For common clinical use the various histological types of lung cancer can be clustered into two groups: small cell carcinomas (20%) (most often metastatic, high initial response to chemotherapy) versus non-small cell carcinomas (80%) (less often metastatic, less responsive)[51]. Non-small cell carcinoma includes the major categories: squamous cell carcinoma (44% in men – 25% in women), adenocarcinoma (28% in men – 42% in women), large cell undifferentiated carcinoma (9%) [9]. Approximately 10% of all lung carcinomas have a combined histology, including two or more types.

Accurate histological classification of lung cancer is essential if patients are to receive appropriate therapy. Histological classification in current use is presented in table 6.

The strongest relationship to smoking is with squamous cell and small cell carcinoma (the central localization), adenocarcinoma is the most common type in nonsmokers, particularly women [2;16] (Figure 8).

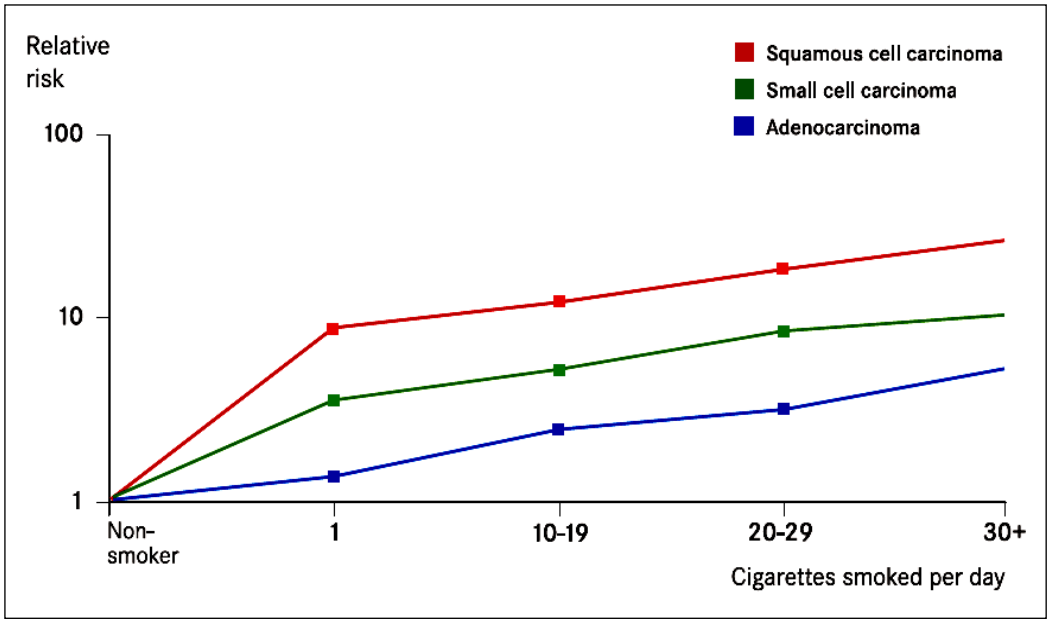


Figure 8. All lung carcinomas are strongly associated with tobacco smoking, the risk being highest for squamous cell carcinoma, followed by small cell carcinoma and adenocarcinoma [16].

Table 6. WHO histologic classification of malignant epithelial lung tumors.

Squamous cell carcinoma
Small cell carcinoma - Combined small cell carcinoma
Adenocarcinoma - Acinar - Papillary - Bronchioloalveolar carcinoma - Solid adenocarcinoma with mucin - Adenocarcinoma with mixed subtypes
Large cell carcinoma - Large cell neuroendocrine carcinoma
Adenosquamous carcinoma
Carcinomas with pleomorphic, sarcomatoid or sarcomatous elements
Carcinoid tumor - Typical carcinoid - Atypical carcinoid
Carcinomas of salivary gland type
Unclassified carcinoma

2.3.1.1 Adenocarcinoma

Epidemiology: Adenocarcinoma is the predominant histological subtype of lung carcinoma in many countries (among men in North America, China, Japan, in women almost everywhere) representing approximately 40 % of all cases [9]. In Europe the most common type of lung cancer is still squamous cell carcinoma, but the incidence of adenocarcinoma has increased significantly by 10% in the last 20 years; it is now the most prevalent form of lung cancer in younger males (<50 years old), in women of all ages, in never smokers and in former smokers [11]. The basis for this change is unclear. One interesting postulate is that changes in cigarette type (tobacco blends, filter tips, lower tar and nicotine) have caused smokers to inhale more deeply and thereby expose

more peripheral airways and cells (with a predilection to adenocarcinoma) to carcinogens [16]. Adenocarcinomas are less frequently associated with a history of smoking (however, greater than 75% are found in smokers) than are squamous or small cell carcinomas (>98%) [2]. The precursor lesion for adenocarcinoma is considered to be atypical alveolar hyperplasia (AAH) [2;30].

Localization, imaging: Compared to other lung cancers the lesions are usually located in the periphery of the lung in the minor airways and tend to be smaller (Figure 9). It is often subpleural and asymptomatic because of its peripheral location. Therefore, they are not readily amenable to detection by sputum cytology or other types of cytology in their early stages; however, they may become apparent on computed tomography (CT) scan in the earliest stages and then on chest radiograph [30].

Spread: Adenocarcinomas grow more slowly than squamous cell carcinomas but tend to metastasize widely and earlier. Local recurrence after resection is less common in adenocarcinoma than in other types [2].

2.3.1.2 Squamous cell carcinoma (epidermoid carcinoma)

Epidemiology: Squamous cell carcinoma is most commonly found in men and over 90% of occur in cigarette smokers. This type represents approximately 30% of all lung cancers [16;52].

Localization, imaging: The majority of squamous cell lung carcinomas arise centrally in the mainstream, lobar, segmental or subsegmental bronchi via progression through stages of dysplasia (Figure 9). Because there is exfoliation of the malignant cells from the bronchial surface, squamous cell carcinoma can be often detected by sputum cytology or bronchoalveolar lavage fluid at its earliest stage, before it is evident on chest radiograph. With further grows extends into parenchyma and bronchial lumen producing obstruction with resultant atelectasis or pneumonia [30]. Not inconsiderable of cases may arise in small peripheral airways, the incidence of squamous cell carcinoma of the peripheral lung is increasing [2]. The primary tumor and its thoracic extension are best demonstrated by CT scan. PET scan is now the method of choice to identify metastases (excluding brain metastases which may require MRI) [52].

Spread: Squamous cell carcinoma tends to be local aggressive. Metastases to distant organs occur at a later phase. Locoregional recurrence after surgical resection is more common in squamous cell carcinoma. This type is associated with the best prognosis [16;52].

2.3.1.3 Large cell undifferentiated carcinoma

This is an undifferentiated malignant epithelial tumor that lacks the cytologic features of small cell carcinoma and glandular or squamous differentiation.
Large cell carcinomas probably represent squamous cell carcinomas and adenocarcinomas that are so undifferentiated that they can no longer be recognized by light microscopy [2].

Epidemiology: Large cell carcinoma represents approximately 9% of all lung cancers. Average age at diagnosis is about 60 and most patients are male. This type generally has poor prognosis and has a strong association with smoking. One histologic variant, large cell neuroendocrine carcinoma (LCNC), has aggressive behavior and can have a similar prognosis to small cell carcinoma [16;52].

Localization, spread: Large cell carcinomas typically present as a large peripheral mass (Figure 9) frequently identified on chest radiographs, with rapid grows and early metastases, especially to mediastinum and brain [52].

2.3.1.4 Small cell lung cancer

Epidemiology: Small cell lung cancer (SCLC) accounts for approximately 20% of all lung cancers diagnosed annually and for up to 25% of lung cancer deaths each year [15].The etiology of SCLC is strongly associated to tobacco use with almost 98% of patients with SCLC having a history of smoking.
The incidence rates has decreased over the last decade in men, but continuing to rise in women in most countries [11]. Recent evidence suggests that women of

all ages are more likely to present with SCLC than men, and that younger women are more likely to present with SCLC than older women [15].

Localization, diagnosis: Most SCLCs are centrally located and present as a hilar and perihilar mass often with extensive mediastinal lymphadenopathy (Figure 9). SCLCs are typically situated in peribronchial location with infiltration of bronchial mucosa and peribronchial tissue. Diagnosis is usually made by bronchoscopy and cytology. Often, the primary tumor is not detected on radiographic methods. Approximately 5% of SCLCs present as peripheral small lesions [13].

SCLC was previously called “oat cell carcinoma” for the small, round cell shape of the cancer cells. SCLC is of a high-grade morphology and pathologic diagnosis is usually made on light microscopic findings. Electron microscopy can show neuroendocrine granules in two thirds of cases and immunohistochemistry for neuroendocrine markers (chromogranin A, neuron specific enolase, gastrin releasing peptide, insulin-like growth factor 1, synaptophysin) is positive in most (75%) cases [13;15;52]. There is no known preinvasive phase or carcinoma in situ, these findings are frequently found in non-small cell lung carcinomas (NSCLC) [2].

Spread: SCLC is the most aggressive of lung tumors often presenting with generalized symptoms and distant metastases. It is cancer most commonly associated with various paraneoplastics syndromes. Although these tumors respond initially to chemotherapy, most patients develop drug resistance [52].

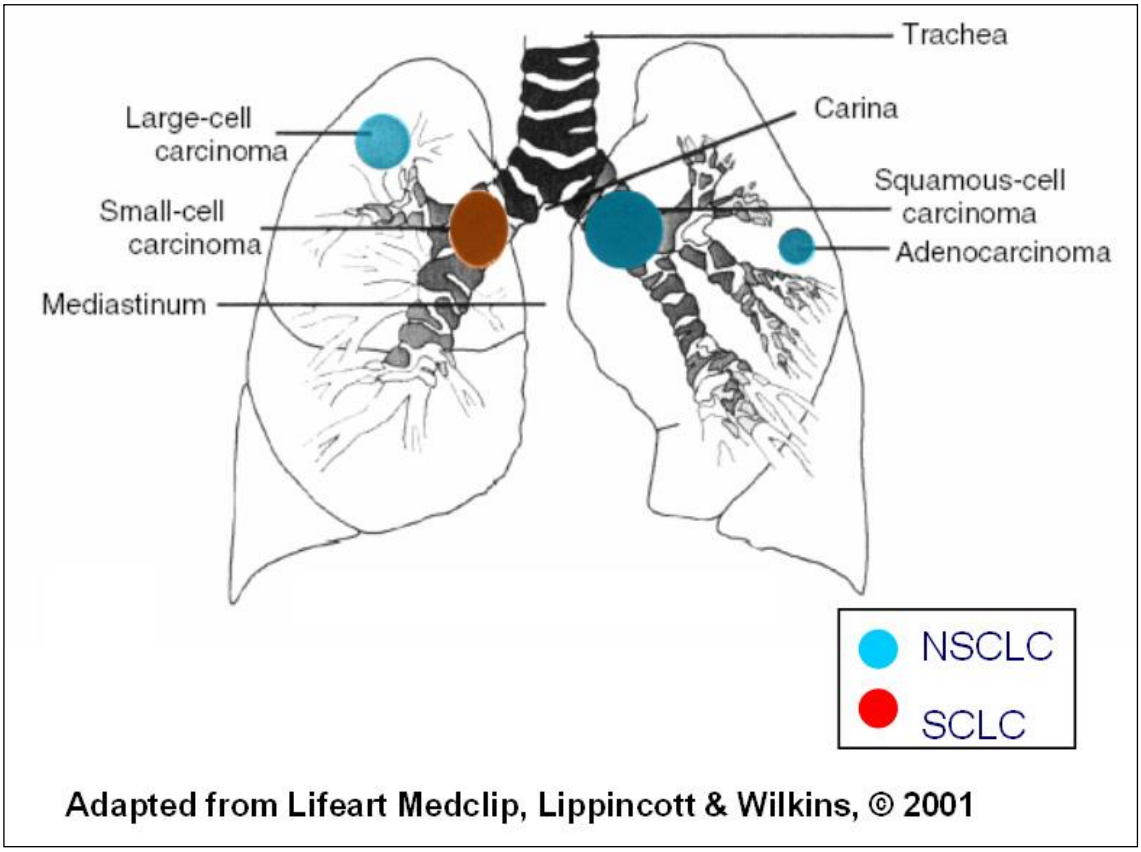


Figure 9. Localization of the major histological types of lung cancer.

2.3.1.5 Tumours with neuroendochrine morphology.

Neuroendocrine tumours of the lung include small cell lung cancer (SCLC), large cell neuroendocrine carcinoma (LCNC), typical and atypical carcinoid. These tumors share morphologic, ultrastructual, immunohistochemical and molecular characteristics. Carcinoid tumors in contrast to most other lung cancers show no relationship to smoke exposure [13;16].

Neuroendocronine differentiation can be show by immunohistochemistry in 10-20% of non-small cell carcinomas (NSCLC), in squamous cell carcinomas, adenocarcinomas and large cell carcinomas, it is seen most often in adenocarcinomas [16].

2.3.2 TNM classification (staging)

Lung cancer staging provides information about the anatomical extent and histological nature of disease, which helps to plan therapy, informs on prognosis and allows assessment for possible resection [53].

2.3.2.1 The international staging system for NSCLC

The current staging system for non small cell lung cancer (NSCLC) is based on the tumor, node and metastasis (TNM) classification (Table 7). The 7th edition of TNM system in lung cancer has been published in 2009 by the International Union Against Cancer and the American Joint Committee on Cancer (AJCC). The changes to the 6th edition were based upon proposals from the International Staging Project of the International Association for the Study of Lung Cancer (IASLC) [54-56].

Table 7. The current TNM system for NSCLC (2009) [54].

TX	The primary tumor cannot be assessed OR the presence of a tumor was only proven by the finding of cancer cells in sputum or other non-imaging tests or bronchoscopy
T0	No evidence of a primary tumor
Tis	"In situ" - cancer is only in the area where the tumor started and has not spread to nearby tissues
T1	The tumor is less than 3 cm (just slightly over 1 inch), has not spread to the membranes that surround the lungs (visceral pleura), and does not affect the main branches of the bronchi
T1a	The tumor is less than 2 cm
T1b	The tumor is larger than 2 cm but less than 3 cm
T2	The tumor is larger than 3 cm but less than 7 cm OR involves the main bronchus or visceral pleura. The tumor may partially block the airways but has not caused the entire lung to collapse (atelectasis) or to develop pneumonia
T2a	The tumor is larger than 3 cm but less than or equal to 5 cm
T2b	The tumor is larger than 5 cm but less than or equal to 7 cm
T3	The tumor is more than 7 cm OR touches an area near the lung (such as the chest wall or diaphragm, or sac surrounding the heart- pericardium) OR has grown into the main bronchus but not the area where the windpipe (trachea) divides OR has caused one lung
T4	The tumor is of any size AND has spread to the medistinum, heart, trachea, esophagus, backbone or the place where the windpipe (trachea) branches OR there is a separate tumor(s) in a different lobe of the same lung

NX	Regional lymph nodes cannot be assessed
N0	No cancer found in the lymph nodes
N1	Cancer has spread to lymph nodes within the lung or to the area where the bronchus enters the lung, but only on the same side of the lung as the tumor (ipsilateral)
N2	Cancer has spread to lymph nodes near where the windpipe (trachea) branches into the left and right bronchi or near the mediastinum, but only on the same side of the lung as the tumor
N3	Cancer has spread to lymph nodes found on the opposite side of the lung as the tumor (contralateral) or lymph nodes in the neck

MX	Cancer spread cannot be assessed
M0	Cancer has not spread
M1	Cancer has spread
M1a	Cancer has spread: Separate tumor(s) in a lobe in the opposite lung from the primary tumor (contralateral) , OR malignant nodules/effusion in the pleura or pericardium
M1b	Cancer has spread to distant part of the body such as brain, kidney, bone

The TNM staging system provides the characteristics of primary tumor (T), regional lymph node involvement (N) and distant metastasis (M). The primary tumor is subdivided into four categories (T1 to T4) depending on site, size and local extent. Lymph node involvement is subdivided into bronchopulmonary (N1), ipsilateral mediastinal (N2) and contralateral or supraclavical disease (N3). Metatases are absent (M0) or present (M1).

The two most commonly types of stage assessment are **clinical staging** (the stage determined using all information available prior to any treatment) and **pathologic staging** (determined after a surgical resection, particularly on the pathologic exam of the tissue). Clinical staging is used to select the primary treatment, pathologic staging is used to estimate prognosis and to evaluate the outcome [56].

The extent of clinical staging can vary from a clinical evaluation alone (history and physical examination) to extensive imaging (CT/PET scans) or invasive staging techniques (mediastinoscopy).

Clinical stage is denoted by the prefix cTNM and pathologic stage by the prefix pTNM [53].

Staging procedures for NSCLC include medical history and physical examination, laboratory tests (complete blood count, levels of electrolyte, calcium, hepatic transaminases, and alkaline phosphatase). From noninvasive imaging metods are routinely performed chest radiograph, chest and upper abdomen computed tomography (CT) that may reveal hilar and mediastinal adenopathy and liver or adrenal involvement [36].

Evaluation of the mediastinal nodes is a key step in the further staging. Fluoro-deoxyglucose (FDG)-PET and PET/CT imagines are significantly superior to CT for the detection of nodal disease and can be used as an initial assessment of the hilar and mediastinal nodes for N-staging. However, despite the encouraging results with the use of PET/CT imaging currently the gold standard for mediastinal nodal staging remains lymph-node biopsy by means of bronchoscopy or the more invasive mediastinoscopy which provides near-perfect specificity and high sensitivity (more than 93%). Endoscopic esophageal ultrasonography (EUS) and endobonchial ultrasonography (EBUS) to guide biopsies are newer techniques with high sensitivity and specificity for mediastinal staging. These techniques are minimally invasive and can be used instead of invasive staging procedures [36;38;50;57].

Brain magnetic resonance imaging (MRI) and bone scanning should be performed in patients with stage II, stage III, and stage IV diseases to rule out metastatic disease if aggressive combined-modality therapy is being considered [50;57].

Stage grouping. Using the TNM system, four stages of lung cancer have been identified (Table 8) [54] that are associated with significant differences in 5-year survival depending on the stage of disease at diagnosis [30] (Figure 10).

Table 8. Stage grouping.

Overall stage	T	N	M
Stage 0	Tis (in situ)	N0	M0
Stage IA	T1a, b	N0	M0
Stage IB	T2a	N0	M0
Stage IIA	T1a, b	N1	M0
	T2a	N1	M0
	T2b	N0	M0
Stage IIB	T2b	N1	M0
	T3	N0	M0
Stage IIIA	T1, T2	N2	M0
	T3	N1, N2	M0
	T4	N0, N1	M0
Stage IIIB	T4	N2	M0
	Any T	N3	M0
Stage IV	Any T	Any N	M 1a, b

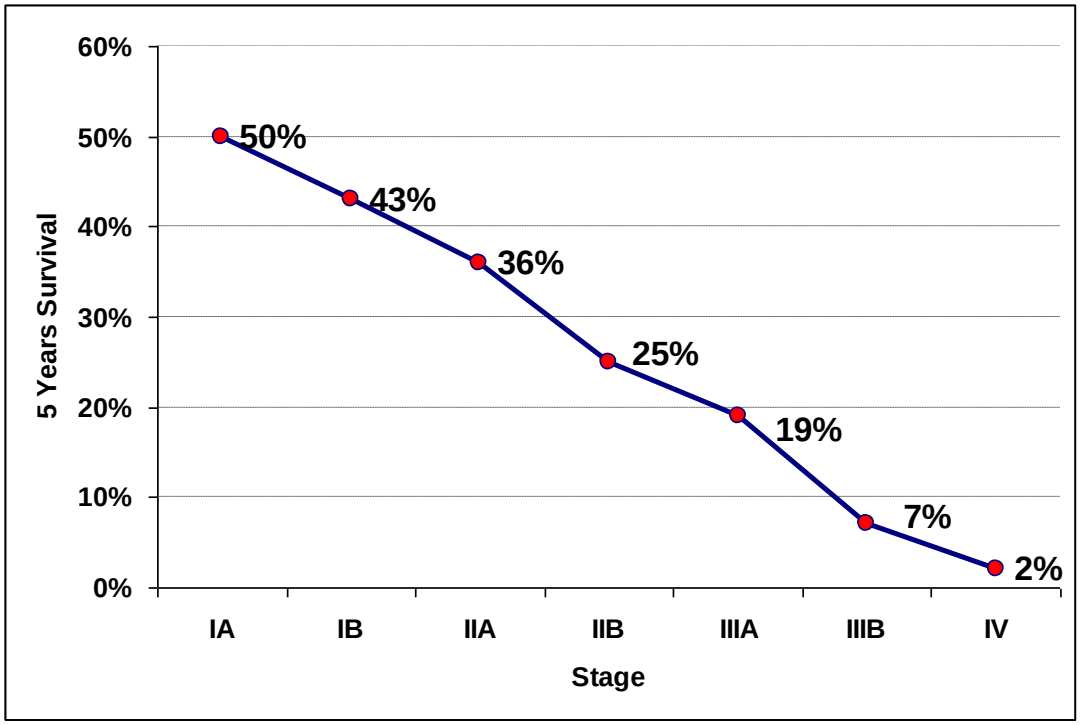


Figure 10. Five years overall survival by clinical stage (Modified from Goldstraw P, Crowley J, Chansky K, et al.) [58].

2.3.2.2 The international staging system for SCLC

The TNM staging classification is not utilized in small cell lung cancer (SCLC), which is normally regarded as a systemic disease. At the time of presentation surgical resection is viable in less than 5% of cases, as up 80% cases have already metastasized [53].

For purposes of practical management of patients, SCLC is classified into a two-stage system, limited (LD) and extensive disease (ED), proposed by the Veterans Administration Lung Study Group (VALG) and modified by International Association for the Study of Lung Cancer (IASLC) [59]. This distinction is important because patients with ED are treated with palliative chemotherapy and/or radiotherapy, whereas patients with LD are treated with curative intent [15]. Approximately one third of SCLC patients are diagnosed with LD.

Limited disease (LD) is restricted to one hemithorax with regional lymph node metastases, including ipsilateral and contralateral mediastinal and supraclavicular nodes. Limited disease is equivalent to stage I – III of the TNM system.

Extensive disease (ED) is any disease outside of the hemithorax, equivalent to stage IV in the TNM system [59].

Staging procedures for SCLC include medical history and physical examination, a basic laboratory evaluation, bronchoscopy and cytologic analysis, chest radiograph, chest and abdominal CT scans including the liver and adrenal glands, a bone scintigram and cranial CT or MRI scan [52;57]. Ongoing studies are investigating the role of PET/CT in disease staging [15].

2.3.3 Histologic grade of lung cancer (grading)

Based on the microscopic appearance of cancer cells, pathologists commonly describe tumor grade by four degrees of severity: Grades 1, 2, 3 and 4. The cells of grade 1 tumors resemble normal cells and tend to grow and multiply

slowly. Grade 1 tumors are generally considered the least aggressive in behavior. Conversely, the cells of grade 3 or grade 4 tumors do not look like normal cells of the same type. Grade 3 and 4 tumors tend to grow rapidly and spread faster than tumors with a lower grade.

Table 9.

The American Joint Commission on Cancer recommends the following guidelines for grading tumors [56].

GX	Grade cannot be assessed (Undetermined grade)
G1	Well-differentiated (Low grade)
G2	Moderately differentiated (Intermediate grade)
G3	Poorly differentiated (High grade)
G4	Undifferentiated (High grade)

2.4 CLINICAL PRESENTATION OF LUNG CANCER

More than 90% of patients with lung cancer are symptomatic at the time of diagnosis. The clinical presentation include the nonspecific systemic symptoms of fatigue, anorexia, and weight loss, or direct signs and symptoms caused by the primary tumor and intrathoracic or extrathoracic spread. The first manifestations in a minority of cases are paraneoplastic syndromes [13;36](Table 10). Less frequently patients present with an asymptomatic lesion discovered incidentally on chest radiograph [30] (Table 11).

Table 10. Lung cancer manifestations [16].

Systemic symptoms Weight loss, loss of appetite, malaise, fever **Local /direct effects** From endobronchial growth and/or invasion of adjacent structures including chest wall and vertebral column Cough, dyspnoea, wheeze, stridor, haemoptysis Chest pain/back pain Obstructive pneumonia (+/- cavitation) Pleural effusion **Extension to mediastinal structures** Nerve entrapment : recurrent laryngeal nerve (hoarseness), phrenic nerve (diaphragmatic paralysis), sympathetic system (Horner syndrome), brachial plexopathy from "superior sulcus" tumours Vena cava obstruction: superior vena cava syndrome Pericardium: effusion, tamponade Myocardium: arrythmia, heart failure Oesophagus: dysphagia, bronchoesophageal fistula Mediastinal lymph nodes: pleural effusion **Metastatic disease** Direct effects related to the organ(s) involved **Paraneoplastic syndromes** Dermatomyositis/polymyositis Clubbing Hypertrophic pulmonary osteoarthropath Encephalopathy Peripheral neuropathies Myasthenic syndromes (including Lambert-Eaton) Transverse myelitis Progressive multifocal leukoencephalopathy	**Endocrine syndromes** Parathormone-like substance: hypercalcemia Inappropriate antidiuretic hormone: hyponatremia ACTH: Cushing syndrome, hyperpigmentation Serotonin: carcinoid syndrome Gonadotropins: gynecomastia Melanocyte-stimulating hormone: increased pigmentation Hypoglycemia, hyperglycemia Hypercalcitonemia Elevated growth hormone Prolactinemia Hypersecretion of vasoactive intestinal polypeptide (VIP): diarrhea **Hematologic/coagulation defects** Disseminated intravascular coagulation Recurrent venous thromboses Nonbacterial thrombotic (marantic) endocarditis Anemia Dysproteinemia Granulocytosis Eosinophilia Hypoalbuminemia Leukoerythroblastosis Marrow plasmacytosis Thrombocytopenia **Miscellaneous (very rare)** Henoch-Schönlein purpura Glomerulonephritis, Nephrotic syndrome Hypouricemia, Hyperamylasemia Amyloidosis Lactic acidosis Systemic lupus erythematosus

Table 11. Frequency of symptoms in 1277 lung cancer patients (a retrospective study) [60].

Symptomatic pattern	All series n (%)
Non-symptomatic patients (incidental diagnosis)	158 (12.4)
Symptomatic patients	1119 (87.6)
Patients with	
Cough	639 (50.0)
Systemic symptoms	630 (49.3)
Dyspnoea	433 (33.9)
Chest pain	402 (31.5)
Bloody sputum	381 (29.8)
Symptoms of local or distant dissemination	298 (23.3)
Chest infection	252 (19.7)
Mean number of symptoms per patient	2.38

2.4.1 Symptomatology due to local growth of the primary tumor

The most frequent presenting symptoms in patients with lung cancer are cough, hemoptysis, dyspnea and chest discomfort. Cough is the most common local manifestation and usually is mildly productive or dry. Cough secondary to an endobronchial mass or postobstructive pneumonia occurs in up to 75% of patients. Most patients also present with a chronic productive cough due to chronic bronchitis. A change in the character of cough or the appearance of blood-tinged sputum is in these patients the initial manifestation of lung cancer. Hemoptysis is the second common alarming symptom, but is rarely massive bleeding, typically is bloody sputum. Dyspnea may be caused by a tumor occluding the airway and usually is associated with the loss of lung function. Intermittent, aching chest discomfort occurs in approximately 50% of patients at diagnosis [13;61].

2.4.2 Symptomatology due to the intrathoracic spread of the primary tumor

Intrathoracic spread of lung cancer is caused by direct extension of the tumor or via lymphatics and produces a variety of symptoms and signs including characteristic syndromes.

Pancoast's syndrome and Horner's syndrome

Pancoast's tumor is a superior pulmonary sulcus tumor that develops at the apex of the upper lobes and usually invades the lymphatics and the following structures: the lower roots of the brachial plexus, the intercostals nerves, the stellate ganglion, the sympathetic chain, and the adjacent ribs and vertebrae. Its initial clinical presentation is the shoulder pain, later the pain extend down the arm along the involved nerve roots. When invades sympathetic chain and the stellate ganglion, Horner's syndrome (enophtalmus, pupillary constriction, palpebral ptosis and anhidrosis) develops on the ipsilateral side of the face [13].

Superior vena cava syndrome

It is the clinical syndrome resulting from the reduction of venous return from the head, neck and upper extremities. Clinically, it presents with head, facial, neck, upper thorax, and upper extremity edema and venous distension, headache, cyanosis and collateral circulation [13].

Recurrent laryngeal and phrenic nerve paralysis

Compression or invasion of the recurrent laryngeal nerve leads to hoarseness, rarely to dysphagia, and is often observed with advanced left upper lobe tumor [13;30].

Phrenic nerve paralysis leading to hemidiaphragmatic paresis may manifest with dyspnea, chest radiograph shows a hemidiaphragmatic elevation [61].

Chest wall invasion

The primary tumor can invade the chest wall, producing stabbing or burning radicular pain and pleural effusion [30].

Pleural effusion

Lung cancer is the leading cause of malignant pleural effusion. During the course of the disease at least 50% of patients with disseminated disease develop pleural effusion. The mechanism can be direct (pleural metastasis, obstruction of the lymphatic vessels, decrease of pleural fluid drainage, thoracic duct interruption) and indirect (hypoproteinemia, pneumonitis, pulmonary embolism, postradiation therapy) [13].

Heart involvement

The mechanisms by which lung cancer leads to pericardium and heart involvement include: retrograde lymphatic migration of tumor cells, hematogenous dissemination and direct tumor invasion. The pericardial involvement usually presents as pericardial effusion, cardiac tamponade or constrictive pericarditis. It is often asymptomatic and discovered by imaging or at autopsy [13].

Esophageal involvement

Esophageal obstruction manifesting with dysphagia may be caused by the primary tumor or by nodal involvement [13].

2.4.3 Symptomatology due to distant extrathoracic spread of the primary tumor (distant metastases)

Approximately one-third of patients with lung cancer present with symptomatology of extrathoracic spread at presentation. Small cell and poorly differentiated carcinomas have a higher tendency to metastasize, followed by the adenocarcinomas, large-cell carcinomas and squamous cell carcinomas [13;36]. The most common sites of distant metastases are:

- **the brain**, where metastases may manifest with symptoms and signs of increased intracranial pressure, neurologic deficits or personality changes.

- **the bones**, with symptoms include pain and pathological fractures, elevated alkaline phosphatase level, usually involves the long bones or vertebrae,
- **the liver**, where metastases may manifest with fever, biochemical abnormalities, pain and general symptoms such as anorexia, weakness, and weight loss,
- **the vertebrae and the epidural tissues**, where metastases manifest with spinal cord compression syndromes,
- **the adrenal glands**, which are clinically silent,
- **the lymph nodes.**

Nonspecific systemic symptoms of extrathoracic spread such as fatigue, weakness, anorexia, and weight loss occur in at least 20% of patients with advanced disease and contribute considerably to poor performance status [13].

2.4.4 Paraneoplastic syndromes associated with lung cancer

Approximately 10% of patients with lung cancer develop systemic symptoms related to paraneoplastic syndromes. This is caused by the immunologic response or the ectopic production of peptide proteins (hormones) by the tumor or its metastases and by other not clear causes. Symptoms may precede the diagnosis, appear late in the disease course or suggest recurrence of cancer [13;36].

Common paraneoplastic syndromes include:

Ectopic cushing's syndrome. The ectopic production of adrenocorticotropic hormone (ACTH) by lung carcinoma cells is most commonly in small-cell carcinoma or in carcinoid tumor. This syndrome has been associated with decreased survival in lung cancer patients, decreased chemoresponsiveness and an increase in chemotherapy-related complications including opportunistic infections. Clinically manifest mainly with weight loss, peripheral edema, proximal myopathy, and moon face. Drowsiness, confusion, depression and psychosis may also occur. Hypokaliemia, alkalosis and hyperglycemia are the common biochemical alterations observed [13;61].

Syndrome of inappropriate antidiuretic hormone. The ectopic secretion of clinically significant levels of ADH by lung cancer (commonly small cell carcinoma) manifests with hyponatremia, decreased plasma osmolality and “inapropiate” natriuresis. When severe it presents with confusion, lethargy and coma. The syndrome resolves with chemotherapy and reappears when cancer reoccurs [13].

Hypercalcemia. Hypercalcemia may affect up to 40% of patients in the disease course. Hypercalcemia is due to either osteolytic bone destruction or ectopic hormone production of parathyroid hormone related peptide (PTHrP) by the tumor (mainly squamous cell). The clinical manifestation includes neurological and gastrointestinal manifestations as well as dehydratation. Fatigue, irritability, confusion, headache, lethargy and coma may simulate cerebral metastases [13].

Carcinoid syndrome. Carcinoid syndrome has been described mainly in patients with small-cell or undifferentiated lung carcinoma. The tumor secretes either 5-hydroxytryptamine or 5- hydroxytryptophan and high levels of 5-hydroxyindoleacetic acid can be detected in the urine. The syndrome is characterized by episodes of explosive diarrhea, cutaneous flushing, bronchospasm, tachycardia, anorexia and weight loss [13].

Neurologic paraneoplastic syndromes. The most common include: Lambert-Eaton myasthenic syndrome, peripheral neuropathy, paraneoplastic encephalomyelitis, and cancer associated retinopathy. Small cell lung cancer is the most common histological type associated with these syndromes [13;36].

Lambert-Eaton myasthenic syndrome. Lambert-Eaton myasthenic syndrome is the most common neurologic paraneoplastic syndrome, with a prevalence of 3% in patients with small cell lung cancer. This syndrome is mainly characterized by proximal muscle weakness, fatigue and depression of the deep tendon reflexes. It is associated with autoantibodies against calcium channels [13].

Cutaneous paraneoplastic manifestations. The more common manifestations include: hypertrichosis, hyperkeratosis and pruritus on the palms and soles (Bazex's disease), erythema gyratus repens, hyperpigmented seborrhoic keratoses, acanthosis nigricans [13].

Paraneoplatic rheumatic syndromes. Lung cancer is occasionally associated with a variety of rheumatological syndromes, including dermatomyositis-polymyositis, vasculitis and carcinoma polyarthritis [13].

Hypertrophic pulmonary osteoarthropathy is characterized by the coexistence of finger clubbing, subperiosteal new bone formation and arthritis. It is one of the most commonly occurring paraneoplastic syndromes in lung cancer and is usually observed in squamous cell and adenocarcinomas, extremely rare in SCLC. Clinically is manifested as bone and joint pain, serum alkaline phosphatase level is often elevated [13;30].

2.5 SCREENING OF LUNG CANCER

Lung cancer is a disease that appears to be an appropriate candidate for a screening program. It is the number one cancer killer for both men and women, it has long preclinical phase and it has curative treatment for the minority of patients who are diagnosed early. It also has a target population at risk (smokers and ex-smokers) and is a major economic burden [62]. The result of a lung cancer screening program should be fewer lung cancer-specific deaths in the screened population [63]. Yet current guidelines don't recommend screening strategy for lung cancer, reflecting the negative results of several trials showing not clear reduction in lung cancer mortality following screening programs using chest X-ray and sputum cytology [64-66]. The most recent recommendation (2004) of the US Preventive Services Taskforce (USPSTF) state that – "The evidence is insufficient to recommend for or against lung cancer screening" [67].

2.5.1 Low dose computed tomography (LDCT)

has been recently assessed as a possible screening method in observational studies suggesting better impact than the one obtained with chest X-ray and sputum cytology. LDCT produce a rapid image at a much lower radiation dose than standard CT and has the ability to detect small peripheral nodules [62]. LDCT screening can increase the diagnosis of early-stage lung cancer with excellent survival data. Data from The International early lung cancer action program (I-ELCAP) showed that stage I lung cancer can be detected in 85% of diagnosed cases,10-year survival rate was 92% for stage I patients whose cancers were promptly removed. However, it is still unknown if such detection can reduce lung cancer mortality [50]. For this reason, five randomized controlled trials of LDCT (RCTs) are currently under way to evaluate low dose computed tomography as a screening tool for lung cancer, with a total of 133 000 subjects. Conclusive data from ongoing trials are necessary to define the benefits and risks of low dose CT in lung cancer screening. Until these results become available, there is insufficient evidence to recommend for or against routine lung cancer screening [65].

2.5.2 Novel screening methods

Several screening methods to increase the sensitivity and specificity of LDCT are under investigation. Fluorescence bronchoscopy, detection of gene mutations or DNA methylation markers in sputum, blood, exhaled breath, and bronchial lavage fluids, are possible alternative methods for early detection of lung cancer in high- risk people and are an active area of research [68;69]. For example, mutations of the k-ras gene have been found in the sputum of up to 25% of patients with non-small cell lung cancers and may be detected in sputum more than one year before the clinical diagnosis (range 1–46 months). Some studies show that dual screening including sputum biomarkers evaluation provides additional benefits over CT scan screening alone.

One of the limitations of LDCT is that it does not detect central lung lesions, characteristic of squamous and small cell cancers, as readily as peripheral

ones. The best way to view early lesions from the trachea to the subsegmental bronchi is via fluorescence bronchoscopy. Fluorescence bronchoscopy is a technique that allows detection of premalignant and in-situ lesions in bronchi, and it might be very useful as a secondary screen in individuals found to have molecular abnormalities in a sample of sputum or blood [62;70-72].

Table 12. Evolution of screening tools for lung cancer [73].

1950s		1960–1980s		1990s		2000s and over
Chest X-ray	→	Chest X-ray Sputum cytology	→	Chest X-ray Sputum cytology Low-dose computed tomography (LDCT)	→	Chest X-ray Sputum cytology LDCT Positron emission tomography (PET) Bronchoscopy (LIFE) Biomolecular markers

2.6 PROGNOSIS OF LUNG CANCER

Although there have been made advances in diagnosis and treatment strategies in the last decade, the prognosis of lung cancer patients is poor, with a 5-year overall survival of 10 – 15 %. This is mainly due to a lack of early diagnosis tools, with a majority of the patients diagnosed with advanced or metastatic disease and therefore not eligible for a curative surgical resection [74;75]. Another probable reason for poor survival among lung cancer patients is the effect of smoking, in that smoking-related comorbidities such as cardiovascular diseases or chronic obstructive pulmonary disease may have an additional negative impact on survival [76;77]. There is also some evidence that current or previous smoking reduces the effectiveness of radiotherapy or chemotherapy when treating lung cancer [78;79]. Lung cancer survival is generally, but not always, better for females and for younger patients. Possible explanations include gender-related differences in tumor biology and/or hormonal factors [80-82].
Prognosis is dependent on the stage and histological type of the tumor as well as clinical factors [83] (Fig. 10).

Prognosis for SCLC is usually inferior compared with NSCLC. The 5-year survival rate by type of lung cancer is between 15 - 20 % for patients with NSCLC and only
6 -15% for those diagnosed with SCLC [84]. Within the non-small cell subtypes prognosis is better for squamous carcinoma than for other lung subtypes. Adenocarcinoma shows the next best prognosis, particulary bronchoalveolar carcinoma. Large cell carcinoma prognosis is generally poor [83;85].
Survival for patients with lung cancer has shown only modest, if any, improvement over the last two or three decades. Improving survival requires focusing attention on smoking cessation, early detection, and research into the genetic profile of lung tumors and developing more effective and well-tolerated forms of therapy [86;87].

2.6.1 Prognostic factors

The prognosis for patients with lung cancer is a function of numerous factors. Identification of prognostic factors is essential in optimizing treatment for patients with lung cancer and may be used in clinical trials for patient stratification [88;89]. Prediction of a patient's prognosis or response to therapy could be improved by combining standard clinical variables (i.e., tumor size, differentiation, or stage), with intrinsic genetic or biochemical characteristics of the tumors. These characteristics have been defined by evaluating the gene expression or levels of selected candidate molecules. Hundreds of studies have evaluated prognostic factors in lung cancer [90].

2.6.1.1 Prognostic factors in non-small cell lung cancer

Certain prognostic factors are predictive of survival in patients with NSCLC.
The major **clinical prognostic factors** are performance status and comorbidity at diagnosis and the disease extension reflected by the TNM and stage in NSCLC [16]. Good prognostic factors include early-stage disease at diagnosis, good performance status ([PS] Eastern Cooperative Oncology Group 0, 1, or 2),

no significant weight loss (not more than 5%), and female gender. Age and histologic subtype have little prognostic significance [50].

Histologic parameters that correlate with unfavourable prognosis include high histologic grade and vascular invasion [16].

Biologic prognostic factors (molecular markers of prognosis), including mutations of the tumor suppressor gene (p53), the activation of k-ras oncogenes, and other biologic markers, may have significant value in predicting a poor prognosis. Patients with stage I lung adenocarcinoma who have specific genetic abnormalities, such as k-ras oncogene activation, have a poor prognosis and disease-free survival [50]. Well-designed studies suggest that overexpression of epidermal growth factor receptor (EGFR), particularly in conjunction with human epidermal growth factor receptor 2 (HER2/neu, also known as erbB2) correlates with diminished survival in lung cancer patients after curative resections. Loss of FHIT (fragile histidine triad) expression as well as overexpression of COX-2 have also been reported to correlate with poor prognosis in lung cancer patients [30].

Unfortunately, none of these prognostic biomarkers is sufficiently robust for use in the clinical management of lung cancer patients at the present time [30].

2.6.1.2 Prognostic factors in small-cell lung cancer

Adverse **clinical prognostic factors** include extensive stage of disease, poor performance status, elevated lactate dehydrogenase LDH or alkaline phosphatase, low plasma albumin and low serum sodium levels, weight loss (more than 5%), and male gender (Table 13). For extensive disease, certain metastatic sites, such as liver, brain, bone marrow, and bone, as well as the total number of metastatic sites involved, have been found to be of prognostic significance for patients. For limited disease, the absence of mediastinal or supraclavicular node involvement is a favorable sign [30;91].

The development of Cushing's syndrome as a paraneoplastic manifestation in SCLC has been correlated with a poor response to therapy and short survival. Continued use of tobacco during the administration of combined modality therapy was identified as an adverse prognostic factor. Multiple series have reported that an abnormal LDH is found in 33% to 57% of all patients with

SCLC and up to 85% of patients with extensive-stage disease and that it is a strong predictor of poor outcome. Other serum markers shed from tumor that have been proposed to have prognostic significance include neuron-specific enolase, chromogranin, and precursors of gastrin-releasing peptide [30].
No **histologic or genetic factors** are predictive of prognosis in SCLC [16].

2.6.2 Serum tumor markers as prognostic factor

In patients with lung cancer, several tumor markers, such as NSE and ProGRP in SCLC as well as CEA and cytokeratins CYFRA 21-1, TPA, TPS in NSCLC, have shown considerable prognostic potential [92]. Several reports suggest that the combined use of cytokeratin markers may provide additional information for prognosis of NSCLC [93;94].

Table 13. Prognostic factors in SCLC [91].

I	Patient factors
	1 Performance status (0–2 favorable)
	2 Gender (female favorable)
II	Tumor factors
	1 Tumor type (SCLC versus NSCLC)
	2 Extent of disease (limited or extensive)
	3 Metastatic evaluation
	a Limited disease
	– Presence/absence of involvement of mediastinal or supraclavicular lymph nodes
	– Presence/absence of pleural effusion
	b Extensive disease
	– Number of organ systems with metastases
	– Presence/absence of hepatic or CNS metastases
III	Screening tests
	1 Elevated serum LDH; alkaline phosphatase unfavorable
	2 Elevated liver enzymes unfavorable
	3 Low serum sodium unfavorable

2.7 TUMOR MARKERS IN ONCOLOGY

2.7.1 Tumor markers – introduction

Tumor markers are substances that indicate presence of malignancy. They can be detected in body fluids (blood, urine, exudate), cells or body tissues. A tumor marker may be produced by a tumor itself, by a surrounding normal tissue in response to the presence of tumor or by a tissue of metastases. There are different types of tumor markers including proteins, membrane antigens, hormones, enzymes, cytokines, DNA, or mRNA measured qualitatively or quantitatively by immunoassays, polymerase chain reaction (PCR), western or northern blot, immunohistochemical test, and more recently microarrays (genomic and proteomic) and mass spectrometry [95;96]. Tumor markers are present in higher quantities in cancer tissue or in blood of cancer patients than in benign tumors or in the blood of healthy people [95]. The concentration of serum tumor marker depends on several parameters, including mass of the tumor, its extent, expression, the releasing of the tumor marker, as well as blood supply to the tumor [97]. Tumor markers are not used alone for the diagnosis because most markers can be found in elevated levels in people who have benign disease or renal failure (false positive), and because no tumor marker is yet specific to a particular cancer. Not every tumor will cause an elevation in the tumor marker test, especially in the early stages of cancer (false negative). With these limitations tumor markers may be however useful for clinical purposes [96].

The diagnostic value of a tumor marker depend on the sensitivity and specificity of the tumor marker, which may be defined as follows:

- Specificity: The percentage of healthy persons or persons with benign diseases from whom a negative result is obtained. The greater the specificity, the fewer the false positive results.
- Sensitivity: The percentage of the test which are correctly positive in the presence of a tumor. The greater the sensitivity, the fewer the false negative results.

Specificity is highly dependent on the choice of control subjects (and patients) and the establishment of an appropriate analyte cut-off level. Sensitivity is dependent on tumor stage, site of recurrence, and histologic differentiation. These factors contribute to a wide range of published sensitivity and sometimes specificity for tumor markers [97].

Table 14. The different categories of tumor markers in oncology according to their structures and biological properties.

HUMORAL TUMOR MARKERS
Oncofetal antigens
• Carcinoembryonic antigen (CEA) • Alpha-fetoprotein (AFP) • Human chorionic gonadotropin (hCG)
Tumor antigens
• CA 242 • CA 125 • CA 19-9 • CA 72-4 • CA 125 • CA 50
Enzymes
• Prostatic acid phosphatase (PAP) • Lactate dehydrogenase (LDH) • Neuron specific enolase (NSE) • Prostate specific antigen total (TPSA) • Prostate specific antigen free (FPSA) • Thymidine kinase (TK) • Matrix metalloproteinases (MMP) • Cathepsin • Tumor M2-pyruvate kinase (M2-PK)
Hormones
• Adrenocorticotropic hormone (ACTH) • Antidiuretic hormone (ADH) • Thyreoglobulin (TG) • Calcitonin (CT) • Parathormon (PTH) • Pro-gastrin-releasing peptide (ProGRP)
Cytokeratins
• Tissue polypeptide antigen (TPA) • Tissue polypeptide specific antigen (TPS) • Cytokeratin 19 fragments (CYFRA 21-1) • Squamous cell carcinoma antigen (SCCA)
Non-specific
• Ferritin • Beta-2 microglobulin • Imunoglobulines • Cytokines
CELLULAR TUMOR MARKERS
• Steroid receptors • Adhesion molecules • Cytokine receptors • Oncoproteins • Products of supresor genes

2.7.2 Historical background

The association of biological markers with cancer has been recognized for many decades. The first identified cancer marker was in 1846 Bence – Jones protein in urine of patients with multiple myeloma and clinicians still use it today. Many years after Bence – Jones‘ discovery, in the mid 1960s, two major tumor markers alpha-fetoprotein (AFP) for hepatoma and carcinoembryonic antigen (CEA) for colorectal cancer were discovered.

In 1960, the Nobel Prize – winning discovery of radioimmunoassay, revolutionized the measurement of trace amounts of analytes in biological fluids. Currently, most tumor markers are measured this way.

In 1975, another Nobel Prize – winning technology, monoclonal antibodies, was developed, and facilitated the discovery of many new tumor markers, including the carbohydrate antigens CA 125, CA 15-3, and CA 19-9. In 1980, prostate - specific antigen (PSA) was discovered.

In the 1970s and 1980s, new concepts – oncogenes and tumor suppressor genes – paralleled the discoveries of radioimmunoassay and monoclonal antibody technologies.

In 2000s many new research fronts in biotechnology are emerging, such as nucleic acid and protein microarrays, mass spectrometry, multiparametric analysis, circulating cancer cells. In the future, these newer techniques, may be used for the measurement of tumor markers [95;98;99].
Some historical milestones in the development of tumor markers appear in Table 15.

Table 15. Tumor markers: historical overview

YEAR	TUMOR MARKER
1846	Bence-Jones protein
1957	TPA
1960	Immunoassay
1963	AFP
1965	CEA
1970-1980	Oncogenes and tumor suppresor genes
1975	Monoclonal antibodies
1977	SCCA
1980s	Carbohydrate antigens CA 125, CA 15-3, CA 19-9, CA 72-4
	PSA
	NSE
1990	TPS
1993	Cyfra 21-1
2000s	Microarrays, mass spectrometry, multiparametric analysis, circulating cancer cells, bioinformatics

2.7.3 Current applications of tumor markers and their limitations

The measurement of tumor markers is currently one of the most rapidly growing areas in laboratory medicine. A tumor marker can be defined as a molecule indicating the likely presence of cancer or providing information about the likely future behaviour of a cancer (e.g., ability to metastasise or to respond to therapy) [98]. Tumor markers are potentially useful in:

1. Screening for early malignancy. Lack of sensitivity for early malignancy and lack of specificity preclude the use of most existing tumor markers for early detection of malignacy [100]. Most circulating tumor markers (with the exception of PSA) are elevated significantly in the late stage disease, thus diagnostic sensitivity is usually low for early stage disease. Most tumor markers (with the exception of PSA) are not specific for a particular tumor or organ and elevations may be due benign and inflammatory diseases. Thus, diagnostic specificity may be low, leading to many false positives [95].

False positive results appear unacceptable from the ethical and economical point-of-view, because the clinical confirmation of positive results is usually

invasive. Despite these limitations, number of tumor markers have either undergone or are currently undergoing evaluation as potencial cancer screening test [98]. These markers include a use of vanillymandelic acid and homovanilic acid in screening for neuroblastoma in newborns [101], AFP in screening for hepatocellular cancer in high-risk patients [102],
CA 125 in combination with transvaginal ultrasound in screening for ovarian cancer [103] and PSA in screening for prostate cancer [104]. To date, there is no conclusive evidence that screening with the use of any tumor marker reduces mortality from cancer [98].

2. Aiding cancer diagnosis. As with screening, lack of diagnostic sensitivity and specificity generally preclude the use of tumor markers for the primary diagnosis. In certain situations, however, selected markers may aid detection [98]. For example, for selected subgroups of high-risk patients, in whom the chance of cancer is high (high prevalence), tumor marker analysis may aid the clinician in ordering more elaborate testing, e.g., imaging or laparoscopic investigations [95]. Other of these situations is the unknown primary origin tumor diagnosis. Metastasis of unknown origin accounts for 5-10 % of all cancers and is defined as a metastasis for which the primary site remains occult. A number of treatable of unknown primary origin tumors give rise to incresed serum marker levels that can be helpful in their diagnosis, but it is important to note that tumor markers have limitations in the diagnosis of unknown primary origin cancers, since markers are not organ-specific and none of the markers currently available is elevated in all patients with a specific malignancy. However, a panel of tumor markers should help to establish the origin of the tumor [105].
Tumor markers may also help in distinguishing between different histological types of a tumor [98].

3. Determining prognosis of malignacy. For optimum patients management, realible prognostic and predictive factors are necessary. Prognostic markers are factors that predict likely outcome of disease in absence of systemic adjuvant therapy. In contrast, predictive markers are factors that are associated with either response or resistance to a specific therapy [98]. Most tumor markers have prognostic value, but their accurancy is not good enough to warrant specific

therapeutic interventions [95]. However, they may be useful as prognostic indicators in malignancy if they provide prognostic information, additional to or independent of conventional prognostic factors. Prognostic markers may help differentiate between patients who should or should not receive adjuvant chemotherapy within subgroups defined by traditional criteria [106] . For example, EGTM [107], ASCO and NACB panels support the use of urokinase plasminogen activator (uPA) and plasminogen activator inhibitor 1
(PAI-1) for determining prognosis in lymph node-negative breast cancer patients. Assay of uPA and PAI-1 may thus help identify low-risk node-negative patients for whom adjuvant chemotherapy is unnecessary [108;109].
Tumor markers for determining prognosis can be measured in either serum or tumor tissue (Table 16). Serum markers are of potential prognostic value as their concentration tends to reflect the extent of tumor bulk or the possible presence of occult metastatic disease. The tissue-based prognostic factors are likely to be molecules causally involved in cancer progression, such as enhancing cell proliferation or mediating metastasis [98].

Table 16. Tumor markers shown to be prognostic in malignancy [98].

Cancer	Prognostic marker(s)
Breast	*Estrogen receptor, *HER-2, *uPA, *PAI-1
Prostate	PSA
Colorectal	CEA
Ovarian	CA 125
Lung	CYFRA 21-1
Non-seminomatous germ cell	AFP, HCG, LDH
Trophoblastic disease	HCG
Neuroblastoma	*N-myc gene amplification

* Denotes that marker determination must be carried out on tumor tissue.
All other markers can be determined in serum.

4. Predicting therapy response. Predictive markers are important in oncology as cancers vary widely in their response to therapy. For most type of cancer, only a minority of patients benefit from a particular form of systemic treatment. Being

able to prospectively select those patients likely to respond would both save patients from unnecessary side effects and allow them to receive therapy that is more likely to be useful [95;110]. Despite the importance of using biomarkers in predicting response to
specific therapies, very few known markers have such predictive power [95] (Table17)

Table 17. Predictive markers in clinical use [98].

Cancer	Therapy	Marker
Breast	Endocrine	Estrogen receptor (ER), progesterone receptor (PR)
Breast	Trastuzumab (Herceptin)	HER-2
Lung	Iressa (gefitinib), erlotinib	EGFR (specific mutations)

Clinically useful therapy predictive markers are estrogen and progesterone receptors to select patients with breast cancer for treatment with hormonal therapy and human epidermal growth factor receptor 2 (HER-2) to select patients with advanced breast cancer for treatment with trastuzumab (Herceptin) [106;107]. Specific mutations and amplification of the EGFR gen appears to be predictive of benefit from EGFR tyrosine kinase inhibitors (TKIs) gefitinib or erlotinib in patients with NSCLC [111;112].
We need more predictive markers to individualize therapy and maximize clinical response [95].

5. Post-operative surveillance. One of the main potentially uses of tumor markers at present are assessing the completeness of tumor removal and the post-operative follow up care for the early detection of recurrent disease.
Control of therapy efficacy. The velocity and the completeness of tumor marker decrease after surgery is indicative of further outcome of the patients. After a short-term increase immediately after therapeutic intervention, due to marker

release from operatively damaged normal and tumor tissue, the decline depends on both biological marker half-life and residual tumor cells . Following curative resection, the levels of tumor markers are expected to decrease rapidly reaching the range of healthy persons within few days, in some markers decrease occurs with some delay depending on the initial marker level. If renal or liver dysfunction, which can prolong the half life of tumor markers are excluded, a slowed marker decrease and/or an elevated plateau is indicative for the presence of residual tumor cells and predict early the recurrence of disease. Decreasing levels after surgery are the first sign of curative resection and therefore an indication of good prognosis [113;114].

Detection of recurrent disease. This practice is based on the assumption that the early detection of recurrent or metastatic disease enhances the chance of cure or results in an improved survival.

Tumor markers are sensitive indicators for recurrence of disease, often with a lead-time of several months as compared to imaging methods. For the early detection of recurrent disease serial determinations of the appropriate tumor marker is mandatory during follow up [98]. Monitoring should be performed using the same tumor marker method. Changing methods should include one to two serial measurements with both methods in parallel [95].

There are some limitations of using tumor markers to detect cancer relaps:

- lead time is short (weeks to a few months) and does not significantly affect outcome, even if therapy is institutued earlier,
- terapies for treating recurrent disease are not effective at present,
- sometimes markers provide misleading information, e.g., clinical relapses occur without biomarker elevation, or biomarker is elevated non-specifically, without progressive disease, leading to either overtreatment or discontinuation of a current treatment protocol [95].

6. Monitoring of therapy response. For patients with advanced disease, who are treated with various modalities, it is important to know if therapy works. In this regard, tumor markers usually provide information that is readily interpretable and more economical, more sensitive, and safer than radiological or invasive procedures [95]. Tumor markers are particularly useful in monitoring those

patients who have disease that cannot be evaluated using conventional criteria (e.g., CA 125 in patients with ovarian cancer) [115].

Generally, the same marker or markers that are used during follow up after surgery for primary cancer are used for monitoring treatment in advanced disease. Serial measurement of these markers can result in the early detection of recurrent disease as well as indicate the efficacy of therapy. Consistently increasing levels suggesting treatment failure should result in discontinuation of ineffective therapy. On the other hand, consistently decreasing levels suggesting tumor regression would allow continuation of treatment. It should be pointed out that during therapy monitoring in advanced cancer, changes in serial marker levels may occur that are unrelated to increases or decreases in tumor load [98]. For example, after the initiation of chemotherapy, transient increases or surges in serum marker levels have been reported and they are likely to result from therapy-mediated tumor cell apoptosis or necrosis rather than from cancer progression [116].

2.7.4 Currently available serum tumor markers for lung cancer

Over the last two to three decades, several serum tumor markers in lung cancer have been described. Clinically useful lung tumor markers include carcinoembryonic antigen (CEA), cytokeratin marker CYFRA 21-1, squamous cell carcinoma antigen (SCC), neuron specific enolase (NSE), and progastrin–releasing peptide (ProGRP). Additional markers have also been proposed, including chromogranin A (CgA), markers of proliferation including thymidinkinase (TK), and cytokeratins MonoTotal, tissue polypeptide antigen (TPA), and tissue polypeptide specific antigen (TPS), carbohydrate antigen 125 (CA 125), tumor M2 pyruvate kinase (Tumor M2-PK), and others.

In table 18, there is a summary of useful and potentially useful markers for lung cancer.

Table 18. The widestly investigated serum tumor markers in lung cancer (National Academy of Clinical Biochemistry Guidelines for the Use of Tumor Markers in Lung Cancer).

Cancer marker	Proposed use/uses
NSE	Differential diagnosis of lung masses when biopsy is not available: in high levels high specificity for small cell carcinoma; in SCLC, additive information to ProGRP Assessing prognosis. High levels predict adverse outcome in SCLC Assessing prognosis. High levels predict adverse outcome in NSCLC Monitoring therapy in SCLC Monitoring therapy in advanced disease (NSCLC) Detection of recurrent disease. Increasing kinetics indicate progressive disease in SCLC
CEA	Differential diagnosis of lung masses when biopsy is not available; in high levels high specificity for adenocarcinoma; in NSCLC, additive information to CYFRA 21-1 Assessing prognosis. High levels predict adverse outcome in early and advanced stage NSCLC Monitoring therapy in advanced disease (NSCLC and SCLC) Detection of recurrent disease. Increasing kinetics indicate progressive disease in NSCLC, part. in adeno cancer.
CYFRA 21-1	Differential diagnosis of lung masses when biopsy is not available: in high levels high specificity for squamous cell carcinoma; best marker for NSCLC Assessing prognosis. High levels predict adverse outcome in early and advanced NSCLC Assessing prognosis. High levels predict adverse outcome in SCLC Monitoring therapy in advanced disease (NSCLC) Early prediction of therapy response in advanced disease (NSCLC) Detection of recurrent disease. Increasing kinetics indicate progressive disease in NSCLC, part in squamous cell cancer.
ProGRP	Differential diagnosis of lung masses when biopsy is not available: in high levels high specificity for small cell carcinoma; best marker for SCLC; additive information to NSE Assessing prognosis. High levels predict adverse outcome in SCLC Monitoring therapy in SCLC Detection of recurrent disease. Increasing kinetics indicate progressive disease in SCLC.

Cancer marker	Proposed use/uses
SCCA	Differential diagnosis of lung masses when biopsy is not available: in high levels high specificity for squamous cell carcinoma; in SQC additive information to CYFRA 21-1 Abnormal levels are associated with a high probability of NSCLC, mainly squamous tumors Assessing prognosis. High levels predict adverse outcome in NSCLC
CA125	Differential diagnosis of lung masses when biopsy is not available; in high levels relative specificity for adenocarcinoma, large cell carcinoma Assessing prognosis in NSCLC. High levels predict adverse outcome in NSCLC Monitoring therapy in advanced disease (NSCLC) Early prediction of therapy response in advanced disease (NSCLC)
Chromogranin A	Differential diagnosis of lung masses when biopsy is not available; particularly for neuroendocrine tumors Assessing prognosis. High levels predict adverse outcome in SCLC and in neuroendocrine tumors Monitoring therapy in neuroendocrine tumors
TPA	Differential diagnosis of lung masses when biopsy is not available Assessing prognosis. High preoperative levels predict adverse outcome in NSCLC
TPS	Assessing diagnosis (inferior to CYFRA 21-1 and TPA); correlation with stage Assessing prognosis. High levels predict adverse outcome in NSCLC Assessing prognosis. High levels predict adverse outcome in SCLC Monitoring therapy in advanced disease (NSCLC) Early prediction of therapy response in SCLC Detection of recurrent disease. Increasing kinetics indicate progressive disease in NSCLC.
TU M2-PK	Assessing diagnosis; inconsistent data are available Monitoring therapy in NSCLC and SCLC Detection of recurrent disease. Increasing kinetics indicate progressive disease in NSCLC and SCLC

The search for new ones continues in the hope of finding a marker which alone or in combination with other markers could be helpful in prognosis estimation, staging or post-surgery monitoring of patients with lung cancer.

Numerous potential biomarkers are available and clearly the list of new members is likely to expand with the elucidation of the complex pathological pathways involve in tumorogenesis, tumor invasion and metastasis, but their clinical value remains uncertain. For their clinical use it is important to analyze their respective utility according to the lung cancer. Some of them have been studied in experimental part of this work.

The properties of the most frequently used serum tumor markers in NSCLC are described below and their potential uses in the diagnosis, prognosis and post-operative follow up of patients with NSCLC will be discussed in experimental part of this work.

Carcinoembryonic antigen (CEA)

CEA is an oncofetal protein normally produced by the gastrointestinal tract, pancreas, and liver during embryonal and fetal development. In adults, however, CEA is produced in low amounts by normal secretor cells of the gastrointestinal tract and overexpressed in adenocarcinomas. Increased CEA production in cancer is caused by the derepression of CEA encoding genes [97].
CEA is a glycoprotein complex that is associated with the plasma membrane of tumor cells, from which it may be released into the blood. CEA shows no organ specificity for the lung, it is typically used for tumors with different localizations. Elevated CEA levels are found in a variety of cancer such as colon, breast, lung, pancreas, stomach, biliary tract, and ovary. It is also detected in benign conditions including smoking, peptic ulcer disease, cirrhosis, hepatitis, pancreatitis, inflammatory bowel diseases, gastritis, Crohn's disease and ulcerative colitis, polyps of the colon and rectum, diverticulitis, myocardial infarction, benign prostatic hypertrophy, renal disease, inflammatory pulmonary diseases, emphysema and chronic obstructive pulmonary diseases. These conditions usually produce transient and only modestly elevated CEA levels, rarely above 10 ng/ml, that decrease as the condition improves [96;97;117]. CEA is

metabolized primarily by the liver. Hepatic diseases, including extrahepatic biliary obstruction, intraheptic cholestasis and hepatocellular disease, may alter clearence rates and artificially increase serum concentrations of CEA. The highest concentrations of the marker are found in patients with liver metastases from colorectal cancer [96;97].

The recommended manufacturer cut off value for CEA in serum is 5,0 ng/ml for healthy persons (at 95% specificity) [97].

Cytokeratins (CKs) are intermediate filament proteins of the cytoskeleton expressed in cells of epithelial origin. At present, more than 20 different cytokeratins have been identified, of which cytokeratins 8, 18, and 19 are the most abundant cytokeratin proteins found in simple epithelial cells, including the bronchial epithelium, and in carcinomas derived from these cells. In healthy individuals, the levels of cytokeratins are low in the circulation, but rise significantly in patients with epithelial cell-associated carcinomas. Upon cell death in a growing tumor, they are released into the serum and others body fluids in the form of soluble fragments, where may be detected using monoclonal anti-cytokeratin antibodies. Cytokeratins are generally called as proliferation tumor markers. By following patients with repeated testing during post-treatment monitoring, the oncologist may obtain information regarding the growth activity of tumor and disease status before conventional mehods. The three most applied cytokeratin markers used in the clinic are TPA (CK 8, 18, 19 fragments), TPS (CK 18 fragment), and CYFRA 21-1(CK 19 fragment). TPA is a broad-spectrum test that measures the soluble cytokeratin fragments 8, 18, and 19, while TPS and Cyfra 21-1 measure CKs 18 and 19, respectively. Recently, a new cytokeratin assay MonoTotal has been introduced, which also measures cytokeratins 8, 18, and 19, but using a different combination of antibodies [93;94;118-121]. A lung alveolar cells express both CK 18 and CK 19, thus TPS, TPA, Cyfra 21-1, and MonoTotal are useful markers for lung cancer [119].

<u>Cytokeratin 19 fragments (CYFRA 21-1)</u>

Cyfra 21-1 is a water soluble fragment of cytokeratin 19. It is a relatively new tumor marker which the assay uses two monoclonal antibodies directed against a cytokeratin 19 fragment. Since Cyfra 21-1 determines only fragments of CK

19, the test shows a higher specificity than TPA, which determines a mixture of CK 8,18 and 19 [93]. In healthy individuals, the level of cytokeratins in the circulation is low. Reason for elevated serum Cyfra 21-1 levels are renal insufficiency, liver cirhosis, trauma of cytokeratin-rich tissues, and benign pulmonary diseases such as fibrosis, tuberculosis, acute inflammatory diseases, and chronic obstructive pulmonary disease. Levels rise significantly in patients with epithelial cell-associated carcinomas, particularly in tumors of squamous origin. According to Sugama et al. [122], increased serum CYFRA 21-1 is the result not only of cytokeratin release as a consequence of cell lysis or necrosis, but also of the degradation of cytokeratin filaments by activated protease in tumor cells. Cyfra 21-1 is significantly elevated in lung cancer, irrespective of cell type, althrough it is more sensitive in NSCLC, and especially in the squamous cell cancers. Cyfra 21-1 is also elevated in urological, gastrointestinal, gynaecological and in head and neck cancers. Cyfra 21-1 levels do not differ between smoking and nonsmoking subjects [93;95;97;120;123-125]. The recommended manufacturer cut off value for Cyfra 21-1 in serum is 3,3 ng/ml for healthy persons (at 95% specificity) [97].

Tissue polypeptide antigen (TPA)

TPA is one of the oldest tumor markers discovered by Bjorklund in 1957. TPA assays measure a mixture of CKs 8, 18, and 19. Elevated serum levels of TPA are observed in patients with various types of cancer, correlating with important clinical parameters such as the mass of tumor, its proliferating activity, and the consequent prognosis [120]. TPA has long been used as a serological marker in breast, stomach, colorectal, lung, bladder, and head and neck cancer [93]. Benign reasons for increased TPA levels include hepatitis, liver cirrhosis, cholecystitis, pregnancy, bacterial and viral infections, autoimmune disorders, and diabetes mellitus. In healthy people and in patients with benign lung disease, TPA-possitive sera occur only with low frequency [95;120;126]. Serum TPA has been shown to be increased in patients with lung cancer irrespective to the histological type [97]. There is no consistent relationship between production of this marker and lung tumor type.

Tissue polypeptide- specific antigen (TPS)

TPS is a specific cytokeratin-based assay, which detects a defined M3 epitope structure on CK 18 using the specific M3 monoclonal antibody [93;119]. This epitope is proposed as specific for cell proliferation. TPS has been claimed to be highly correlated with the proliferation rate of cancer cells, thus, marking more closely the presence of tumor and its clinical behavior [120]. TPS is a well documented cytokeratin tumor marker in various epithelial cell associated carcinomas, increased TPS has been reported in breast, lung, ovarian, prostate, bladder, head and neck, and gastrointestinal cancer [93;94].

Squamous cell carcinoma antigen (SCC)

SCC is considered a structural protein that reflects the differentiation grade of squamous cell carcinomas. SCC is a tumor associated antigen whose circulating concentrations may be elevated in squamous cell carcinomas involving the cervix, head and neck, esophagus, and lung [127]. Elevated levels of SCC antigen have been described in patients with dermatological diseases such as psoriasis, eczema and in patients with hyperkeratotic skin diseases associated with an inflammatory component [97]. 40% of patients with benign pulmonary diseases (i.e., chronic bronchitis, chronic obstructive pulmonary disease, tuberculosis, silicosis) demonstrated increased SCC concentration [97]. Smoking does not influence serum concentrations of SCC [95]. SCC has superior specificity for squamous cell lung cancer and can be used for histological subtyping of lung cancer [123]. One of the most important applications of SCC measurements in lung cancer is as an aid to histological diagnosis [128].
The recommended manufacturer cut off value for SCC in serum is 1,5 ng/ml for healthy persons (at 95% specificity) [97].

Thymidine kinase (TK) is considered to be an important proliferation tumor marker that can be detected in the serum of patients diagnosed with different types of solid tumors and haematological malignancies [129-132]. There are two thymidine kinases in human cells. As tumor marker is useful the first isoenzyme, thymidine kinase 1 (TK1), a pyrimidine metabolic pathway enzyme involved in salvage DNA synthesis and therefore a cell cycle and proliferation-dependent marker [129-131;133]. In healthy people, the amount of TK in serum is low. Tumor

cells release this enzyme into the circulation, probably in connection with a disruption of dead or dying tumor cells [131]. The thymidine kinase level in serum therefore serves as a measure of malignant proliferation, indirectly as a measure of the aggessiveness of the tumor [130].

TK represents a secondary tumor marker which is particularly useful for cancer disease monitoring and in the diagnosis of haematological malignancies [131]. In interpretation of this marker it is always necessary to exclude the possibility of false positives results, which may be a consequence of viral infections, pernicious anemia, inflammatory or autoimmune diseases [133;134].

The second isoform, thymidine kinase 2 (TK2) is the key enzyme in mitochondrial DNA (mtDNA) synthesis. TK2 is cell cycle independent and the concentration of this enzyme in tissues is not correlated with proliferation. TK2 is involved in certain forms of mitochondrial diseases but not in diseases related to cell proliferation [135]. TK2 deficiency in affected tissues leads to progressive myopathy, hepatopathy and/or encephalopathy [136;137].

3. THE EXPERIMENTAL PART

3.1 THE AIM OF THE DOCTORAL THESIS

The aim of this thesis is to measure a large spectrum of biomarkers in serum or plasma of patients with operable stage of NSCLC and to evaluate and compare the clinical utility of these biomarkers in the three most important clinical applications for NSCLC:

- The evaluation of disease extent at the first clinical presentation (Diagnosis)
- The evaluation of postsurgery status (Postsurgery follow up care)
- The prediction of the clinical outcome (Prognosis)

3.2 PATIENTS AND METHODS

3.2.1 Patients

The present prospective study was conducted from November 2004, there were enrolled 108 patients with primary diagnosis of lung cancer who had undergone radical lung surgery (complete R0 resection) between 2004-2007 at the Department of Surgery, University Hospital in Pilsen. All patients were followed up until death or the last day of follow up (December 31, 2009). The average length of follow up was 28,2 month (range, 1- 60 months). There were excluded 13 patients, whose final diagnosis was different from NSCLC (4 small cell lung carcinoma, 2 carcinoid tumors, 2 sarcomas, 3 lung metastases originating from other cancers, 2 combinations of small cell lung carcinoma and non-small cell carcinoma), and 2 NSCLC patients with metastatic disease (stage IV). The studied group incorporated 93 patients with NSCLC diagnosis stage I-IIIa. For all patients, the diagnosis of NSCLC was confirmed by histological examination of biopsy and cytologic specimens and classified according to World Health Organization (WHO) criteria [51]. Clinical staging (cTNM) was determined on the base of the international TNM staging system and the procedure included chest radiography, computed tomography (CT) scans of the chest and upper abdomen, bronchoscopy, ultrasonography, and brain magnetic resonance imaging (MRI) and bone scintigraphy to rule out metastatic disease. Fluoro-deoxyglucose (FDG)-PET/CT image was employed in selected cases. Mediastinoscopy was performed to exclude N2 disease if suspicious mediastinal lymph nodes were identified by CT or PET/CT. The postsurgical stage (pTNM) of each tumor was determined according to the international staging system for NSCLC [58].

In all patients, the following clinical parameters were studied: age, gender, smoking habit, histological type of tumor, TNM classification, stage, treatment strategy, first relapse, clinical status (remission, progression, stable disease) during follow up and at the time of last control, and cancer- related death (Table 19).

Table 19. The clinicopathological characteristics of NSCLC patients and control group.

		STUDY GROUP NSCLC n (%)	CONTROL GROUP BENIGN n (%)
Total		93 (100)	20 (100)
Age, years - median (range)		62 (43-77)	58 (39-68)
Gender			
	Male	71 (76,4)	9 (45)
	Female	22 (23,6)	11 (55)
Smoker			
	Yes	86 (92,5)	13 (65)
	No	7 (7,5)	7 (35)
Histology			
	Adenocarcinoma	34 (36,6)	
	Squamous	59 (63,4)	
Stage			
	Ia	19 (20,4)	
	Ib	30 (32,3)	
	IIa	4 (4,3)	
	IIb	15 (16,1)	
	IIIa	25 (26,9)	
Follow up			
Disease free interval	Mean (range) months	34,1 (28,5 - 39,7)	
Overall survival	Mean (range) months	41,4 (36,2 - 46,5)	
Initial therapy			
	No therapy	23 (24,7)	
	Adjuvant	56 (60,2)	
	Paliative	14 (15,1)	
Therapy response			
	Remission	45 (48,4)	
	Stable diesease	5 (5,4)	
	Progression	43 (46,2)	
Survival			
	Survivors	48 (51,6)	
	Cancer death	45 (48,4)	

The response to therapy was objectified by clinical and laboratory examinations and imaging methods (chest radiography, bronchoscopy, computed tomography (CT), PET/CT scan, ultrasonography, and bone scan) during follow up period of at least half year. The outcome was classified according to the WHO criteria, defining “complete remission” as the disappearance of the tumor, “partial remission” as tumor reduction ≥ 50 %, “progression” as tumor increase ≥ 25%

or appearance of new tumor manifestations, and ‘‘no change’’ (stable disease) as tumor reduction <50% or increase <25%.

As a control group we used 20 individuals with lung benign disease (Table 20) and no history of cancer disease, whose median age (58 years, range 44-77) corresponded to the median age of the patients with NSCLC (62 years, range 39-68). Nobody had renal failure, liver disease, and benign skin diseases, well known causes for false positive results of routine tumor markers, at enrolment.

The clinicopathologial characteristics of NSCLC patients and control group are shown in table 19.

Table 20. Benign diseases characteristics of control group (N= 20).

Benign diseases	n
Postinflammatory fibrosis	5
Tuberculosis	4
Chondrohamartoma	4
Interstitial fibrosis	2
Lung fibroma	2
Lung granuloma	1
Bronchial cyst	1
Aspergiloma	1

3.2.2 Measurement of serum / plasma biomarkers

We conducted a prospective study evaluating different serum and plasma markers (listing of markers in table 21) measured by immunoassays.

Venous blood samples were obtained from patients before surgery, 1-2 weeks after surgery and each 6 months during follow up period (i.e. 6, 12, 18 and 24 months after surgery). The peripheral blood was drawn between 6 – 8 in morning using VACUETTE® tubes (Greiner Bio-One, Austria) from cubital vein. Serum samples were left to clot. Sera and plasma were separated by

centrifugation at 1300g and all specimens were immediately aliquoted. Analyses of routine markers were performed immediately or the aliquots were frozen and stored at -20^{o}C until analysis. Aliquots for multiplex immunoassays were frozen and stored at -80^{o}C. No more than 1 freeze-thaw cycle was allowed before an analysis. Before multiplex analyses the aliquots were centrifuged for 5 min. at 10000*g* to remove any clots or particles.

For biomarker measurement in presented study there were used conventional immunoanalytic routine methods (IRMA, REA, CLIA, MEIA, TRACE, ELISA) and multiplex immunoanalytic method: bead-based Multi-analyte profiling technology (xMAP). Complete listing of used methods and kit manufacturers is in table 21.

The xMAP technology as a novel technology is nowadays used in a couple of oncologic research projects focused on biomarker development and has enabled new era of multiparametric panel studies [138-141]. In our laboratory this technology has been used since 2005 for assessment of biomarkers associated with tumor diseases, our pilot study focused on ovarian cancer multiplex xMAP technology panel was published recently in 2009 [142].

The xMAP technology combines sandwich immunoanalysis with flow cytometry. It is based on binding of studied proteins to the antibodies linked to microspheres with internal spectral code referring to protein identity. The amounts of the bound proteins are determined by a second antibody connected with fluorescent molecule. The measurement is performed on special flow cytometer, which determines the spectral code of microspheres – the identity of proteins – after an excitation by first laser and after an excitation by second laser it detects the amount of second antibodies on microspheres – the quantity of protein in sample. The concentrations of proteins are assessed according to standard calibration curves.

In our study circulating levels of IL-6, IL-8, MCP-1, VEGF, ICAM-1, VCAM-1, MMP-9, PAI-1, MMP-1 in plasma/serum were measured by xMAP technology using commercially available kits: Human Cytokine/chemokine Milliplex MAP kit, Human Cardiovascular Disease Panel 1 (both Linco-Millipore, USA), and Fluorokine MAP Human MMP kit (R&D Systems, USA).

Table 21. Listing of used biomarkers, methods and kit manufacturers.

Parameter	Abbreviation	Material	Assay	Producer	Unit
Thymidine kinase	TK	Serum	REA	Immunotech	IU/L
Carcinoembryonic antigen	CEA	Serum	CLIA	Beckman	ng/ml
Insulin-like growth factor 1	IGF-1	Serum	IRMA	Biosource	ng/ml
Cytokeratin-19 fragment	CYFRA 21-1	Serum	TRACE	Brahms	ng/ml
MonoTotal	MT	Serum	IRMA	IDL	U/L
Tissue polypeptide-specific antigen	TPS	Serum	IRMA	IDL	IU/L
Tissue polypeptide antigen	TPA	Serum	IRMA	DiaSorin	IU/L
Squamous cell carcinoma antigen	SCC	Serum	MEIA	ABBOTT	ng/ml
Tissue inhibitor of metalloproteinase 1	TIMP-1	Plasma	ELISA	R&D Systems	ng/ml
Tissue inhibitor of metalloproteinase 2	TIMP-2	Plasma	ELISA	R&D Systems	ng/ml
Matrix metalloproteinase 1	MMP-1	Plasma	LUMINEX	R&D Systems	pg/ml
Matrix metalloproteinase 2	MMP-2	Serum	ELISA	R&D Systems	ng/ml
Matrix metalloproteinase 7	MMP-7	Serum	ELISA	R&D Systems	ng/ml
Matrix metalloproteinase 9	MMP-9	Plasma	LUMINEX	Linco-Millipore	ng/ml
Monocyte chemotactic protein-1	MCP-1	Serum	LUMINEX	Linco-Millipore	pg/ml
Interleukin 6	IL-6	Serum	LUMINEX	Linco-Millipore	pg/ml
Interleukin 8	IL-8	Serum	LUMINEX	Linco-Millipore	pg/ml
Vascular endothelial growth factor	VEGF	Serum	LUMINEX	Linco-Millipore	pg/ml
Intracellular adhesion molecule 1	ICAM-1	Plasma	LUMINEX	Linco-Millipore	ng/ml
Vascular cell adhesion molecule 1	VCAM-1	Plasma	LUMINEX	Linco-Millipore	ng/ml
Plasminogen activator inhibitor 1	PAI-1	Plasma	LUMINEX	Linco-Millipore	pg/ml
Chromogranin A	CgA	Serum	IRMA	Cisbio	ng/ml

3.2.3 Statistical analysis

To test the association of marker levels with disease free survival and overall survival of the patients, Cox proportional hazard regression model was used to evaluate relation with markers without dichotomization by cut off. Additionally also Kaplan-Meier analysis of time-to-an-event and log-rank test for comparison of survival (OS or DFS) curve were used for markers dichotomized by different cut offs.

Overall survival (OS) was calculated as the time from the date of the surgery to the date of death or last follow up examination. Disease free survival (DFS) was calculated as the time from the date of the surgery to the evidence of cancer relapse**.** The markers baseline levels and levels during follow up (6, 12,18 and 24 months after surgery) were evaluated on its power to univariately discriminate between NSCLC and non malignancy, histology types and patients with progression and non progression by means of Wilcoxon Rank Sum test. To identify the best cut-off for differentiating benign disease and NSCLC, progression and remission, receiver operating characteristic (ROC) curves and corresponding areas under the curve (AUC) were calculated. In addition, the standard measures of diagnostic test validity, such as sensitivity calculated at

95% specificity, accompanied by 95% confidence intervals were calculated. Sensitivity was considered as the ratio between the number of patients with malignancy (or clinically positive – D+) whose marker levels were elevated over the total number of patients with malignancy. Specificity was calculated as the ratio between the number of patients without malignancy (or clinically negative – D-) and normal markers values by the total number of patients without malignancy. Positive predictive values were calculated as the ratio among the cases with elevated markers and malignancy (or clinically positive – D+) and the sum of all the cases with elevated markers. The negative predictive value was calculated by the ratio among the patients with negative results and without malignancy (or clinically negative – D-) and the total number of patients with negative results. All univariate and a multivariate time-to-an-event (OS and DFS) analysis was calculated by Kaplan Meier and Cox regression analyses (stepwise selection from all significant predictors from univariate analysis and fixed combinations). Finally, to measure the statistical dependence/correlation between two variables, the Spearman's rank correlation coefficient was used. Level of statistical significance 5% (0.05) was used. Due to non-gaussian (non-normal) distributions of marker levels the non-paramatric statistical methods were used as it was described above. All statistical analyses were done with software SPSS for Windows (version 15.0, 2006; Chicago: SPSS Inc) and SAS (version 9.1; SAS Institute).

3.3 RESULTS

3.3.1 Presurgery levels of biomarkers in benign group and patients with NSCLC

Table 22 (see tables and figures in pp. 148-185) presents descriptive characteristics of presurgery biomarker levels and the differences between control group (patients with benign lung disease) and cancer group (NSCLC patients). Figure 11 shows the box-plots of significantly different markers. We observed significantly higher levels of CYFRA 21-1, TPA, TPS, MonoTotal, SCC and TIMP-1 in the group of NSCLC patients.

3.3.1.1 Correlations of presurgery marker levels with clinicopathological features of NSCLC.

3.3.1.1.1 Presurgery tumor marker levels in relation to histological type

Within NSCLC group (Table 23), we observed differences in relation to histology with significantly higher levels of Cyfra 21.1, TK, MonoTotal and SCC in squamous cell carcinoma compared to adenocarcinoma. Table 24 and 25 show the comparison of the tumor marker levels between benign and NSCLC histologic subgroups. Significantly higher levels of CEA and CYFRA 21-1 were observed in adenocarcinoma patients compared to benign group. Patients with squamous cell carcinoma have significantly higher levels of CYFRA 21-1, TPA, TPS, MonoTotal, SCC, TIMP-1 and IL-6 in comparison to benign group.
Fig. 12a-b show the box-plots of significantly different markers in benign lung disease and NSCLC histology subgroups.

3.3.1.1.2 Presurgery tumor marker levels in relation to tumor stage

Tables 26-28 show the correlation of presurgery tumor marker levels with stage in NSCLC patients. It is interesting to point out that significantly higher presurgery levels of TPA and MonoTotal were associated with more advanced stages in squamous cell carcinoma patients (III vs. I, p= 0,0404, p= 0,0140,

respectively). In patients with advanced stages of adenocarcinoma were observed significantly higher levels of CEA (III vs. I, p= 0,0474).

3.3.1.1.3 Presurgery tumor marker levels in relation to tumor size (T)

Tables 29-31 show the correlation of presurgery tumor marker levels with tumor size (T) in NSCLC patients. Presurgery levels of CEA, CYFRA 21-1, TPA, TK, MonoTotal, MMP-9 and MCP-1 were significantly higher in larger tumors (T4) when compared with smaller ones (T1), T4 vs. T1, p= 0,0414, p= 0,0156, p= 0,0057, p= 0,0370, p= 0,0077, p= 0,0156, and p= 0,0379, respectively. These differences were found in NSCLC patients and except TK and MMP-9 in patients with squamous cell carcinoma, T4 vs. T1, CEA (p= 0,0351), CYFRA 21-1 (p= 0,0291), TPA (p= 0,0128), MonoTotal (p= 0,0240), and MCP-1 (p= 0,0196). It was not possible to find statistical differences in patients with adenocarcinoma, due to the low number of adenocarcinoma patients with large tumor size (T3, T4).

3.3.1.2 Presurgery marker levels in NSCLC patients versus benign controls: Evaluation of sensitivity and specificity.

Table 32 shows the cut off levels and the sensitivity at 95% specificity of all markers comparing patients with benign lung disease and NSCLC. The receiving operating curves (ROC) of the markers with the highest sensitivity (Cyfra 21-1, TPA, MonoTotal, IL-6 and CEA) to distinguish patients with NSCLC and benign lung disease are shown in figure 13. The area under ROC curve (AUC) higher than 0,70 was observed for cytokeratin markers Cyfra 21-1, TPA, and MonoTotal.

Table 33 shows the cut off levels, the sensitivity at 95% of specificity, and AUC of markers comparing patients in benign group and NSCLC histologic subgroups. Sensitivity of Cyfra 21-1, TPA, and IL-6 was the highest in squamous cell carcinomas (63,6%, 42,6%, 41,8%, respectively), wheraes sensitivity of CEA (46,7%) was the highest in adenocarcinoma.

3.3.1.3 The combination of biomarkers for increasing the sensitivity for diagnosis of NSCLC

In our study we found that the best combination to distinguish between benign disease and NSCLC was achieved using CEA, CYFRA21-1, IL-6 and VEGF, with a 75,6% sensitivity and 86,7% specificity, with a high predictive positive value of 97%. When CEA was excluded the sensitivity decreased to 65,9% with a 93% specificity and a positive predictive value of 98,2%.

CYFRA (>2,0 ng/mL) + CEA (>3,7 ng/mL) + IL6 (>9,8 pg/mL) + VEGF (>405 pg/mL)	
- Sensitivity (One or more positive):	**76,5%**
- Specificity (All negative):	**86,7%**
- PPV:	**97,0%**
- NPV:	**37,5%**

CYFRA (>2,0 ng/mL) + IL6 (>9,8 pg/mL) + VEGF (>405 pg/mL)	
- Sensitivity (One or more positive):	**65,9%**
- Specificity (All negative):	**93,0%**
- PPV:	**98,2%**
- NPV:	**31,0%**

3.3.2 Postsurgery follow up monitoring of NSCLC patiens

The biomarkers results during follow up monitoring of NSCLC patients were divided into the remission and progression subgroups according to the clinical status of the patients at the time of blood sampling.

Table 34 shows the mean and median levels of remission and progression samples during follow up. CEA, CYFRA 21-1, TPA, TPS, TK, MonoTotal, SCC, Chromogranin A, TIMP-1, MMP-1, MMP-7, MCP-1, IL-6, VEGF, and ICAM-1 showed significantly higher levels in NSCLC patients with disease progression. At the time of progression significantly higher levels of CEA were found in adenocarcinoma, and SCC higher levels in squamous cell carcinoma (Table 35).

Figures 14a-b show box-plots of significant markers related to response and histology during follow up (Figure 15).

Table 36 presents ROC analysis of follow up results. This table shows the cut off levels and the sensitivity at 95% specificity of all markers for detection of progression. The receiving operating curves (ROC) of the markers with the highest sensitivity (MonoTotal, Cyfra 21-1, CEA and TPA) are shown in figure 16. The area under ROC curve (AUC) higher than 0,70 was observed for all routine markers and for TIMP-1, MMP-1, MMP-7, MCP-1, IL-6 and VEGF.

3.3.3 The correlation between biomarker levels in a control group, NSCLC group and during follow up

Table 37 shows Spearman's rank correlation coefficient (r) in relation to a control group, NSCLC group, and during follow up of the related markers used in this study.

Table 38 summarise the correlation of all markers. It is important to remark that no significant correlation was found between CEA, SCC, Chromogranin A, IGF-I, MMP-9, ICAM-1 and any of other biomarkers. All the rest show correlation in different manners. All cytokeratins correlate one to each other in a control group, NSCLC group and during follow up. Some metalloproteinases and their inhibitors, as TIMP-1/ MMP-1 and TIMP-2/ MMP-2 are related to each other. Furthermore, proinflammatory and proangiogenic cytokines, IL-6/IL-8 and IL-6/VEGF correlate in NSCLC group and during follow up, and IL-8/MCP-1 correlate in control group. It is interesting to point out that cytokeratins and TK show correlation with some metalloproteinase, proinflammatory and adhesion biomarkers, but only in control group of patients. Other biomarkers, like VCAM-1 and PAI-1 correlates with cytokeratins TPA and TPS, in addition PAI-1 correlate with TIMP-1 and VEGF, mostly in the control group.

3.3.4 Correlation between pretreatment serum marker levels and prognosis

3.3.4.1 Presurgery marker levels in relation to NSCLC outcome

Presurgery marker levels were compared between groups of patients divided according to the status in last control: group 1 – patients in remission in last

control, group 2 - patients in progression in last control. The patients with the stable disease were excluded.

Table 39 shows presurgery marker levels in patients with NSCLC in relation to status in last control. It is interesting to point out that Cyfra 21-1, TPA, MonoTotal, TIMP-1, MMP-1, IL-6 and IL-8 presurgery levels were related to outcome. Significantly higher levels of these markers were found in patients with disease progression in last control (Fig. 17).

3.3.4.2 The prognostic value of biomarkers, relation with disease free survival and overall survival of patients with NSCLC

Tables 40-44 show the univariate and multivariate analyses of the presurgery markers and the main clinical and pathologic parameters related to time to progression and overall survival. The variables that were significant predictors of survival ($p < 0,05$) in univariate analyses were evaluated in multivariate analyses to know the effects of these variables on survival. Figures 18a-b and 19a-b show the DFS and OS curves of the univariate analysis significant markers and the significant clinical and pathologic parameters.

Significant relation with DFS via univariate model showed MonoTotal (p-value by means of Cox model is <0,0001 and after dichotomization by cut off 200 p-value of log-rank test is 0,0113), TPA (Cox model p= 0,0007; by cut off 77 log-rank test is 0,0081), MMP-2 (Cox model p= 0,0201; by cut off 295,15 log-rank test is 0,0022), MMP-7 (Cox model p= 0,0427; by cut off 10,52 log-rank test is 0,0340), CEA (Cox model p= 0,0021), TPS (Cox model p= 0,0228) and MMP-1 (by cut off 1481,48 log-rank test is 0,0225 because some highly elevated levels of MMP-1 were related with long DFS it was not confirmed by Cox model). Also tumor stage (Cox model p= 0,0002; log-rank test p= 0,0002) and size of tumor (T) (Cox model p= 0,0113; log-rank test p= 0,0002) were significantly related with DFS. Stepwise variant of Cox multivariate model selected MonoTotal and MMP-2 as independent predictors of DFS. Multivariate analysis of DFS by histology: in adenocarcinoma MMP-2 only; in squamous cell carcinoma MonoTotal only. Cox multivariate model using cut offs showed association of one cytokeratin (TPA or MonoTotal) with one MMP (MMP-1 or MMP-7) and tumor stage as independent prognostic factors for DFS.

Significant relation with OS via univariate model showed MonoTotal (p-value by means of Cox model is <0,0001 and after dichotomization by cut off 200 p-value of log-rank test is 0,0018), CEA (Cox model p= 0,0009; by cut off 4,75 log-rank test is 0,0457), TPA (Cox model p= 0,0044; by cut off 77 log-rank test is 0,0020), TPS (Cox model p= 0,0315; by cut off 153,5 log-rank test is 0,0116), Chromogranin A (Cox model p= 0,0070), MMP-7 (Cox model p= 0,0177), and MCP-1 (by cut off 876,63 log-rank test is 0,0042 because some highly elevated levels of MCP-1 were related with long OS it was not confirmed by Cox model). Also tumor stage (Cox model and log-rank test p<0,0001), tumor size (T) (Cox model p= 0,0195; log-rank test p= 0,0013) and lymph nodes (N) (Cox model p= 0,0322; log-rank test p= 0,019) were significantly related with OS. Stepwise variant of Cox multivariate model selected MonoTotal, N and MMP-7 as independent predictors of OS. Multivariate analysis of OS by histology: in adenocarcinoma CEA only; in squamous cell carcinoma MonoTotal only. Cox multivariate model using cut offs showed association of one cytokeratin (TPA or MonoTotal) with MMP-7, MCP-1, chromogranin A and tumor stage as independent prognostic factors for OS.

3.4 DISCUSSION

The current study is the first attempt to compare comprehensively the well-known lung serum tumor markers with some of promising serum biomarkers and analyze their clinical utility in the hope of finding a marker which alone or in combination with traditionaly used lung serum tumor markers could be helpful in management of patients with NSCLC. It is particularly important and recommended by European Group on Tumor Markers (EGTM) that potential new markers are compared with existing markers.

Selection of biomarkers was based on published reports for each biomarker showing value for at least one of the following functions: NSCLC diagnosis, staging, prognosis, and post-surgery surveillance [128;143-152] or involvement in biologic processes implicated in disease progression [96;153;154]. Based on these criteria, we found 22 biomarkers with the most promising profiles : 8 standard tumor markers (cytokeratines Cyfra 21-1, TPA, TPS, and MonoTotal, CEA,

SCC, TK, Chromogranin A) and 14 potential useful biomarkers including pro-inflammatory cytokines IL-6, IL-8, MCP-1, pro-angiogenic cytokine VEGF, matrix metaloproteinases MMP-1, MMP-2, MMP-7, MMP-9 and their inhibitors TIMP-1 and TIMP-2, adhesion molecules ICAM-1, VCAM-1, growth factor IGF-1, and PAI-1 stimulating tumor growth and angiogenesis.

With a view of evaluating the clinical relevance of these markers for NSCLC we measured serum or plasma levels of these 22 markers in group of 93 patients with NSCLC undergoing radical surgery and in group of 20 patients with benign lung disease. Based on means of Wilcoxon Rank Sum test, CYFRA 21-1, MonoTotal, TPA, TPS, CEA, SCC, Chromogranin A, TIMP-1, MMP-1, MMP-7, IL-6, MCP-1, VEGF, TK, and ICAM-1 were found to be significantly higher in patients with NSCLC in the moment of diagnosis or during follow up than in control individuals with benign lung disease. The mean serum levels of MMP-2, IL-8, TIMP-2, MMP-9, PAI-1, IGF-1 and VCAM-1 did not differ in the sera of NSCLC patients as compared with controls.

We have demonstrated that in the sera of NSCLC patients several circulating biomarkers were frequently increased, but only some of them were of clinical relevance. Owing to the number of markers analyzed and for to show potential clinical relevance of each biomarker for NSCLC our results will be discussed below one by one in related groups.

In recent years there has been growing evidence of the usefulness of cytokeratin tumor markers in the management of NSCLC patients. High levels of these markers have been reported in patients with this malignancy and several studies have suggested that cytokeratins such as CYFRA 21-1, TPA, and TPS are prognostically significant markers in patients with NSCLC [93;94;97;149;155;156].

Cytokeratin 19 fragments (Cyfra 21-1) has been the most studied cytokeratin marker. The diagnostic value of Cyfra 21-1 in NSCLC has been established. Our results related to Cyfra 21-1, as other authors reported [97;157-165], confirm that Cyfra 21-1 is significantly elevated in NSCLC, and it is the most sensitive tumor marker for NSCLC, particularly squamous cell tumors. A sensitivity of

54% at specificity 95%, as we determined, was equivalent to those reported by previous reports (the sensitivity varied between 23% and 78%).

The sensitivity of the marker Cyfra 21-1, when used to detect lung cancer, is dependent on histological type and stage of the disease [97;128;147]. In our study Cyfra 21-1 was closely related to histology, with higher levels (median value 2,7 ng/ml) and sensitivities (64 % at 95% specificity) in squamous cell carcinomas. In comparison, patients with adenocarcinomas had median value 1,6 ng/ml and sensitivities 37% at 95% specificity. These findings are in agreement with published data [128;160;166-173]. One of the first and largest evaluations of Cyfra 21-1 was a European multicentre study, showing 57% sensitivity at 96% specificity for squamous cell carcinomas, which was higher than for others included markers, SCC, CEA, and TPA [174].

In our study Cyfra 21-1 serum levels were related to tumor stage, with significantly higher levels in stage II-III, suggesting that the Cyfra 21-1 level reflects the tumor burden. Statistical difference was found for T1 tumors versus T2 and for T1 tumors vs. T4 in group of NSCLC patients ($p= 0,0360$, $p= 0,0156$, respectively) and in squamous cell carcinoma group ($p< 0,0001$, $p= 0,0291$, respectively). Similar data were obtained by other investigators [160;167;170;171;173;175], who also suggested that CYFRA 21-1 might be considered as a marker of tumour mass and provide an important adjunct to the clinical staging. In addition, they pointed out that it is impossible to predict operability of NSCLC (difference between stage IIIa and stage IIIb) using the serum level of this marker. Satoh at al. [176] reported that measurement of serum Cyfra 21-1 provides discriminative information between metastatic and non-metastatic NSCLC. If Cyfra 21-1 levels are more than 3,4 ng/ml, distant metastasis should be examined extensively.

Several recent studies have suggested that cytokeratins 8, 18 and 19 have a role in tumor progression. The increased expression of these CKs was found during progression of some human tumors including lung cancer [177;178]. In accordance with the data of other autors [170;179-183], we also found that serum Cyfra 21-1 levels increased with disease progression, with 52% sensitivity at 95% specificity. From our results we suggest that Cyfra 21-1 is a useful cytokeratin marker for the postoperative follow up and has a diagnostic value in the early detection of recurrent disease in NSCLC patients.

Our study showed that patients with pretreatment elevated CYFRA 21-1 levels had shorter disease free and overall survival time than patients with normal serum CYFRA 21-1 levels, but the tendency did not reach statistical significance. Similar data were obtained by other autors [184;185]. In contrast to our results, other studies demonstrated that Cyfra 21-1 was an independent prognostic factor in both early and late stages of NSCLC [149;156;173;186-193], elevated levels of this marker at diagnosis were associated with a poor prognosis and short patients survival time. There could be several reasons for the contradictory results in our study and studies from other authors on prognostic value of Cyfra 21-1: the heterogenity of the study population (mixture of early and advanced stages, mixture of various histological types), the use of different methods to determine cut off values, the heterogenity of studied co-parameters such as disease stage, TNM, performance status. The exact type of used immunoassay for Cyfra 21-1 measurement varied widely.

Other cytokeratins: TPA, TPS and MonoTotal were evaluated as well.

Tissue polypeptide antigen (TPA) is a circulating complex of polypeptide fragments of CKs 8, 18, and 19. Serum levels of TPA have been found to correlate with important clinical parameters: the burden of tumor, its proliferating activity and the consequent prognosis in different types of cancer.

In NSCLC, TPA is inferior to Cyfra 21-1, with respect to its sensitivity and specificity for diagnosis and the detection of progression of NSCLC [160;186;194]. We reported TPA sensitivity for diagnosis of NSCLC of 39.3% at 95% specificity, in comparison with studies of other authors showing sensitivity in a wide range between 39,4 and 76 % at 95% specificity [155;160;194;195]. This lack of TPA sensitivity in our study might be due to a fact that 73% of enrolled patients showed early lung cancer stages (Ia - IIb) while in other studies showing high sensitivity were assayed the sera of patients mostly in advanced stages.

Our investigation revealed, in accordance with the published data [196-198], a dependence on tumor stage with TPA levels increasing with stage II and III in NSCLC patients. We also observed significantly higher levels for stage III in compare with stage I ($p= 0,0404$) and for T4 tumors in compare with T1 tumors ($p= 0,0128$) in patients with squamous cell carcinoma. These findings suggest that the pretreatment TPA level correlates with tumor burden and may

contribute to staging of NSCLC. In comparison with other conventional staging methods Buccheri at al. [126;199] reported that using appropriate threshold values of TPA it should be possible to predict NSCLC resectability with a diagnostic accuracy similar to that routinely achieved by CT.

Increasing TPA serum levels indicate disease progression with sensitivity of 50% at 95% specificity. As other autors [196-198;200] we also suggest that TPA might be considered as an early indicator of relapse during follow up in NSCLC. Changes of TPA often precede detection of relapse by other conventional methods. Increasing of TPA levels during follow up should be indicator for clinicians to examinate the patients deeply including imaging methods. This could help in early diagnosis and treatment of tumour relapse.

Tissue polypeptide antigen has been proposed also as a tool for predicting the course of disease. The prognostic capability of TPA may be anticipated by the knowledge of the molecule metabolism. The substance is synthetized during the S- to M-phase of the cell cycle and released upon proliferation into the bloodstream. Thus, the concentration of the antigen is an indicator of the rate of cell division and tumor aggressiveness, and, therefore, of the host survival [196].

In our study univariate survival analyses demonstrated that TPA elevation was predictive of poor survival expectancy (DFS: p= 0,008, OS: p= 0,002). Taking into account other significant variables (histology, stage), TPA was confirmed as independent prognostic variable for disease free and overal survival time (DFS: hazard ratio 2,36; confidence interval 1,13 – 4,94, and for OS: hazard ratio 3,07; confidence interval 1,36 – 6,94). In accordance with the published data [155;186;196;201-203] we found that TPA serum level at the time of diagnosis was reliable predictor of disease free and overall survival, high value of this marker being associated with worse prognosis. Our findings suggest that in completely resected NSCLC, TPA preoperative serum levels better than CYFRA 21-1 levels might provide a useful tool for stratifying subgroups of patients with different chances of disease recurrence after surgery.

Our results concerning to Cyfra 21-1 and TPA confirm Buccheri et al. studies [118;155], that both CYFRA 21–1 and TPA are valuable markers in the evaluation of disease extent at the first clinical presentation, the evaluation of posttreatment status, and the prediction of the outcome.

Tissue polypeptide-specific antigen (TPS) is an assay that detects one major epitop on cytokeratins 18 by the M3 monoclonal antibody. This M3 epitope is proposed as more specific for cell proliferation. TPS is not so commonly studied marker in conjuction to lung cancer, but the published results seems to be clinically valuable.

In compare with other CKs markers TPS demonstrated low sensitivity, around 30%, for assessing diagnosis of NSCLC and histologic type, when we used the cut off values providing 95% specificity in patients with benign lung disease. Studies of other authors showed that TPS sensitivity ranges between 13-54% [194;204-207]. In our study sensitivity-specificity curves (ROC) demonstrated a higher accuracy of Cyfra 21-1 (0,80) and TPA (0,73) in comparison with TPS (0,67) to distinguish patients with NSCLC and benign lung disease. Sensitivity of Cyfra 21-1 and TPA was the highest in squamous cell carcinomas (63%, 42%, respectively), wheraes sensitivity of TPS did not vary according to histology. TPS distributions as for the others cytokeratins varied significantly according to stage of disease, being more elevated in stage II and III. Our results on TPS were comparable to the reported by other authors [194;204-208].

The usefulness of TPS for detection of disease progression showed in our study a sensitivity of 44% at 95% specificity, and was inferior than CYFRA 21-1 and TPA, 52% and 50% respectively; similar results were reported by others [205;206;209].

As was showed in some studies [191;205;206;209;210], we also observed that presurgery high levels of TPS were related to adverse outcome in NSCLC. Univariate analysis of disease free and overal survival showed that patients with increased plasma levels of TPS had reduced both survivals. In multivariate analysis TPS was not found to be independent predictor of survival contrary to TPA.

MonoTotal

Our work was also focused on MonoTotal, a new cytokeratin-based tumor marker utilizing a combination of three monoclonal antibodies directed against soluble fragments of cytokeratin 8, 18 and 19. There is lack of published data on this novel marker. The existing few studies demonstrate its utility in esophageal carcinoma [211]. They show correlation to increased tumor burden

and this marker might, in conjunction with other clinical parameters, help the clinician in estimating the prognosis of the individual patient for this diagnosis.
There is only one published study showing its usefulness in NSCLC [121]. MonoTotal seems to be a potentially very interesting serum marker that, in conjunction with other clinical data, might be used for monitoring of patients with NSCLC.
We have observed in our study MonoTotal sensitivity for diagnosis comparable with TPA and TPS, the levels of this marker were correlated to tumour burden as all the other cytokeratins. The sensitivity for diagnosis of progression during follow up is reaching the comparable values as other cytokeratins and CEA app. 50 % at 95% specificity. Both used variants of cox multivariate model showed MonoTotal as independent predictor of DFS and OS.
We also demonstrated that serum MonoTotal levels in squamous cell carcinoma are significantly higher than that of adenocarcinoma. We observed that MonoTotal serum levels were related to tumor stage, with significantly higher levels for stage III in compare with stage I in patients with squamous cell carcinoma (p= 0,0140). Analysis of patient survival also suggested that serum MonoTotal levels in squamous cell carcinoma demonstrated tendency to be more beneficial in the prediction of disease free and overall survival than in adenocarcinoma. Based on these findings, preoperative serum MonoTotal levels appear to be more valuable in squamous cell carcinoma than for adenocarcinoma in the prediction of disease progression and prognosis.
In our opinion MonoTotal in combination with other markers (will be dicussed further), may be used for help in the diagnosis for NSCLC patients, for early detection of relapse and as survival factor. On the other hand we have shown also that for diagnosis there are only marginal and non significant differences between MonoTotal, Cyfra 21-1, and TPA, but MonoTotal seems to be promising marker for prognosis. Our results, confirm the study of Eriksson et al [121].

In summary, a cytokeratin marker assay should be performed both before treatment (to exploit their capability for giving an insight into the severity of the illness and its possible outcome), and, serially, during and after treatment (to help to decide on the status of the disease and its response to the treatment).

While there are mostly comparable results among cytokeratins and because they all monitor the same biologic process they should not be combined together but with the markers from another group.
Today the choice of the cytokeratine subspecies depends on many nonclinical factors, such as the traditional use of a particular laboratory or most important the cost of the kits in each particular country [155].

Carcinoembryonic antigen (CEA) was one of the first markers measured in patients with NSCLC. In our study we reported CEA sensitivity for diagnosis of NSCLC of 34,1% at 95% specificity, in comparison with studies of other authors showing sensitivity in a wide range between 17% to 78 % at 95% specificity, typically around 45% [147;160;169;205;212;213]. This lack of CEA sensitivity in our study, as commented before, might be due a fact that an important proportion of enrolled patients showed early lung cancer stages (I - IIb). In our study analysis of receiver operating characteristic (ROC) curves demonstrated less diagnostic accuracy of CEA (0,61) in comparison with cytokeratin tumor markers in distinguishing patients with NSCLC from benign lung disease when using cut off value of 3,70 ng/ml.
On the other hand the usefulness of CEA for detection of disease progression during follow up showed in our study a sensitivity of 50% at 95% specificity that was analogous to cytokeratins CYFRA 21-1, TPA and MonoTotal sensitivity, 52%, 50%, and 52%, respectively. As other autors [160;169;205;214] we also suggest that CEA might be considered as a useful indicator of relapse during follow up of NSCLC patients.
Preoperative CEA serum levels seem to be of prognostic interest in NSCLC patients, but there are no unanimous opinions. In our study univariate analysis demonstrated that NSCLC and adenocarcinoma patients with preoperative CEA levels >4,75 ng/ml had a significantly unfavourable prognosis. Most studies using univariate analysis showed a significant relationship between high preoperative CEA levels and poor prognosis [189;191;202;214;215], but the studies by Blankenburg at al. [184] and Foa at al. [187] do not confirm these data. According to our results, in multivariate analysis CEA was not an independent prognostic factor in NSCLC patients, while this tumor marker was an independent prognostic factor in patients with adenocarcinoma and elevated pretreatment

levels (> 4,75 ng/mL) (data are not shown). Several studies reported that CEA preoperative serum levels might provide a useful tool for stratifying subgroups of patients with different chances of disease recurrence after surgery, mainly in patients diagnosed with lung adenocarcinoma [157;175;189;191;214-219].

In conclusion, as stated at the 1980 consensus conference of the National Institutes of Health at Bethesda, CEA assays are useful in lung cancer clinical management.

Squamous cell carcinoma antigen (SCC antigen)

In the present study, the diagnostic sensitivity of the SCC antigen at 95% specificity for NSCLC patients was 16,5% (cut off 1,8 ng/ml) and 23,6% for squamous cell carcinoma (cut off 1,9 ng/ml). Similar rates were reported by other authors [128;163;169;212;213]. Other tumor markers such as cytokeratins and CEA are more sensitive in NSCLC than SCC, but their relationship with the histology is not so clear. As Molina at al. reported [128;147;163], the combination of these tumor markers with SCC, a tumor marker mainly found in squamous tumors, improves their diagnostic utility and aids to suggest the histological diagnosis in NSCLC patients.

We found no correlation between increased SCC levels and extent of disease. Comparable observations have also been reported by others [163;169;204]. Elbert at al. [220] reported that the SCC concentration depends on tumor size, but there were no such dependencies between the concentration of this marker and nodal status.

Contradictory results have been published concerning the prognostic effect of pretreatment SCC levels in patients with NSCLC, in particular in squamous cell carcinoma patients. In our study preoperative SCC level had no prognostic significance for survival of NSCLC patients. Whereas Moro et al. [214] concluded that SCC had no prognostic value, Sanchez de Cos et al. [221] and Kulpa at al. [169] found it to be a predictor of survival. De Bruijn et al. [222] suggested that determinant factor of the prognostic value of the SCC antigen may result from biochemical properties of this antigen as an inhibitor of proteases. Serine and cysteine proteases and their inhibitors are thought to be involved in the degradation of components of the extracellular matrix and play an important role in the process of tumor invasion and metastases.

In term of detecting relapse, we found that serum SCC levels during follow up increased with disease progression, with significantly higher levels in squamous cell carcinoma patients (p= 0,0262)..

Thymidine kinase (TK) is considered to be an important proliferation tumor marker that can be detected in the serum of patients diagnosed with different types of cancer [129-131] . Several studies have suggested that determination of thymidine kinase helps to monitor the follow up of solid tumors and haematological malignancies as well as indicating the efficacy of adjuvant and palliative chemotherapy [130;132-134;223]. In a recent study Chen [129] et al. reported that serum TK values correlated with the clinical stage in patients with lung, esophagus, thyroid, and gastric carcinomas. In the same study they reported that serum TK declined in all tumor groups after treatments. The TK was low or decreasing during treatment in patients with complete response or partial response, but high or increasing in patients with stable disease or progressive disease. Li et al. [224] reported that serum TK had a prognostic value and was a reliable marker for monitoring the response to surgery of NSCLC patients.

Agree to these results, we observed significantly higher levels of serum TK during follow up in patients with disease progression. Sensitivity in this case (52% in 95% specificity) was reaching the comparable value as cytokeratins TPA, Cyfra 21-1, and MonoTotal and CEA. Our study showed that the elevation of TK serum levels during follow up was a helpful marker in predicting relapse during follow up, but it is necessary to note that TK serum levels did not correlate with prognosis in our group of patients during the time of the initial diagnosis. We observed also a trend with significantly higher levels of TK in patients with squamous cell carcinoma; conclusion that was not reported before.

In our opinion serum TK could be useful for postsurgery disease monitoring. It is important to point out that elevated levels of TK must always be interpreted together with a detailed knowledge of the patient's condition because all other possible non-specific causes (viral infections, pernicious anemia, inflammatory or autoimmune diseases) of elevated serum levels must be excluded [133]. This could be the reason that in our study were not found statistical significant differences in presurgery TK levels between NSCLC patients and control group.

Chromogranin A (CgA) is well established as a serum marker for neuroendocrine tumours and has also been associated with some non-neuroendocrine tumours including lung cancer [225]. Elevated CgA serum levels found in patients with non-neuroendocrine tumors could indicate neuroendocrine (NE) differentiation in the tumor [226]. Neuroendocrine differentiation were reported in a greater proportion of NSCLC [227;228]. The clinical relevance of NE differentiation in NSCLC has been debated during recent years. Even though some studies have shown a prognostic significance of NE differentiation in subgroups such as adenocarcinoma [229;230], the present opinion is that the finding of some tumor cells with NE features does not seem to influence prognosis or response to treatment [231-233].

Eleveted levels of CgA in NSCLC are reported in a few studies, but no correlation between CgA tumor tissue expression and CgA serum levels has been observed [228;234]. In our study we were not found significant differences in presurgery CgA serum levels between NSCLC patients and control group, but we observed significantly higher levels of serum CgA during follow up in patients with disease progression. In agreement with the study of Nisman et al. [235] and Gregorc et al. [234] we found elevated serum levels of CgA before treatment as an independent indicator of poor prognosis. In our study a multivariate Cox regression analysis identified CgA elevation as independent prognostic variable for overal survival time (hazard ratio 3,93, confidence interval 1,16 – 13,33).

Interestingly, circulating CgA was associated with worse patient conditions and more advanced NSCLC [234]. Zhang et al. [236;237] reported CgA as a strong and independent indicator of prognosis in critically ill patients. It is possible hypothesize that serum CgA in NSCLC patients reflects stress-related systemic neuroendocrine activation associated with worsening of patient condition. The lack of correlation with CgA expression in tumor tissues suggests that increased circulating levels of CgA are more likely related to worse patient conditions than to neuroendocrine differentiation.

In addition, both cardiovascular and respiratory disorders may activate the NE system and increase the circulating levels of CgA [238;239]. Renal and hepatic failure and medication with proton pump inhibitors could have contributed to the

elevated levels of CgA [240;241]. This suggests that CgA may be associated not only with tumors, but also with other inflammatory diseases or organ failure.

Matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidases which generally play an important role in the process of extracellular matrix (ECM) and basal membrane degradation in relation to tumor invasion, metastasis and angiogenesis as well as in numerous other diseases [242]. The MMP family is a continually growing group, now comprising more than 20 enzymes. Based on their substrate specificity, MMPs have been divided into distinct subclasses: collagenases (MMP -1,-8, and -13), gelatinases (MMP-2 and -9), lysins (MMP-3, -7, -10, and -11) and elastases (MMP-12). MMP activity is inhibited specifically and reversibly by endogenous inhibitors known as tissue inhibitors of metalloproteases (TIMPs). To date, four TIMPs have been identified: TIMP-1, -2, -3, and -4 [243-247]. The role of MMPs and TIMPs in tumor growth, metastasis, and angiogenesis has been widely investigated. In tumorogenesis, it is clear that MMP participate in many deregulated signaling pathways that are used by the tumor to promote cancer cell grows and angiogenesis, side-step apoptosis, and for evasion of protective host responses. A positive correlation between tumor progression and the expression of multiple MMP family members in tumor tissues has been demonstrated in numerous human and animal studies. The functions we have already known make MMPs a promising prognostic and diagnostic tumor biomarkers [246-250].

The potential role and regulation of MMPs have been the subject of a number of past studies in lung cancer that have examined MMP expression and/or levels in lung cancer specimens [251-259]. These studies usually focused on one MMP type or a single class of MMPs. The studies on serum/plasma circulating levels of MMPs and their inhibitors in patients with NSCLC are still limited and the results are also heterogenous [260-265]. Future studies that simultaneously measure the relationship between circulating and tumor tissue levels of MMPs in patients with NSCLC to that of clinical outcomes would be warranted.

MMPs in a collected blood sample have shown prognostic potential in several different cancers. For example, plasma MMP levels (particularly MMP-2, -7, and -9) have been studied in a variety of cancers, including colon cancer [266],

breast cancer [267], gastric cancer [268], and renal cell carcinoma [269]. Several studies have reported that plasma/serum levels of MMP-9 and TIMP-1 are elevated in patients with NSCLC when compared in patients with nonmalignant lung diseases or healthy controls [261-264].

In our study we studied serum/plasma levels of a large number of MMP types, from different MMP classes: collagenase MMP-1, gelatinases MMP-2, and -9, and matrilysin MMP-7. Overall, presurgery serum/plasma MMP levels were increased in NSCLC patients compared with control group, but the tendency did not reach statistical significance. In term of detecting relapse, we found that serum MMP levels during follow up increased with disease progression, with significantly higher levels of MMP-1 (p= 0,0180) and MMP-7 (p= 0,0122). We also observed that MMP-1, and MMP-7 serum/plasma levels at diagnosis were reliable predictors of recurrence, only MMP-7 elevation was independent prognostic biomarker for disease recurrence and overal survival time.

Focusing on the MMP-1, that we analyse in this study, Li et al. [270] reported that high plasma MMP-1 levels were associated with advanced stage of the disease and significantly lower overall survival rate of the patients. They conclude that MMP-1 levels in plasma/serum represent a potential and clinically relevant biomarker for the prognosis of patients with lung cancer. Agree to these results, we observed significantly higher levels of plasma MMP-1 during follow up in patients with disease progression (p= 0,0180) and we found elevated plasma levels of MMP-1 before treatment as an independent prognostic variable for DFS in NSCLC patients (hazard ratio 2,93; confidence interval 1,20 – 7,20). We differ with Li et al. in the results related to the histology; however our results are consistent with a recent work Shah et al. [271] examining MMP profile in NSCLC tissue samples. They found significantly higher levels of MMP-1 in NSCLC tissue compared with normal lung tissue, and increased levels of MMP-1 were particularly pronounced in squamous cell carcinoma samples. We observed a trend with higher levels of MMP-1 in squamous cell carcinoma in the moment of progression and with a higher sensitivity value at 95% specificity in presurgery plasma levels in patients with squamous cell carcinoma when is compared with adenocarcinoma.

The role of MMPs produced by endothelial cells, especially MMP-2, appear to be crucial for tumor angiogenesis, which is a requirement for cancer growth and dissemination [242]. There are just few studies focused on MMP-2 plasma/serum levels and lung cancer, more studies evaluated MMP-2 expression or levels in lung cancer specimens. Ylisirniö at al. [265] analyzed the serum levels of MMP-2 in NSCLC patients. In our and in their study, significant difference was not recognized between MMP-2 in NSCLC patients and that in control group.

In past study Zucker et al. [272] compared plasma MMP-2 levels in healthy individuals, patients with various types of cancer including lung cancer, and hospitalized patients with chronic diseases other than cancer. Their results demonstrated that MMP-2 levels are not increased in cancer patients regardless of the type, clinical stage of cancer, and the extent of disseminated malignancy, less than 15% of the cancer patients evaluated had plasma MMP-2 levels above the normal range. These results suggest that MMP-2 is not useful as plasma marker for lung cancer diagnosis.

On the other hand in several studies MMP-2 has been reported to be increased in the NSCLC tissues and MMP-2 expression has been reported to be indicator of poor prognosis in patients with NSCLC [253;255;271;273;274]. Shah et al. [271] reported that the levels were increased by approximately 3-fold in the NSCLC tissues, with no significantly differences between squamous cell carcinoma and adenocarcinoma. In our study univariate analysis demonstrated that NSCLC and adenocarcinoma patients with preoperative MMP-2 levels >295 ng/ml had a significantly unfavourable prognosis, loosing the significance in multivariate Cox model using cut off. However, stepwise variant of Cox multivariate model selected MMP-2 as independent predictor of DFS in NSCLC and adenocarcinoma groups. On the basis of our results a prospective study that measures the relationship between circulating levels of MMP-2 in patients with NSCLC to that of clinical outcomes could be available.

Despite recent progress in this area, there have been few studies on MMP-7 in NSCLC. As far as we know, there are not published works that study the relation between MMP-7 plasma/serum levels and lung cancer. The existing few reports are related to the levels or expression of this marker in lung cancer tissue [252;254;257;271;275-277]. Unlike the other members of the MMP family, MMP-7

is expressed by tumor cells themselves but not by the peritumoral stromal cells [250;276], indicating that MMP-7 could be useful as a tumor-associated biomarker and a target of therapeutic intervention. In the study from Shah et al. [271] several MMPs levels in NSCLC tissue were analysed, and it was shown that tissue levels of MMP-7 were increased by 10-fold in NSCLC compared to normal lung tissue.

The impact of MMP-7 expression on the prognosis in NSCLC has been evaluated by a few studies [252;254;257;275;276]. Liu D. et al. [276] study of NSCLC tissue found that MMP-7 expression was higher in squamous cell carcinomas than in adenocarcinomas and correlated with significantly lower overall survival in NSCLC patients. In a similar study [254] was reported that the MMP- 7 status was a significant predictor for the overall survival in NSCLC and correlated inversely with overall response to chemotherapy. These results support our study findings suggesting that MMP-7 serum levels are closely related to NSCLC prognosis. In our work a multivariate analysis identified MMP-7 elevation as one of the prognostic biomarker for overal survival time (hazard ratio 4,82; confidence interval 1,14 – 20,46) and disease free survival (hazard ratio 2,25; confidence interval 1,12 – 4,51). However, this marker showed low sensitivity at 95% specificity for the diagnosis of NSCLC (15,3%), for adenocarcinoma (10%) and squamous cell carcinoma (18,2%). These findings suggest that MMP-7 expression and MMP-7 serum levels in patiens with NSCLC may be significant prognostic factors. To our knowledge, our study appears to be the first study to identify a correlation between MMP-7 serum levels and the clinical outcome in NSCLC patients.

Future studies may support our hypothesis that the pretreatment serum level of MMP-7 is a new powerful prognostic marker and can help stratify NSCLC patients with stage I-III disease into low- and high-risk groups. These results need to be confirmed by further prospective trials studying prognostic factors in NSCLC.

Furthermore, we studied the utility of plasma MMP-9 levels. Several studies have reported increased circulating MMP-9 levels in patients with NSCLC when compared with those in patients with nonmalignant lung diseases or healthy controls [261-264]. In our study no significant differences in MMP-9 plasma levels

between NSCLC patients and those in benign control group were observed. However, the increase in MMP-9 plasma levels was correlated with increased T stage, statistical difference was found for T2 tumors versus T1 (p= 0,0055) and for T4 tumors vs. T1 (p= 0,0156). This suggests a specific role for MMP-9 in tumor proliferation and in the progression of NSCLC. Accordingly, Gouyer et al. [278] reported that MMP-9 tumor expression was correlated with an increase in T stage. In the study of Iizasa et al. [261] they also reported that levels of plasma MMP-9 in NSCLC patients could be a beneficial adjunct for assessing the tumor burden of NSCLC. They investigated the relationship between circulating plasma MMP-9, its expression in tumor samples, and other clinical features in 73 patients with NSCLC. The plasma concentration of MMP-9 was significantly elevated compared to that of healthy control group ($p < 0.0001$). However, this elevation did not seem to correlate with MMP-9 production by cancer and stromal cells. They suggest that macrophages, which physiologically produce MMP-9, may be responsible for the increased MMP-9 levels in the tumor burden of NSCLC, and that tumor tissues may contribute to the stimulation of these cells through the production of regulatory factors, including cytokines.

The reports about the prognostic signification of MMP-9 plasma levels in NSCLC are comparatively few and some of them are controversial. In Ylisirnio et al. [265] study high MMP-9 plasma levels correlated to a poor survival in lung cancer patients and they suggest that MMP-9 could serve as a prognostic marker, whereas in the study of Laack et al.[264] the pretreatment MMP-9 serum levels in patients with metastatic NSCLC did not correlate with overall survival [264]. Similarly in our study pretreatment MMP-9 plasma level had no prognostic value in NSCLC patients. Our finding of a missing correlation between MMP-9 serum levels and survival in patients with NSCLC could be the explanation for the negative results of randomized trials with matrix metalloproteinase inhibitors [279]. Further studies should evaluate whether matrix metalloproteinase inhibitors can prolong survival as adjuvant treatment in patients with early disease who have increased MMP-serum levels pre- or/and postsurgery.

MMP-9 has been reported to be increased in lung cancer tissue [252;256;271;280] and MMP-9 expression has been releated to poor outcome in patients with NSCLC [258;281] , whereas some studies did not give the same results [252;282]. In Cox et al. [283] study, included 169 NSCLC patients with stage I–IIIA, expression of MMP-9

in tumour tissue was identified as an independent prognostic factor. Contradictory results exist in a similar study [252] that consisted of 212 patients with resected NSCLC, showed that high MMP-9 expression indicates aggressive tumor behaviour, however MMP-9 expression had no prognostic value in NSCLC patients.

It is important to note that the measurement of MMPs in body fluids, in particular serum or plasma, can be influenced by the type of fluid and method of collection and storage. For example, basal MMP-9 levels in serum/plasma can be influenced by the use of EDTA or heparin [284], a problem that can be alleviated by using sodium citrate instead [285]. Another issue to be considered is a sample storage. For example, it has been reported that plasma MMP-9 is unstable and degrades rapidly even when stored at -80°C [286].

For all these reasons, in our opinion, methods standardization and multicentric prospective studies are needed before reach reliable conclusions.

Tissue inhibitors of metalloproteinases (TIMPs) are well-known as inhibitors of tumor growth and metastasis by inhibiting MMP activity. However, increasing evidence indicates that TIMPs are multifunctional proteins, with apparent paradoxical effects on tumor progression. Elevated TIMPs levels are reported in association with cancer progression and identified as poor prognostic indicators in several tumor types including colorectal, prostate, breast, ovarian and lung cancer [287;288]. The mechanism explaining a paradoxical effect of TIMPs in tumor progression is not fully understood and currently is under intense investigation. It has been shown that both TIMP-1 and TIMP-2 promote cell grows [289-291] and TIMP-1 has antiapoptotic activity [292], which may partially explain the paradoxical role in tumor progression.

In our study we also considered that TIMP-1 plasma levels were significantly associated with the progression of NSCLC patients. In agreement with our findings, Ylisirnio at al. [265] and Suemitsu at al. [293] reported that serum concentration of TIMP-1 was significantly higher, whereas the serum TIMP-2 was lower in patients with lung cancer than in control group. In a recent study, Safranek at al. [257] reported that the expression of TIMP-1 mRNA was enhanced in the lung tumor tissue but the expression of TIMP-2 mRNA was not increased in the carcinoma or in the benign disease or the normal lung tissues.

In accordance with previous study of Suemitsu et al. [293], we observed significantly higher levels of TIMP-1 in squamous cell carcinoma when compared with the control group and a trend with higher results in the plasma of patients with squamous cell carcinoma than in those with adenocarcinoma. In analysing of T factors, the plasma TIMP-1 levels were significantly higher for T3 tumors in comparison with T1 tumors (p= 0,0325).

According to previous studies [278;294-296] , elevated TIMP-1 levels and TIMP-1 RNA expresion were associated with a poor prognosis and shorter survival time in NSCLC patients. A study from our laboratory (in press) has confirmed a relationship between TIMP-1 mRNA expression in NSCLC tumor tissue and prognosis. We found that higher tissue level of TIMP-1 is related to an adverse prognosis of NSCLC patients. However, our results did not show a relationship between TIMP-1 plasma levels and prognosis. In addition, no statistically significant correlation between TIMP-1 mRNA expression and TIMP-1 plasma levels was recorded either.

Our present study showed that patients with elevated TIMP-1 levels at diagnosis had shorter survival time than patients with normal plasma levels, although the difference was not significant. The value of TIMP-2 did not have any effect on survival.

Despite the promising positive results and also the light shed on the functions and molecular pathways of TIMP-1, the value of assessment of serum/plasma TIMP-1 for the prediction of survival in NSCLC patients is still uncertain for routine clinical use and need to be further investigated in large sample-size studies.

To date, there are only few reports addressing the level of TIMP-2 in the sera and/or plasmas of lung cancer patients and the potential value of TIMP-2 as a serological marker of lung cancer. Therefore, whether TIMP-2 level is increased in lung cancer patients' sera/ plasmas needs to be further investigated.

In summary, the results of our study highlight the important role of MMPs and TIMPs in the neoplastic progression of NSCLC. Our finding that MMP-1, MMP-2 and MMP-7 could serve as prognostic markers to predict aggressive behaviour of NSCLC may be relevant clinically for identifying patients with NSCLC who

have a greater risk of disease recurrence after surgery and who may benefit from adjuvant chemotherapy.

The immune system and inflammation are implicated in the pathogenesis of cancer [297;298]. Cytokines, due to the accumulating evidence of their involvement in the development and progression of lung cancer [299-301], may be potencially useful as serum tumor markers in patients with NSCLC. To explore this hypothesis we measured the serum levels of proinflammatory and proangiogenic cytokines interleukin (IL) 6, IL-8, monocyte chemotactic protein-1 (MCP-1), and vascular endothelial growth factor (VEGF). Serum concentrations of these proinflammatory and proangiogenic cytokines and their clinical implications in NSCLC patients have been analyzed in several studies [121;143;145;146;150;264;302-316]. They have reported that serum levels of these cytokines are elevated in patients with NSCLC when compared in patients with nonmalignant lung diseases and in healthy controls. In agreement with these studies we also found elevated presurgery concentrations of these cytokines in the sera of NSCLC patients compared to a control group, but the tendency did not reach statistical significance. In addition, we observed a trend with significantly higher levels of IL-6 in patients with squamous cell carcinoma, in agreement with other authors [145;317]. In our study no difference was found in cytokines levels between the various stages in NSCLC. In term of detecting relapse, we found that serum cytokines levels during follow up increased with disease progression, with significantly higher levels of IL-6 (p= 0,01), MCP-1 (p= 0,0059), and VEGF (p= 0,0062). We also observed that high values of MCP-1 at diagnosis were associated with a worse prognosis.

The data available on the relationships between cytokine levels and clinicopathological parameters of NSCLC are fragmentary and often inconsistent. Inconsistent results may have been due to differences in genetics, tumor characteristics (e.g. stage, tumor size or histology), race, or other exposures, including infection, cigarette smoking or inflammatory diseases.

One should be aware that elevated serum cytokine concentrations frequently accompany various diseases, particularly those with an inflammatory component, and also cigarette smoking is known to influence the levels of circulating cytokines [318]. To minimize the influence of accompanying

inflammatory conditions on the measured cytokine concentrations, patients with any signs of infection or obstructive pneumonia were not enrolled. Cigarette smoking has presumably had a comparable effect in our patients, as nearly all of them were former chronic smokers.

Focusing on interleukin 6 (IL-6), that we analyse in this study, IL-6 levels have been reported to increase with NSCLC stage [145;305;308;312;319;320] as a marker of tumor advancement. However, other authors [317] and our study found no correlation between IL-6 and the stage of the disease. Our observation that tumor progression was associated with an increase of IL-6 levels was consistent with previous reports [305;319]. These data suggest that NSCLC patients with high levels of IL-6 have a worse clinical outcome.

We could not confirm the findings of other authors who reported that pretreatment serum IL-6 levels were associated with lung cancer prognosis [145;308;312]. Martin et al. [308] reported that association of IL-6 with lung cancer survival was independent prognostic factor but only within the first 3 years of follow up. It is possible that we were unable to find these associations because of the relatively small number of advanced stages of disease in the study enrolled.

Several studies suggest possible biological mechanisms for increased IL-6 in serum from cancer patients. The tumor cells themselves might have been a source of IL-6; a recent study examined the expression of cytokines from 31 lung cancer cell lines and reported that 55% of the lines expressed IL-6 [321]. In addition, results from several studies indicated that IL-6 may function in angiogenesis [321;322].

Interleukin 8 (IL-8) is expressed and secreted by a variety of cells including lung cancer cells [323]. IL-8 has been shown to play a role in cancer growth and progression. Most research work focuses on the role of IL-8 as an angiogenic factor. Highly vascularized tumors and progression to metastatic disease are associated with the ability of cancer cells to produce IL-8 [324-327]. The mechanisms by which IL-8 may favor cancer growth and progression remain unclear and currently is under intense investigation.

Our results on IL-8 confirmed those of Orditura et al.[310], who reported elevated serum IL-8 levels in advanced NSCLC patients, but without prognostic significance for survival of NSCLC patients. We observed that IL-8 levels were related to tumor size, with significantly higher levels for T4 tumors in compare with T2 tumors in NSCLC patients (p= 0,0084) and in patients with squamous cell carcinoma (p= 0,0104). This suggests a specific role for proinflammatory cytokine IL-8 in tumor proliferation and in the progression of NSCLC. Moreover, higher cancer stage may be associated with greater inflammation, ulceration, and greater inflammatory response, and patients at higher cancer stage were more likely to have higher serum IL-8 levels that those at lower stages.

Despite recent progress in this area, there is a lacking of in vivo studies on the roles of monocyte chemotactic protein -1 (MCP-1) on lung cancer development. MCP-1 is a member of the chemokine family that plays a critical role in the recruitment and activation of monocytes during acute inflammation and angiogenesis. MCP-1 has been shown to induce angiogenesis and plays role in tumor growth and progession [328;329]. The role of MCP-1 in lung cancer remains controversial, with evidence of both protumorigenic and antitumorigenic effects. MCP-1 may activate the cytostatic function of monocytes against tumor cells but also has been reported to enhance tumor invasion and metastasis through increased neovascularisation. The data from several animals models of NSCLC show that MCP-1 blockade, as mediated by neutralizing antibodies, can inhibit the tumor growth of primary and metastatic disease [329;330].
It has been reported that various tumor cells such as prostate cancer, breast cancer, and myeloma cells produce MCP-1 and express its receptor CCR2 [329;331;332]. In patients with breast, ovarian and cervical carcinoma has been reported that increased serum MCP-1 levels are associated with the tumor stages [333-335]. As far as we know, there is just one published work of Cai et al. [303] that studies the relation between MCP-1 serum levels and lung cancer. This study observed that MCP-1 levels were elevated in patients with localized lung cancer compared with those in healthy donors. They found that serum MCP-1 levels were increased in lung cancer patients with bone metastases compared with those in patients with localized cancer. These results suggest that MCP-1 could be used as biomarker for the tumor progression, specifically for bone

metastases. Consistent with this report, we found that serum MCP-1 levels during follow up increased with disease progression, with significantly higher levels of MCP-1 (p= 0,0059) in patients in the moment of progression. Increasing MCP-1 serum levels indicated disease progression with sensitivity of 24% at 95% specificity. In addition, the increase in presurgery MCP-1 serum levels was correlated with increased T stage, statistical difference was found for T4 tumors versus T2 and for T4 tumors vs. T1 in group of NSCLC patients (p= 0,0153 , p= 0,0370, respectively) and in squamous cell carcinoma group (p=0,0002, p=0,0196, respectively). This suggests a specific role for MCP-1 in tumor proliferation and in the progression of NSCLC. An interesting novel finding of our study is that high pretreatment levels of MCP-1 are associated with decreased overall survival in patients with NSCLC. To our best knowledge, this is the first report about the correlation between MCP-1 serum levels and poor clinical outcome in NSCLC patients. Our study suggests that MCP-1 could potentially serve as a prognostic tumor marker to predict aggressive behavior of NSCLC. These results need to be confirmed by further prospective studies.

Furthermore, we studied the clinical utility of serum vascular endothelial growth factor (VEGF) in NSCLC patients. VEGF is angiogenic mediator with important effects in tumor growth and metastasis [311]. It has been reported that VEGF is the most potent and specific growth factor for endothelial cells (e.g. proliferation and migration) and also increases vascular permeability. [336] . High levels of expression of VEGF are found in many solid tumor types [337;338]. VEGF is secreted by various tumor cells, vascular endothelial cells and inflammatory cells [339]. Although VEGF is involved in angiogenesis of various cancers, the clinical utility of serum concentration of this cytokine in NSCLC has not yet been elucidated.

Confirming previous reports [145;146;150;304;309;313-315;340], we have found an increased concentration of serum VEGF in NSCLC patients than in controls, but the tendency did not reach statistical significance. Our observation that serum VEGF levels are not associated with disease stage comes in agreement with previous authors [145;304;313;315], whereas others have reported that circulating VEGF increases significantly according to disease stage progression [146;309;340;341]. Takigawa et al. investigated 70 patients (45 patients with NSCLC

and 25 patients with small cell lung cancer) and did not observe a correlation between serum level of VEGF and tumour stage, distant metastasis or tumour histology. Choi et al. also did not find a significant association between VEGF concentration and various clinicopathologic characteristics including age, gender, histologic type, tumour stage and median survival in 41 patients with NSCLC. Matsuyama et al. predict that in lung cancer, VEGF production differs depending on the stage of progression of disease. In agreement with previous authors who have suggested a strong association of VEGF with tumor progression, in our study we also considered that VEGF serum levels were significantly associated with the progression of NSCLC patients (p= 0,0062).

Several investigative studies of tumor tissues suggested VEGF as a poor prognostic factor for NSCLC [341;342]. However, the prognostic influence of serum VEGF levels still remains unclear. In our study and in some published studies, serum VEGF levels have no prognostic influence on survival [145;304;315;316;341;343;344], while others reported a prognostic importance for patients with NSCLC [143;146;264;302;314]. This discrepancy can be explained by the difference in population of the stage examined: our study and Brattstrom's [343] or Kaminska's [145] study contain a large number of patients with early disease and operable locally advanced disease in comparison with Laack's [264] and Kaya's [146] study with large number of inoperable stages. This hypothesis needs to be confirmed with larger studies of patients with different TNM stages. Moreover, it is possible that serum VEGF depends on factors other than tumor secretion; hypoxia and inflammation may also alter circulating VEGF concentrations [316]. In addition, VEGF is released from platelets and other blood cells during clotting, therefore serum VEGF concentrations increase with platelet count and duration and temperature of clotting. The negative outcomes in some of the studies including our study are possibly related to the lack of adjustments for platelet and white blood cell counts. It is suggested that, although the serum VEGF levels are affected by blood platelets, platelet-derived VEGF also reflect biology of cancer cells, and that serum instead of plasma would be the more useful specimen for measurement of circulating VEGF in cancer patients for prognosis [345-347].

In summary in respect to VEGF, although in our study VEGF levels have no prognostic importance on survival, there is growing evidence that high VEGF

levels in tumours and blood of NSCLC patients are negative prognostic indicators for survival. These facts support anti-VEGF treatment strategies like anti-VEGF antibodies (e.g bevacizumab) or inhibitors of the VEGF receptors to improve survival of NSCLC patients. The circulating levels of VEGF may in the future be used for planning therapy, evaluating treatment effect, and monitoring patients for relapse post therapy. Thus, further large-scale studies should evaluate its use in clinical practice.

In conclusion, the present study provides evidence that concentrations of selected proinflammatory and angiogenic cytokines IL-6, IL-8, MCP-1 and VEGF are elevated in the sera of NSCLC patients before treatment and in the time of disease progression during follow up measurement of cytokines. These cytokines may allow earlier identification and treatment of disease relapse. Cytokines are not expected to reach the value of high specificity markers, but those frequently related to clinicopathological features and survival may possess a prognostic value. We revealed that the most valuable cytokine to be assessed in NSCLC patients is MCP-1, since its serum levels independently influence prognosis, and its elevated concentrations may be an indication for more aggressive treatment to prolong survival.
There is limitation in the specificity of serum cytokines as markers in NSCLC, in that cytokines can become elevated for other reasons, including infection, inflammation and cigarette smoking. Therefore, before cytokines are accepted as NSCLC markers, smoking and such common conditions as thrombosis, heart disease, hypertension or other diseases should be examined as to whether they influence circulating cytokine levels independently of tumor burden.

Several studies suggest that cellular adhesion molecules play a role in the process of tumour progression and metastasis. To evaluate the role of these molecules as possible tumor markers in patients with NSCLC, we examined the plasma levels of soluble adhesive molecules intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1).
Cell adhesion molecules play an important role in the immune response and mediate a variety of cell-cell and cell-extracellular matrix interactions in the

process of tumor growth and the formation of metastases [348]. ICAM-1 and VCAM-1 are transmembrane glycoproteins that have been isolated from most tissues and cells. ICAM-1 is expressed on the surface of endothelial cells, lymphocytes, and monocytes, whereas VCAM-1 is known to be expressed on activated endothelial cells, dendritic cells and renal proximal tubule cells [348;349]. Recently, the existence of soluble forms of ICAM-1, VCAM-1 has been described in human serum [350]. The biological and clinical significance of circulating ICAM-1, VCAM-1 has not yet been elucidated. Increased levels of ICAM-1 and VCAM-1 have been detected in variety of malignancies, including hepatocellular carcinoma, colorectal, breast, gastric, ovarian, pancreatic, lung and bladder cancer, malignant melanoma, and lymphomas [351]. Our study is one of the few studies focused on ICAM-1 and VCAM-1 serum/plasma levels and lung cancer, more studies evaluated their expression in lung cancer specimens. In our study we were not found significant differences in presurgery ICAM-1, VCAM-1 plasma levels between NSCLC patients and control group, but we observed significantly higher levels of plasma ICAM-1 during follow up in patients with disease progression. In concordance with our study, these levels have also been associated with tumor progression in breast [352], colorectal [353;354], gastric carcinoma [355], and melanoma [356]. In the study of De Vita et al. [306] they reported that serum concentration of ICAM-1 correlated with clinical stage and tumor progression of NSCLC patients. Accordingly, Sprenger at al. [357] observed that advanced tumour stages and NSCLC patients with progressive disease tended to be associated with higher ICAM-1 levels. Taguchi et al. [358] also found association of advanced, metastatic tumor stages with an elevation of ICAM-1 in the sera of NSCLC patients. In the study of Grothey et al. [359] they concluded that serum levels of ICAM-1 in NSCLC patients could be a beneficial adjunct for assessing the tumor burden of NSCLC and may serve as a useful indicator of advanced disease. They found the correlation of ICAM-1 serum levels and tumor expression of ICAM-1 suggesting a release of soluble ICAM-1 by tumor cells. Our results are in agreement with the study of Shin et al. [360] and Guney et al. [351] that reported no difference in serum ICAM-1 concentration among different stages and histological tumor type of NSCLC. In contrary to our study, Shin et al. [360] found that high levels of serum ICAM-1 reflect poor prognosis for NSCLC patients. However, other studies [351;359;361] have shown

that pretreatment serum ICAM-1 levels were not correlated with prognosis of NSCLC patients. Further investigations are necessary to evaluate ICAM-1 as a marker for monitoring disease activity in patients with NSCLC.

It is important to point out that elevated levels of ICAM-1 must be interpreted together with knowledge of the patient's condition because all other possible non-malignant causes (infections, inflammatory or autoimmune diseases) of elevated serum/plasma levels must be excluded. The levels of serum ICAM-1 could be increased in patients with benign lung diseases such as tuberculosis, pneumonia, acute bronchitis, chronic asthma, and chronic obstructive lung disease [357]. This could be the reason why in our study were not found statistical significant differences in presurgery ICAM-1 levels between NSCLC patients and control group.

As far as we know, there are not published works studying the relation between VCAM-1 circulating levels and lung cancer. The existing few reports [362-364] are related to the expression of this marker in lung cancer tissue. Jiang et al. [363] study showed major differences in the expression of ICAM-1 and VCAM-1 in tumor cells from pulmonary adenocarcinoma. ICAM-1 was expressed in NSCLC tissue, while VCAM-1 expression was not identified in tumor cells, it was expressed only in pulmonary lymphocytes and interstitial fibroblastic cells. Accordingly, Staal-van den Brekel et al. [364] reported that VCAM-1 was clearly expressed on NSCLC cells just in 4 of the 43 cases and on lymphocytes and fibroblasts. These studies confirm our results that plasma VCAM-1 did not show any significant elevation in NSCLC patients. However, there is an evidence that VCAM-1 may be involved in tumor progression and metastasis in other malignances including colorectal and gastric cancer patients [354;355;365;366].

In summary, a number of studies in a variety of malignant diseases suggest a role for ICAM-1 in the process of tumour growth and metastasis. VCAM-1 is also emerging as an important adhesion molecule in malignancy. Our findings support the suggestion that serum levels of ICAM-1 may be of importance for monitoring tumor progression in NSCLC patients. Further longitudinal studies in large numbers of cancer patients with measurement of circulating ICAM-1 and VCAM-1 during the course of the disease and during active treatment are needed in order to define the emerging clinical significance of these molecules.

Furthermore, we studied relationship between circulating serum levels of insulin-like growth factor (IGF)-I and NSCLC. IGF-1 is a circulating hormone and tissue growth factor, which regulates cell growth, differentiation, and apoptosis [367]. IGF-1 has been implicated in the development and progression of several cancers including breast, prostate, colorectal and lung cancer [368]. Higher IGF-1 levels have been associated with an increased risk of lung and other cancers, although four prospective studies observed null associations with respect to lung cancer risk [369]. In a meta-analysis study Chen et al. [370] concluded that the associations between circulating IGF-I levels and the risk of lung cancer were not statistically significant. A recent review demonstrated that IGF-1 levels are positively associated with the risk of non-smoking related cancers including prostate, colorectal, and premenopausal breast cancer, but not with lung cancer [371;372]. Although a lot of studies evaluated the association of circulating levels of IGF-1 with lung cancer risk, little is known about the prognostic role of IGF-1 in patients with NSCLC. Han et al. [373] concluded that high plasma levels of IGF-1 were associated with good prognosis in patients with advanced NSCLC. In our study serum IGF-1 did not show any significant elevation in NSCLC patients. In agreement with our results, in other studies [373;374] neither a histological type of NSCLC nor clinical staging had any effect on the serum levels of IGF-I. To verify the clinical significance of circulating IGF-1 levels in patiens with NSCLC further studies are needed.

We investigated the clinical importance of plasminogen activator inhibitor-1 (PAI-1) plasma levels in group of NSCLC patients. PAI-1 is thought to play an important role in cancer progression, presumably via mediating extracellular matrix degradation and tumor cell migration during angiogenesis [154;375]. PAI-1 has been shown to promote and inhibit tumor growth and angiogenesis. Low concentrations of PAI-1 can stimulate tumor angiogenesis while treatment of animals with high doses of PAI-1 inhibits angiogenesis and tumor growth [376]. PAI-1 levels are elevated in a number of malignancies and have been correlated with a poor prognosis, particularly in breast cancer, colon cancer, and renal cell carcinoma [377-385]. The earlier tissue findings were extended to plasma studies and similar results were seen [379;386;387] . As far as we know, there are not published works that evaluate the relation between PAI-1 plasma levels and lung cancer. The existing few reports are related to the levels of this

marker in lung cancer tissue. These studies have reported significantly higher PAI-1 levels in tumor tissue as compared to normal lung tissue [154;375;380-382;388-392]. Studies on the prognostic value of PAI-1 in NSCLC tissue are limited. To our knowledge, seven studies have investigated the prognostic value of PAI-1 in NSCLC [380-382;385;389;391-393]. In four of them, PAI-1 was correlated to poor survival [380-382;385] . This discrepancy in results can be explained by the small sizes of histological subgroups, the difference in the patient groups, and the use of different methodology (quantitative ELISA and semiquantitative immunohistochemistry). In our study plasma PAI-1 did not show any significant elevation before surgery and during follow up in NSCLC patients. However, we observed that PAI-1 plasma levels were related to tumor stage, with significantly higher levels for stage III in comparison with stage I (p=0,0035) and for stage III vs. stage II (p=0,0173) in patients with adenocarcinoma. Likewise, Pavey at al. reported a positive relationship between PAI-1 levels in tumor tissue and tumor stage in NSCLC, but not in adenocarcinoma. Furthermore, we found correlation between levels of PAI-1/TPS and PAI-1/VEGF in control group and PAI-1 and TIMP-1 in NSCLC group of patients. At present, it is difficult to explain the biological basis for these correlations, and it is possible that they are incidental.

In summary, recent findings suggest that PAI-1 tissue levels in patiens with NSCLC may be significant prognostic factor. To our knowledge, our study appears to be the first study to investigate a correlation between PAI-1 plasma levels, clinicopathological features and the clinical outcome in NSCLC patients. Further studies that compare the tissue levels of PAI-1 with the circulating levels and correlate these factors with the clinical outcome of patiens with NSCLC are needed.

3.4.1 The combination of biomarkers

Diagnosis

Several studies have demonstrated that the combination of two or more markers increases the sensitivity for the diagnosis of NSCLC and improve the early detection of progression, although the most useful combination remains unclear [128;162;163;207;212;394]. In our study we analyzed the utility of several markers with different characteristics and different sensitivities. Some of them are related one to each other and their combination does not improve the sensitivity. Some of them with good sensitivity, when used in combination with others the specificity is reduced. We propose that the combination of the more sensitive markers without relationship in the Spearman's rank correlation test could be useful for increase the sensitivity for diagnosis of NSCLC. In the group of our study, we found that the best combination to distinguish between benign disease and NSCLC was achieved using CEA, CYFRA 21-1, IL-6 and VEGF, with a 75,6% sensitivity and 86,7% specificity, with a high predictive positive value of 97%. When CEA was excluded the sensitivity decrease to 65,9% with a 93% specificity and a positive predictive value of 98,2%. We suggest that the use of IL-6 and VEGF like complementary markers to tumor markers commonly applied in NSCLC could be useful. Tamura et al. [395] showed that the combination of plasma VEGF and serum CEA was useful in the early diagnosis of NSCLC, with a sensitivity of 75% and a specificity of 60%. They concluded that the combination of VEGF and CEA was superior to CEA alone in the early diagnosis of lung adenocarcinoma. Molina et al. [163] reported better sensitivity (81,3% in adenocarcinomas, 79,3% in squamous, both stages I-III), similar specificity (85%) and the positive predictive value (98,5%), using a combination of 3 tumor markers : CEA, Cyfra 21-1 in all histologies, and SCC in squamous tumors or CA 15-3 in adenocarcinomas. The lack of sensitivity using the combination proposed in our study might be related to the histological characteristics, the size of the studied groups and high number of enrolled patients in early lung cancer stages.

Prognosis

In relation to the prognosis and the early detection of relapse, we were studying biomarkers that were independent prognostic factor for OS and DFS in the multivariate Cox regression analysis. According to the results of our study we suggest that the combination of one cytokeratin, chromogranin A, MMP-7, and MCP-1 offers a good predictive value as survival predictors and for prediction of recurrence we found a valuable combination of one cytokeratine and one MMP (MMP-1 or MMP-7). The cytokeratins that showed the best association as independent prognostic factors for OS and DFS were MonoTotal and TPA. Several studies have demonstrated the association of high pretreatment TPA serum levels with poor prognosis [93;155;187;196;201;202]. Eriksson et al. [121] published that MonoTotal seems to be promising serum marker for prognosis and might be used for monitoring of patients with NSCLC.

The reports about the prognostic signification of chromogranin A, MMP-7, and MCP-1 levels in NSCLC patients are comparatively few and some of them are controversial. Chromogranin A (CgA) is frequently used as a diagnostic and prognostic serum marker for a range of neuroendocrine tumors. Circulating CgA is also increased in patients with other diseases, including subpopulations of patients with non-neuroendocrine tumors, with important prognostic implications [396]. In agreement with our study Nisman et al. [235] and Gregorc et al. [234] found elevated serum levels of CgA before treatment as an independent indicator of poor prognosis in NSCLC patients. Interestingly, circulating CgA was associated with worse patient conditions and more advanced NSCLC [234]. Zhang et al. [236;237] reported CgA as a strong and independent indicator of prognosis in critically ill patients. It is possible hypothesize that serum CgA in NSCLC patients reflects stress-related systemic neuroendocrine activation associated with worsening of patient condition.

Several reports suggest that matrix metalloproteinases may be useful in the clinical investigation of patients with NSCLC and are also associated with aggressiveness of lung cancer [245;253-255;258;262;263;270;271;274;276;282]. In our study, we analyzed the clinical utility of MMP-1, MMP-2, MMP-7, and MMP-9. We observed that MMP-1, MMP-2, and MMP-7 serum/plasma levels at diagnosis were reliable predictors of recurrence; only MMP-7 elevation was independent

prognostic biomarker for disease recurrence and overall survival time. To our knowledge, our study appears to be the first study to identify a correlation between MMP-7 serum levels and the clinical outcome in NSCLC patients. The existing reports are related to the levels or expression of this marker in lung cancer tissue [252;254;257;271;275-277]. Unlike the other members of the MMP family, MMP-7 is expressed by tumor cells themselves but not by the peritumoral stromal cells [250;276], indicating that MMP-7 could be useful as a tumor-associated biomarker. These findings suggest that MMP-7 expression and MMP-7 serum levels in patiens with NSCLC may be significant prognostic factors.

The last marker that we suggest to include in this panel is the monocyte chemotactic protein-1 (MCP-1), a proinflammatory cytokine that has been shown to induce angiogenesis and plays role in tumor growth and progession [328;329]. An interesting novel finding of our study is that high pretreatment levels of MCP-1 are associated with decreased overall survival in patients with NSCLC. To our best knowledge, this is the first report about the correlation between MCP-1 serum levels and poor clinical outcome in NSCLC patients. There is just one published work of Cai et al. [303] that studies the relation between MCP-1 serum levels and lung cancer. They found that serum MCP-1 levels were increased in lung cancer patients with bone metastases compared to those in patients with localized cancer. These findings suggest that MCP-1 could potentially serve as a prognostic tumor marker to predict aggressive behavior of NSCLC. These results need to be confirmed by further prospective studies.

In conclusion, these results open a new point of view on use the well accepted traditional tumor markers in combination with the new biomarkers. As we showed, it's possible to increase the value of tumor markers as CEA or cytokeratins when they are used in combination with the new ones.

3.5 CONCLUSIONS

Serum levels of 22 serum biomarkers were monitored systematically in 93 patients with operable stage of NSCLC. The presented thesis was designed to compare prospectively conventional tumor markers with novel biomarkers in the three most important clinical applications for NSCLC:

-The evaluation of disease extent and histological type at the first clinical presentation (Diagnosis, staging)

-The evaluation of postsurgery status (Postsurgery follow up care)

-The prediction of the outcome (Prognosis).

Results and conclusions split according to these clinical applications are listed below:

1. Differences in presurgery biomarker levels between studied groups and the relation of the biomarkers to the diagnosis.

- Significantly higher levels of cytokeratin markers (CYFRA 21-1, TPA, TPS, and MonoTotal), SCC and TIMP-1 were observed in NSCLC patients when compared with the control group.
- Significantly higher levels of CEA and CYFRA 21-1 were observed in adenocarcinoma patients when compared with control group.
- Significantly higher levels of CYFRA 21-1, TPA, TPS, MonoTotal, SCC, TIMP-1 and IL-6 were observed in squamous cell carcinoma patients when compared with control group.
- Significantly higher levels of CYFRA 21-1, MonoTotal, TK and SCC were observed in squamous cell carcinoma patients when compared with adenocarcinoma.
- The sensitivities for NSCLC diagnosis were in a wide range from 54,1% to 2,4% at 95% specificity. The highest sensitivity to distinguish between benign lung disease and NSCLC diagnosis was showed by cytokeratin markers (CYFRA 21-1, TPA, and MonoTotal), IL-6, and CEA. The lowest

diagnostic sensitivity was showed by Chromogranin A, PAI-1, IGF-I, ICAM-1 and MCP-1.

- The best sensitivity at 95% specificity for the adenoncarcinoma diagnosis was observed for CEA and CYFRA 21-1 (46,7% and 36,7%, respectively).
- The best sensitivity at 95% specificity for the squamous cell carcinoma diagnosis was observed for CYFRA 21-1, TPA, and IL-6 (63,6%, 42,6%, and 41,8%, respectively).
- The best combination to distinguish between benign disease and NSCLC was achieved using CEA (>3,7 ng/m), CYFRA 21-1 (>2,0 ng/ml),, IL-6 (>9,8 pg/m) and VEGF (>405 pg/ml), with a 75,6% sensitivity and 86,7% specificity, with a high predictive positive value of 97%. When CEA was excluded the sensitivity decrease to 65,9% with a 93% specificity and a positive predictive value of 98,2%.
- Significantly higher levels of TPA and MonoTotal were observed in advanced stages in squamous cell carcinoma patients and significantly higher CEA levels were associated with more advanced stages in adenocarcinoma patients (stage III vs. stage I).
- CEA, CYFRA 21-1, TPA, TK, MonoTotal, MMP-9 and MCP-1 levels were correlated with tumor size in NSCLC patients, with significantly higher levels for larger tumors (T4) compared to smaller ones (T1).

2. <u>Postsurgery follow up monitoring</u>

- Significantly higher levels of CEA, cytokeratins (CYFRA 21-1, TPA, TPS, and MonoTotal), SCC, TK, Chromogranin A, matrix metalloproteinases (MMP-1, MMP-7), TIMP-1, cytokines (MCP-1, IL-6, VEGF), and ICAM-1 were observed in the moment of progression compared to remission during follow up of NSCLC patients. In the time of progression significantly higher levels of CEA were found in adenocarcinoma, and SCC higher levels in squamous cell carcinoma.
- The sensitivities at 95% specificity for detection of disease progression during follow up were in a wide range from 52% to 4,8%. The highest sensitivity to distinguish between progression and remission status was

showed by cytokeratin markers (CYFRA 21-1, TPA, and MonoTotal), TK, and CEA. The lowest sensitivity was showed by IL-6, VEGF, VCAM-1, MMP-2, and PAI-1.

3. Prognosis

- Presurgery levels of cytokeratins (CYFRA 21-1, TPA, and MonoTotal), TIMP-1, MMP-1, and interleukins IL-6 and IL-8 were significantly higher in NSCLC patients with disease progression in last follow up control.
- Presurgery levels of cytokeratin markers (TPA, TPS, and MonoTotal), MMPs (MMP-1, MMP-2, and MMP7), CEA and also tumor stage and tumor size were significant prognostic factors in univariate analysis for DFS.
- Univariate DFS analysis according to the histologic subtypes was showed by cytokeratins (CYFRA 21-1, TPA, and MonoTotal), MMPs (MMP-2, and MMP-7), and TIMP-2 as significant prognostic factors of early recurrence in adenocarcinoma and TPA and MonoTotal in squamous cell carcinoma.
- Multivariate Cox model using cut offs showed one cytokeratin (TPA or MonoTotal), one MMP (MMP-1 or MMP-7) and tumor stage as independent prognostic factors of DFS in NSCLC patients. Stepwise variant of Cox multivariate model selected MonoTotal and MMP-2 as independent predictors of DFS. Multivariate analysis of DFS by histology: in adenocarcinoma MMP-2, in squamous cell carcinoma MonoTotal.
- Presurgery levels of cytokeratins (TPA, TPS, and MonoTotal), CEA, Chromogranin A, and MCP-1 were significant prognostic factors in univariate analysis for OS. Also tumor stage, tumor size (T) and lymph nodes (N) were significantly related with OS.
- Univariate OS analysis according to the histologic subtypes showed CEA, MMPs (MMP-1, and MMP-2), and TIMP-2 as significant prognostic factors of poor prognosis in adenocarcinoma and TPA, MonoTotal, Chromogranin A, and MMP-7 as poor prognostic factors in squamous cell carcinoma.
- Multivariate Cox model using cut offs showed one cytokeratin (TPA or MonoTotal), MMP-7, MCP-1, Chromogranin A and tumor stage as independent prognostic factors for OS in NSCLC patients. Stepwise variant

of Cox multivariate model selected MonoTotal, MMP-7 and lymph nodes (N) as independent predictors of OS. Multivariate analysis of OS by histology: in adenocarcinoma CEA, in squamous cell carcinoma MonoTotal.

- We suggest that the combination of one cytokeratin (TPA or MonoTotal), Chromogranin A, MMP-7, and MCP-1 offers a good predictive value as survival predictors in NSCLC patients and for prediction of disease recurrence we found a valuable combination of one cytokeratin (TPA or MonoTotal) and one MMP (MMP-1 or MMP-7).

4. Biomarker correlations

We observed significant positive correlation between levels of cytokeratin tumor markers, matrix metalloproteinases and their inhibitors, as TIMP-1/ MMP-1 and TIMP-2/ MMP-2, proinflammatory and proangiogenic cytokines, IL-6/IL-8 and IL-6/VEGF, and between PAI-1/TIMP-1 in NSCLC patients group and during follow up. The basis for these correlations may be pathophysiological relationship between these biomarkers in biologic process of disease development.

Tumor markers are not frequently used in patients with lung cancer because previously the clinical advantages have not been clear. In this thesis, we have provided evidence that biomarkers may provide a very helpful aid in the diagnosis, prognosis and early recognition of recurrence in NSCLC patients, especially when biomarkers are combined advisedly.

The important outcome of this thesis for the future development of circulating markers is the observation that the novel studied biomarkers should not be used alone, because of the lack of the sensitivity at an acceptable specificity. However, as we showed, the combination of well accepted tumor markers as e.g.: CEA and cytokeratins with other markers e.g. chromogranin A and new biomarkers: in front of all matrix metalloproteinases MMP-1 or MMP-7, MCP-1, VEGF and IL-6, seems to be useful for improving the power of the conventional markers to aid in the diagnosis proceedings, prediction of the disease course and follow up of NSCLC patients. Nevertheless multicentric prospective studies

with larger populations are needed to demonstrate their utility in clinical practice and evaluate the proper marker combinations, as well as the main cause of false positive results, in order to have the knowledge and asses the objective to give added value to a simple laboratory result. The results of this study encourage the present tendency to use markers panels to improve the prognosis and monitoring of NSCLC patients.

We can conclude that the lack of sensitivity and specificity of the measured biomarkers do not enable their use for screening and primary diagnosis of NSCLC, but some of the biomarkers could be very helpful in consideration of disease severity, treatment efficacy and prognosis estimation.

We believe that elucidating the clinicopathological characteristics of NSCLC patients showing high preoperative values of prognostics tumor markers will increase the understanding of this poor prognostic subgroup that require treatment and follow up strategies distinct from those patients with normal tumor marker levels. These facts could help in proper stratification of patients and design the treatment related to the biologic activity of tumour. Among many prognostic indicators, e.g. pathologic factors, genetic change, tumor markers are simple to measure and can be judged as abnormal clearly and consistently by different institutions.

An early diagnosis and detection of progression, prediction of the disease course and optimization of treatment are among the main challenges for further prospective biomarker studies to improve the management of lung cancer patients.

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5. TABLE OF ABBREVIATIONS

Abbreviation	Definition
A	Adenine
AAH	Atypical alveolar hyperplasia
ACTH	Adrenocorticotropic hormone
ADH	Antidiuretic hormone
ADK	Adenocarcinoma
AFP	Alpha-fetoprotein
AJCC	American Joint Committee on Cancer
ASCO	American Society of Clinical Oncology
AUC	Area under the curve
C	Cytosine
CA	Carbohydrate antigen
CA 125	Carbohydrate antigen 125
CCR2	Chemokine receptor 2
CEA	Carcinoembryonic antigen
CI	Confidence interval
CK	Cytokeratin
CLIA	Chemiluminescent Immunoassay
CNS	Central nervous system
COX-2	Cyclo-oxygenase 2
CgA	Chromogranin A
CT	Computed tomography
CYFRA 21-1	Cytokeratin-19 fragment
CYP1A1	Cytochrome P450 1A1
DFS	Disease free survival
DNA	Deoxyribonucleic acid
EBUS	Endobonchial ultrasonography
ECO	European cancer observatory
ED	Extensive disease
EDTA	Ethylene-diamine-tetra-acetic acid
EGFR	Epidermal growth factor receptor
EGFR-TK	Epidermal growth factor receptor - Tyrosine kinase
EGTM	European Group on Tumor Markers
ELISA	Enzyme-Linked Immunoabsorbent Assay
EUS	Endoscopic esophageal ultrasonography
FDG	Fluorodeoxyglucose
FHIT	Fragile histidine triad protein
G	Guanine
GRP	Gastrin releasing peptide
HER-2	Human epidermal growth factor receptor 2
IARC	International agency for research on cancer
IASLC	International Association for the Study of Lung
ICAM-1	Intracellular adhesion molecule 1
I-ELCAP	International early lung cancer action program
IGF-1	Insulin-like growth factor 1
IL-6	Interleukin 6
IL-8	Interleukin 8
IRMA	Immunoradiometric assay
kDa	Kilodalton

LCNEC	Large cell neuroendocrine carcinoma
LD	Limited disease
LDCT	Low dose computed tomography
LDH	Lactate dehydrogenase
LOH	Loss of heterozygosity
MCP-1	Monocyte chemotactic protein-1
MEIA	Microparticle enzyme immunoassay
MMP	Matrix metalloproteinase
MMP-1	Matrix metalloproteinase 1
MMP-2	Matrix metalloproteinase 2
MMP-7	Matrix metalloproteinase 7
MMP-9	Matrix metalloproteinase 9
MN	Malignant neoplasia
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NACB	National Academy of Clinical Biochemistry
MT	MonoTotal
NE	Neuroendocrine
NPV	Negative predictive value
NSCLC	Non small cell lung cancer
NSE	Neuron specific enolase
OS	Overall survival
p	Probability value
PAHs	Polycyclic aromatic hydrocarbons
PAI-1	Plasminogen activator inhibitor 1
PET	Positron emission tomography
PPV	Positive predictive value
ProGRP	Progastrin–releasing peptide
PS	Performance status
PSA	Prostate-specific antigen
PTHrP	Parathyroid hormone related peptide
r	Correlation coefficient
RB	Retinoblastoma
RCT	Randomized controlled trials
REA	Radioenzymatic assay
RIA	Radioimmunoassay
ROC	Receiving operating curve
RR	Relative risk
SCC	Squamous cell carcinoma antigen
SCLC	Small cell lung cancer
SD	Standard deviation
SQM	Squamous
T	Thymine
TIMP-1	Tissue Inhibitor of Metalloproteinase 1
TIMP-2	Tissue Inhibitor of Metalloproteinase 2
TK	Thymidine kinase
TKIs	Tyrosine kinase inhibitors
TNM	Tumor Node Metastases Classification
cTNM	Clinical Tumor Node Metastases Classification
pTNM	Pathological Tumor Node Metastases Classification
TP53	Tumor protein p53
TPA	Tissue Polypeptide Antigen
TPS	Tissue Polypeptide-Specific Antigen

TRACE	Time Resolved Amplified Cryptate Emission
TU M2-PK	Tumor M2 pyruvate kinase
uPA	Urokinase-type plasminogen activator
USPSTF	US Preventive Services Taskforce
VALG	Veterans Administration Lung Study Group
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WHO	World health organization
xMAP	Multi-analyte profiling technology

6. TABLES AND FIGURES OF THE EXPERIMENTAL PART

TABLES:

FIGURES:

	Control group (n=20)					NSCLC (presurgery) (n=93)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA	2,2	1,2	1,9	1,0	6,4	22,7	120,2	2,4	0,8	1.072,0	nss
CYFRA 21-1*	1,0	0,6	0,9	0,1	2,5	5,6	10,3	2,2	0,4	64,6	<0,0001
TPA*	36,2	21,5	29,0	10,0	71,0	82,4	72,8	61,5	10,0	409,0	0,0017
TPS*	58,6	34,8	62,0	10,0	138,0	106,2	108,4	91,0	10,0	768,0	0,0318
TK	7,9	4,3	7,3	1,5	16,1	8,5	7,9	6,2	1,8	50,2	nss
MonoTotal*	98,8	56,0	93,0	22,3	218,3	261,2	361,1	155,2	10,4	2.167,8	0,0028
SCC*	0,8	1,1	0,2	0,2	4,8	1,7	4,5	0,7	0,1	38,2	0,0427
Chromogranin A	87,8	176,8	37,3	12,5	754,7	74,2	91,2	49,6	10,6	563,7	nss
IGF-I	369,7	138,6	324,8	131,9	591,2	343,6	124,9	328,4	137,9	686,7	nss
TIMP-1*	116,3	38,0	106,5	74,1	208,2	138,3	41,2	134,2	59,1	267,8	0,0255
TIMP-2	74,6	20,6	74,7	46,7	113,1	72,2	19,9	68,6	40,1	123,9	nss
MMP-1	506,8	309,5	520,1	35,7	1.285,0	714,1	803,9	415,6	35,7	3.629,8	nss
MMP-2	174,6	38,5	168,8	111,0	275,8	177,3	43,7	177,2	106,5	296,1	nss
MMP-7	5,0	1,7	4,6	2,6	9,2	5,9	2,5	5,2	2,5	16,8	nss
MMP-9	134,2	83,5	100,4	43,7	364,8	188,3	152,5	144,8	29,3	848,5	nss
MCP-1	445,5	506,1	204,5	77,0	1.760,1	329,3	354,2	207,0	39,5	2.188,1	nss
IL-6	75,1	272,6	3,5	3,2	1.060,6	48,0	128,8	5,5	3,2	765,0	nss
IL-8	91,2	208,7	21,0	3,2	828,8	166,2	559,3	14,7	3,2	4.078,8	nss
VEGF	357,2	812,9	135,3	26,4	3.482,2	280,8	308,8	168,6	16,0	1.595,5	nss
ICAM-1	137,4	93,6	111,5	55,1	459,3	133,8	55,3	121,8	54,7	327,2	nss
VCAM-1	1.141,9	248,0	1.155,7	662,1	1.538,4	1.038,9	319,2	1.029,6	244,9	1.694,9	nss
PAI-1	44.533,0	19.884,2	42.858,3	15.318,9	82.614,9	44.660,8	23.424,6	41.893,7	10.386,1	126.892,4	nss

Table 22. Presurgery tumor marker levels in control group vs NSCLC patients.

	Squamous (n=59)					Adenocarcinoma (n=34)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA	26,0	145,4	2,2	0,8	1.072,0	16,8	49,4	3,5	0,8	264,4	nss
CYFRA 21-1*	6,8	11,9	2,7	0,5	64,6	3,3	6,2	1,7	0,4	34,3	0,0209
TPA	86,2	67,9	66,0	10,0	290,0	75,7	81,8	51,5	11,0	409,0	nss
TPS	111,6	111,6	92,0	10,0	768,0	96,1	103,5	74,0	10,0	550,0	nss
TK*	9,4	9,0	7,0	2,5	50,2	6,7	5,0	5,3	1,8	25,1	0,0372
MonoTotal*	304,0	404,4	165,7	23,6	2.167,8	182,7	251,6	125,4	10,4	1.435,0	0,0450
SCC*	2,4	5,5	1,0	0,1	38,2	0,6	0,5	0,5	0,1	2,0	0,0009
Chromogranin A	68,7	85,2	49,8	10,6	563,7	84,5	102,4	49,4	16,6	405,9	nss
IGF-I	355,8	131,5	330,1	137,9	686,7	322,1	112,1	326,3	141,2	499,2	nss
TIMP-1	143,7	40,8	140,1	72,6	267,8	128,0	40,7	121,0	59,1	239,7	nss
TIMP-2	72,5	19,7	68,4	40,1	123,9	71,8	20,5	69,1	42,2	122,9	nss
MMP-1	802,4	912,2	418,1	35,7	3.629,8	546,5	515,9	323,6	35,7	2.333,3	nss
MMP-2	178,5	44,2	178,6	106,5	296,1	175,1	43,4	171,5	113,0	290,1	nss
MMP-7	6,0	2,7	5,2	2,6	16,8	5,5	2,1	5,3	2,5	11,4	nss
MMP-9	186,1	143,3	143,0	29,3	739,0	192,6	171,0	149,9	33,3	848,5	nss
MCP-1	343,2	397,4	206,6	39,5	2.188,1	303,6	261,4	212,3	46,1	956,2	nss
IL-6	44,9	120,4	8,0	3,2	765,0	53,6	144,8	3,7	3,2	667,5	nss
IL-8	162,8	438,2	16,6	3,2	2.000,0	172,3	740,5	12,5	3,2	4.078,8	nss
VEGF	261,5	283,7	155,7	16,0	1.595,5	316,1	352,6	186,5	39,6	1.551,2	nss
ICAM-1	139,8	58,6	131,5	54,7	327,2	122,5	47,3	106,1	55,1	207,7	nss
VCAM-1	1.025,3	314,3	994,5	244,9	1.694,9	1.064,6	332,2	1.094,7	263,2	1.627,6	nss
PAI-1	44.410,5	20.279,9	41.971,1	12.000,2	90.351,1	45.135,4	28.861,9	36.619,8	10.386,1	126.892,4	nss

Table 23. Presurgery tumor marker levels in NSCLC patients in relation to histology.

	Benign (n=20)					Adenocarcinoma (n=34)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA*	2,2	1,2	1,9	1,0	6,4	16,8	49,4	3,5	0,8	264,4	0,0311
CYFRA 21-1*	1,0	0,6	0,9	0,1	2,5	3,3	6,2	1,7	0,4	34,3	0,0122
TPA	36,2	21,5	29,0	10,0	71,0	75,7	81,8	51,5	11,0	409,0	nss
TPS	58,6	34,8	62,0	10,0	138,0	96,1	103,5	74,0	10,0	550,0	nss
TK	7,9	4,3	7,3	1,5	16,1	6,7	5,0	5,3	1,8	25,1	nss
MonoTotal	98,8	56,0	93,0	22,3	218,3	182,7	251,6	125,4	10,4	1.435,0	nss
SCC	0,8	1,1	0,2	0,2	4,8	0,6	0,5	0,5	0,1	2,0	nss
Chromogranin A	87,8	176,8	37,3	12,5	754,7	84,5	102,4	49,4	16,6	405,9	nss
IGF-I	369,7	138,6	324,8	131,9	591,2	322,1	112,1	326,3	141,2	499,2	nss
TIMP-1	116,3	38,0	106,5	74,1	208,2	128,0	40,7	121,0	59,1	239,7	nss
TIMP-2	74,6	20,6	74,7	46,7	113,1	71,8	20,5	69,1	42,2	122,9	nss
MMP-1	506,8	309,5	520,1	35,7	1.285,0	546,5	515,9	323,6	35,7	2.333,3	nss
MMP-2	174,6	38,5	168,8	111,0	275,8	175,1	43,4	171,5	113,0	290,1	nss
MMP-7	5,0	1,7	4,6	2,6	9,2	5,5	2,1	5,3	2,5	11,4	nss
MMP-9	134,2	83,5	100,4	43,7	364,8	192,6	171,0	149,9	33,3	848,5	nss
MCP-1	445,5	506,1	204,5	77,0	1.760,1	303,6	261,4	212,3	46,1	956,2	nss
IL-6	75,1	272,6	3,5	3,2	1.060,6	53,6	144,8	3,7	3,2	667,5	nss
IL-8	91,2	208,7	21,0	3,2	828,8	172,3	740,5	12,5	3,2	4.078,8	nss
VEGF	357,2	812,9	135,3	26,4	3.482,2	316,1	352,6	186,5	39,6	1.551,2	nss
ICAM-1	137,4	93,6	111,5	55,1	459,3	122,5	47,3	106,1	55,1	207,7	nss
VCAM-1	1.141,9	248,0	1.155,7	662,1	1.538,4	1.064,6	332,2	1.094,7	263,2	1.627,6	nss
PAI-1	44.533,0	19.884,2	42.858,3	15.318,9	82.614,9	45.135,4	28.861,9	36.619,8	10.386,1	126.892,4	nss

Table 24. Presurgery tumor marker levels in benign control group vs. adenocarcinoma patients

	Benign (n=20)					Squamous (n=59)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA	2,2	1,2	1,9	1,0	6,4	26,0	145,4	2,2	0,8	1.072,0	nss
CYFRA 21-1*	1,0	0,6	0,9	0,1	2,5	6,8	11,9	2,7	0,5	64,6	<0,0001
TPA*	36,2	21,5	29,0	10,0	71,0	86,2	67,9	66,0	10,0	290,0	0,0005
TPS*	58,6	34,8	62,0	10,0	138,0	111,6	111,6	92,0	10,0	768,0	0,0117
TK	7,9	4,3	7,3	1,5	16,1	9,4	9,0	7,0	2,5	50,2	nss
MonoTotal*	98,8	56,0	93,0	22,3	218,3	304,0	404,4	165,7	23,6	2.167,8	0,0006
SCC*	0,8	1,1	0,2	0,2	4,8	2,4	5,5	1,0	0,1	38,2	0,0074
Chromogranin A	87,8	176,8	37,3	12,5	754,7	68,7	85,2	49,8	10,6	563,7	nss
IGF-I	369,7	138,6	324,8	131,9	591,2	355,8	131,5	330,1	137,9	686,7	nss
TIMP-1*	116,3	38,0	106,5	74,1	208,2	143,7	40,8	140,1	72,6	267,8	0,0079
TIMP-2	74,6	20,6	74,7	46,7	113,1	72,5	19,7	68,4	40,1	123,9	nss
MMP-1	506,8	309,5	520,1	35,7	1.285,0	802,4	912,2	418,1	35,7	3.629,8	nss
MMP-2	174,6	38,5	168,8	111,0	275,8	178,5	44,2	178,6	106,5	296,1	nss
MMP-7	5,0	1,7	4,6	2,6	9,2	6,0	2,7	5,2	2,6	16,8	nss
MMP-9	134,2	83,5	100,4	43,7	364,8	186,1	143,3	143,0	29,3	739,0	nss
MCP-1	445,5	506,1	204,5	77,0	1.760,1	343,2	397,4	206,6	39,5	2.188,1	nss
IL-6	75,1	272,6	3,5	3,2	1.060,6	44,9	120,4	8,0	3,2	765,0	0,0341
IL-8	91,2	208,7	21,0	3,2	828,8	162,8	438,2	16,6	3,2	2.000,0	nss
VEGF	357,2	812,9	135,3	26,4	3.482,2	261,5	283,7	155,7	16,0	1.595,5	nss
ICAM-1	137,4	93,6	111,5	55,1	459,3	139,8	58,6	131,5	54,7	327,2	nss
VCAM-1	1.141,9	248,0	1.155,7	662,1	1.538,4	1.025,3	314,3	994,5	244,9	1.694,9	nss
PAI-1	44.533,0	19.884,2	42.858,3	15.318,9	82.614,9	44.410,5	20.279,9	41.971,1	12.000,2	90.351,1	nss

Table 25. Presurgery tumor marker levels in benign control group vs. squamous cell carcinoma patients

	Stage I (n=49)					Stage II (n=19)					Stage III (n=25)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA	5,2	9,1	2,5	0,8	47,8	8,4	19,2	2,7	0,8	78,8	67,9	227,6	2,2	0,8	1.072,0
CYFRA 21-1	4,1	7,3	2,2	0,4	40,1	6,0	8,8	2,7	0,5	34,3	8,1	15,3	2,1	0,8	64,6
TPA	69,5	51,5	61,5	10,0	228,0	101,6	107,3	58,0	10,0	409,0	95,5	79,0	68,5	14,0	290,0
TPS	88,7	49,4	90,5	10,0	174,0	165,1	216,4	84,5	10,0	768,0	100,1	69,0	92,0	10,0	277,0
TK	7,7	7,6	5,6	1,8	50,2	8,0	5,3	6,1	2,8	21,8	10,4	9,9	7,7	2,8	49,4
MonoTotal	208,2	319,0	131,6	10,4	2.167,8	300,8	373,5	160,7	19,8	1.435,0	339,6	425,7	184,8	59,8	1.861,8
SCC	1,9	5,6	0,8	0,1	38,2	1,5	2,8	0,8	0,1	11,6	1,6	2,7	0,7	0,2	12,2
Chromogranin A	71,2	83,1	46,9	16,2	405,9	86,2	87,0	59,7	10,6	306,9	71,7	110,7	49,4	22,1	563,7
IGF-I	349,1	102,6	341,8	137,9	605,2	344,5	151,9	339,4	141,2	686,7	331,5	150,4	282,9	160,7	670,9
TIMP-1	132,1	40,0	128,5	72,6	239,7	153,7	43,2	150,2	71,0	245,0	139,7	41,1	129,3	59,1	267,8
TIMP-2	70,4	18,2	68,4	40,1	123,9	75,5	19,3	72,8	47,5	103,6	73,6	23,5	76,0	40,5	122,9
MMP-1	637,4	799,9	404,1	35,7	3.629,8	906,9	919,8	565,0	62,7	3.173,0	730,0	736,3	490,6	35,7	2.659,8
MMP-2	175,7	36,2	177,1	106,5	278,4	165,3	34,3	168,1	115,3	223,2	188,9	59,6	185,0	113,0	296,1
MMP-7	5,7	2,2	5,0	2,6	12,2	5,3	1,9	5,9	2,5	9,1	6,4	3,4	5,3	2,5	16,8
MMP-9	207,2	182,9	155,7	33,3	848,5	186,7	123,2	144,7	29,3	405,6	152,5	91,7	127,6	47,2	383,8
MCP-1	337,0	339,1	212,3	39,5	2.000,0	279,1	240,4	190,0	64,8	956,2	348,7	449,7	206,6	46,1	2.188,1
IL-6	56,9	144,1	4,6	3,2	765,0	57,0	165,0	5,1	3,2	667,5	23,8	37,6	8,6	3,2	141,8
IL-8	187,8	669,4	11,8	3,2	4.078,8	199,9	502,8	13,9	3,2	2.000,0	99,5	314,7	18,1	3,2	1.513,2
VEGF	326,4	366,6	174,9	16,0	1.595,5	251,1	277,0	147,2	74,8	1.199,1	210,1	162,8	155,7	16,0	748,3
ICAM-1	128,6	49,0	113,8	54,7	257,3	134,2	51,3	116,8	61,2	225,5	143,8	69,1	144,8	58,0	327,2
VCAM-1	1.055,3	301,1	1.012,1	533,6	1.694,9	974,3	327,1	1.046,2	263,2	1.502,6	1.051,6	355,2	1.044,8	244,9	1.634,0
PAI-1	46.651,2	22.587,0	42.633,8	10.386,1	126.892,4	43.363,1	27.792,6	36.444,3	12.000,2	124.547,7	41.669,2	22.460,0	41.534,5	12.778,4	80.810,7

Table 26. Presurgery tumor marker levels in relation to stage in NSCLC patients (n= 93).

	Stage I (n=27)					Stage II (n=13)					Stage III (n=19)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA	4,5	7,8	2,3	1,0	41,3	3,6	4,6	2,3	0,8	16,4	70,7	252,7	1,9	0,8	1.072,0
CYFRA 21-1	5,2	9,2	2,3	0,5	40,1	5,6	5,3	4,7	0,8	18,5	10,0	17,0	3,2	1,0	64,6
TPA*	63,3	45,7	56,0	10,0	190,0	106,4	75,5	74,0	10,0	239,0	110,7	83,0	85,0	27,0	290,0
TPS	81,3	45,1	86,0	10,0	166,0	184,1	228,0	118,0	10,0	768,0	116,9	65,8	102,5	41,0	277,0
TK	8,4	8,9	7,0	2,5	50,2	8,6	6,0	6,5	3,7	21,8	11,4	10,7	8,6	2,8	49,4
MonoTotal*	242,8	408,7	132,1	60,0	2.167,8	295,1	249,3	191,5	23,6	859,0	400,8	464,0	200,7	59,8	1.861,8
SCC	2,8	7,2	1,1	0,1	38,2	2,0	3,4	1,0	0,1	11,6	1,9	3,0	0,8	0,2	12,2
Chromogranin A	56,0	45,8	42,6	16,2	248,9	83,9	85,0	61,9	10,6	306,9	79,4	124,5	51,5	22,1	563,7
IGF-I	354,9	116,5	350,3	137,9	605,2	376,9	148,3	339,4	209,4	686,7	341,9	152,5	282,9	185,9	670,9
TIMP-1	134,3	39,1	134,3	72,6	227,3	161,0	45,5	150,2	99,3	245,0	148,2	38,9	137,0	112,0	267,8
TIMP-2	72,6	19,6	68,4	40,1	123,9	71,1	16,9	68,1	48,5	102,5	73,1	22,1	73,1	40,5	118,2
MMP-1	748,5	987,8	409,2	35,7	3.629,8	838,7	1.018,0	368,5	70,4	3.173,0	863,2	770,1	609,1	54,2	2.659,8
MMP-2	175,3	33,5	177,8	106,5	243,8	165,6	35,3	167,3	119,5	223,2	190,6	59,6	187,3	114,0	296,1
MMP-7	5,7	2,2	5,2	2,6	12,2	5,2	2,2	5,4	2,6	9,1	7,0	3,5	5,3	3,9	16,8
MMP-9	211,0	173,6	173,4	40,5	739,0	173,2	119,6	137,3	29,3	405,6	155,8	97,8	134,0	47,2	383,8
MCP-1	347,6	388,2	187,8	39,5	2.000,0	223,4	163,3	177,5	64,8	620,8	403,3	494,8	211,4	76,5	2.188,1
IL-6	67,7	166,6	7,3	3,2	765,0	12,9	18,5	5,6	3,2	62,6	28,3	41,4	12,1	3,2	141,8
IL-8	147,5	415,8	14,2	3,2	1.750,9	301,1	624,7	10,7	3,2	2.000,0	108,9	352,5	18,4	3,2	1.513,2
VEGF	326,8	365,3	189,7	16,0	1.595,5	153,5	116,1	115,6	74,8	470,8	223,7	170,8	163,2	16,0	748,3
ICAM-1	135,0	50,1	127,3	54,7	257,3	136,6	54,1	113,7	61,9	225,5	148,8	73,3	147,7	64,9	327,2
VCAM-1	1.049,8	303,8	986,4	662,8	1.694,9	1.005,9	305,8	1.053,8	550,5	1.502,6	999,3	348,2	1.018,8	244,9	1.634,0
PAI-1	45.666,1	20.319,7	43.267,9	16.357,0	90.351,1	34.897,1	16.848,6	34.850,0	12.000,2	70.347,7	47.812,4	21.369,1	48.316,5	16.990,8	80.810,7

Table 27. Presurgery tumor marker levels in relation to stage in patients with squamous cell carcinoma (n= 59)

	Stage I (n=22)					Stage II (n=6)					Stage III (n=6)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA*	6,1	11,0	3,1	0,8	47,8	16,4	30,7	4,7	0,9	78,8	57,8	115,6	5,0	2,4	264,4
CYFRA 21-1	2,6	2,3	1,7	0,4	7,9	6,8	13,5	1,4	0,5	34,3	1,4	0,5	1,5	0,8	2,1
TPA	78,3	58,9	63,0	13,0	228,0	93,7	155,5	36,0	11,0	409,0	44,0	30,7	38,0	14,0	86,0
TPS	99,3	54,3	103,0	11,0	174,0	133,3	212,2	32,0	10,0	550,0	39,6	44,2	21,0	10,0	117,0
TK	6,5	5,3	5,3	1,8	25,1	7,0	3,9	5,8	2,8	13,9	7,0	6,0	3,5	3,2	17,2
MonoTotal	159,0	93,6	130,9	10,4	384,7	310,3	553,6	108,1	19,8	1.435,0	119,5	66,4	84,9	66,0	219,1
SCC	0,6	0,5	0,5	0,1	2,0	0,5	0,3	0,5	0,2	0,9	0,5	0,5	0,2	0,2	1,2
Chromogranin A	93,9	117,3	49,4	16,6	405,9	90,0	98,4	53,9	38,2	289,7	44,2	17,9	41,6	24,2	71,6
IGF-I	341,1	83,5	326,3	211,6	459,1	279,7	157,7	269,8	141,2	438,1	303,0	162,8	276,0	160,7	499,2
TIMP-1	128,8	42,2	117,1	73,0	239,7	141,4	39,7	157,1	71,0	173,4	109,2	36,7	113,1	59,1	159,8
TIMP-2	67,1	16,0	67,2	46,0	102,5	82,8	22,2	88,5	47,5	103,6	75,4	30,8	76,0	42,2	122,9
MMP-1	470,7	338,4	316,8	84,6	1.056,3	1.020,6	805,0	856,1	62,7	2.333,3	250,5	305,9	101,9	35,7	776,5
MMP-2	176,3	40,7	171,8	115,3	278,4	164,8	35,7	168,1	115,3	216,0	182,9	66,3	159,8	113,0	290,1
MMP-7	5,8	2,2	4,8	2,9	11,4	5,5	1,5	5,9	2,5	6,8	4,4	2,3	3,2	2,5	7,7
MMP-9	201,4	201,0	147,0	33,3	848,5	209,4	137,2	160,3	84,6	391,9	140,6	73,5	118,3	62,8	259,7
MCP-1	321,9	263,3	217,7	47,9	941,9	372,0	329,7	250,2	94,3	956,2	152,3	100,7	105,8	46,1	283,9
IL-6	41,4	106,9	3,6	3,2	415,0	130,5	265,9	4,3	3,2	667,5	7,7	9,0	3,2	3,2	23,7
IL-8	245,0	929,8	8,9	3,2	4.078,8	31,3	35,2	17,7	4,3	100,0	65,7	122,3	5,1	4,1	283,4
VEGF	326,0	378,5	168,6	39,6	1.551,2	413,9	393,8	287,3	130,0	1.199,1	161,4	134,7	86,3	58,4	357,8
ICAM-1	119,1	47,0	102,2	55,1	200,2	130,1	50,9	126,4	61,2	207,7	125,8	53,7	136,3	58,0	186,1
VCAM-1	1.063,6	305,7	1.097,0	533,6	1.627,6	921,7	383,9	984,3	263,2	1.394,9	1.239,8	349,7	1.408,5	841,1	1.541,3
PAI-1	48.128,8	26.174,7	42.586,8	10.386,1	126.892,4	57.473,2	37.736,6	48.685,1	20.705,9	124.547,7	19.553,7	7.251,4	17.672,0	12.778,4	29.891,6

Table 28. Presurgery tumor marker levels in relation to stage in patients with adenocarcinoma (n= 34).

	T1(n=25)					T2(n=50)					T3(n=8)					T4(n=10)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA*	14,1	55,9	1,5	0,8	264,4	7,5	14,4	3,0	0,8	78,8	2,9	2,1	2,3	1,0	7,7	139,8	353,5	4,1	0,9	1.072,0
CYFRA 21-1*	1,8	1,5	1,4	0,4	7,5	6,3	10,2	2,8	0,5	43,9	4,9	6,0	2,2	0,8	18,5	12,2	20,3	3,1	0,6	64,6
TPA*	52,1	36,5	53,0	10,0	174,0	89,3	80,1	64,5	10,0	409,0	83,8	59,7	58,5	32,0	207,0	128,9	90,5	96,0	27,0	290,0
TPS	81,7	53,4	82,0	10,0	174,0	115,4	131,5	92,0	10,0	768,0	107,4	106,5	73,0	16,0	346,0	128,1	85,3	110,0	22,0	277,0
TK*	8,3	6,4	5,7	2,2	25,1	7,7	7,2	6,0	1,8	50,2	6,6	3,3	5,8	2,8	12,9	15,1	13,9	9,1	5,5	49,4
MonoTotal*	214,5	442,2	124,4	10,4	2.167,8	242,3	267,4	163,9	36,2	1.435,0	208,6	121,5	165,1	84,9	404,3	537,7	590,3	234,7	72,4	1.861,8
SCC	2,7	8,0	0,7	0,1	38,2	1,6	2,5	0,9	0,2	12,2	1,3	1,7	0,8	0,2	5,5	0,6	0,6	0,4	0,2	2,1
Chromogranin A	83,9	100,8	48,9	16,2	405,9	65,6	68,4	50,0	16,6	358,7	50,3	38,0	43,6	10,6	133,5	118,9	171,4	67,9	27,5	563,7
IGF-I	353,6	106,3	352,1	137,9	539,3	355,6	120,1	359,7	141,2	686,7	257,1	113,5	235,3	147,0	437,8	337,8	231,0	251,3	177,7	670,9
TIMP-1	123,9	36,6	117,1	71,0	217,4	141,4	38,6	140,0	72,6	239,7	168,3	68,1	153,6	59,1	267,8	134,4	20,9	129,3	112,0	169,0
TIMP-2	70,2	16,0	67,8	47,5	102,7	70,8	19,8	67,6	40,1	123,9	77,7	20,4	81,4	46,1	102,5	82,3	25,7	78,5	48,5	122,9
MMP-1	602,8	935,0	338,8	35,7	3.629,8	705,0	747,6	415,6	35,7	3.516,6	1.106,4	959,6	1.014,3	79,3	2.659,8	749,9	602,5	632,1	54,2	1.740,9
MMP-2	174,7	34,2	178,7	114,0	238,1	171,3	36,3	174,8	106,5	278,4	181,3	46,8	175,7	119,5	255,1	217,5	72,2	215,0	127,4	296,1
MMP-7	5,5	2,4	5,0	2,5	11,4	5,7	2,5	5,2	2,8	16,8	7,2	3,0	6,1	3,3	12,4	6,6	2,4	5,3	4,7	11,6
MMP-9*	117,7	59,3	100,4	33,3	294,8	228,2	184,2	169,1	29,3	848,5	177,2	141,7	114,1	47,2	405,6	189,6	86,8	155,7	97,9	371,0
MCP-1*	336,2	427,0	218,9	39,5	2.000,0	281,0	223,0	187,8	46,1	956,2	252,4	254,0	139,0	76,5	699,0	626,8	624,6	364,2	105,8	2.188,1
IL-6	53,9	120,3	4,1	3,2	415,0	58,3	155,0	5,7	3,2	765,0	16,8	20,1	9,6	3,2	62,6	14,2	18,6	6,0	3,2	57,3
IL-8	192,1	461,4	7,7	3,5	1.750,9	115,1	605,1	13,1	3,2	4.078,8	319,1	698,0	18,7	4,3	2.000,0	237,1	487,5	21,1	10,0	1.513,2
VEGF	232,2	235,0	166,5	16,0	847,4	317,6	364,4	170,7	26,2	1.595,5	227,3	154,4	155,8	86,3	470,8	288,0	285,8	212,3	16,0	792,5
ICAM-1	133,5	51,8	126,7	55,1	257,3	132,8	55,6	115,0	54,7	309,7	115,0	40,9	104,3	68,0	171,4	165,1	66,1	150,7	103,9	327,2
VCAM-1	1.148,8	344,8	1.097,0	263,2	1.694,9	974,8	286,8	875,7	533,6	1.634,0	1.024,2	426,1	1.153,2	244,9	1.502,6	1.114,0	279,8	1.106,2	772,5	1.526,7
PAI-1	38.848,5	23.078,4	29.578,1	10.386,1	87.670,4	45.462,6	22.622,7	41.893,7	16.357,0	126.892,4	44.896,3	24.328,3	52.294,5	12.778,4	80.200,4	58.191,9	24.553,0	63.169,8	16.990,8	90.351,1

Table 29. Presurgery tumor marker levels in relation to tumor size (T) in NSCLC patients (n=93).

	T1(n=15)					T2(n=29)					T3(n=6)					T4(n=9)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA*	1,8	1,1	1,5	0,8	4,3	5,1	8,1	2,6	0,8	41,3	2,2	1,0	2,1	1,0	4,0	156,6	374,1	3,7	0,9	1.072,0
CYFRA 21-1*	1,5	0,8	1,2	0,7	2,8	7,8	11,5	3,8	0,5	43,9	6,0	6,7	3,7	1,0	18,5	13,4	21,3	5,8	0,6	64,6
TPA*	47,0	22,6	53,0	10,0	78,0	90,2	66,9	67,0	10,0	254,0	96,0	65,2	75,5	32,0	207,0	134,3	95,2	120,0	27,0	290,0
TPS	84,4	52,3	82,0	10,0	172,0	115,3	138,8	92,0	10,0	768,0	134,8	110,6	104,5	41,0	346,0	129,5	91,1	108,5	22,0	277,0
TK	8,7	6,2	6,7	2,5	21,8	8,6	8,9	6,2	2,8	50,2	7,7	3,0	6,9	4,9	12,9	14,8	14,8	8,7	5,5	49,4
MonoTotal*	261,9	550,9	130,0	23,6	2.167,8	258,7	235,0	173,7	60,0	921,2	241,2	123,7	240,4	96,4	404,3	577,5	618,0	275,2	72,4	1.861,8
SCC	3,7	10,0	0,9	0,1	38,2	2,4	3,1	1,4	0,2	12,2	1,6	1,9	0,9	0,5	5,5	0,6	0,6	0,4	0,2	2,1
Chromogranin A	78,9	87,2	52,4	16,2	306,9	50,6	22,7	49,8	19,7	108,5	51,8	44,2	40,3	10,6	133,5	124,8	182,3	53,6	27,5	563,7
IGF-I	317,0	116,5	301,5	137,9	539,3	379,3	126,4	373,1	209,4	686,7	308,7	103,8	304,8	187,6	437,8	391,2	250,9	316,7	185,9	670,9
TIMP-1	130,7	36,4	133,2	83,7	217,4	143,9	37,3	145,0	72,6	227,3	190,1	58,7	183,0	123,6	267,8	131,3	19,9	128,7	112,0	169,0
TIMP-2	71,1	14,0	67,8	51,8	102,7	71,1	21,3	66,6	40,1	123,9	75,6	23,7	80,1	46,1	102,5	77,3	22,2	73,3	48,5	118,2
MMP-1	801,1	1.127,2	401,1	74,7	3.629,8	759,5	901,3	409,2	35,7	3.516,6	1.073,1	880,8	1.014,3	242,4	2.659,8	746,5	644,0	609,1	54,2	1.740,9
MMP-2	167,9	30,9	177,2	114,0	222,6	173,8	35,6	178,6	106,5	243,8	184,4	54,4	189,9	119,5	255,1	208,4	71,5	196,3	127,4	296,1
MMP-7	5,2	1,9	5,0	2,6	8,6	6,0	2,9	5,2	2,8	16,8	7,6	3,4	7,4	3,3	12,4	6,5	2,6	5,2	4,7	11,6
MMP-9	127,2	65,7	101,9	57,0	294,8	214,5	175,6	161,4	29,3	739,0	202,4	157,9	168,2	47,2	405,6	180,8	88,4	151,9	97,9	371,0
MCP-1*	369,4	500,6	223,8	39,5	2.000,0	237,3	169,9	179,0	58,1	681,0	294,2	285,5	139,0	76,5	699,0	691,9	634,2	471,9	216,2	2.188,1
IL-6	44,9	109,8	4,1	3,2	405,0	58,8	152,6	8,6	3,2	765,0	21,2	21,8	17,6	3,6	62,6	15,4	19,5	7,0	3,2	57,3
IL-8	280,1	564,2	7,4	4,4	1.750,9	23,6	30,9	13,1	3,2	113,7	424,0	793,3	23,2	7,6	2.000,0	231,3	520,8	20,7	10,0	1.513,2
VEGF	177,4	202,5	137,1	16,0	841,0	302,3	332,2	170,7	26,2	1.595,5	231,7	163,6	155,8	87,9	470,8	293,3	305,1	181,4	16,0	792,5
ICAM-1	145,5	52,7	136,2	76,5	257,3	133,1	60,6	113,6	54,7	309,7	122,9	45,0	132,4	68,0	171,4	165,2	70,7	147,7	103,9	327,2
VCAM-1	1.153,9	298,4	1.071,7	711,2	1.694,9	965,9	296,8	850,0	598,4	1.634,0	943,2	463,3	1.086,4	244,9	1.502,6	1.062,4	249,2	1.075,5	772,5	1.513,4
PAI-1	39.341,3	22.689,5	29.578,1	12.000,2	87.670,4	41.197,2	14.779,1	41.867,4	16.357,0	66.868,4	47.607,0	23.732,1	52.294,5	18.495,5	80.200,4	61.729,4	23.669,7	63.212,8	16.990,8	90.351,1

Table 30. Presurgery tumor marker levels in relation to tumor size (T) in patients with squamous cell carcinoma (n= 59).

	T1(n=10)					T2(n=21)					T3(n=2)					T4(n=1)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
CEA	35,5	92,5	2,4	0,8	264,4	11,1	20,3	3,4	1,0	78,8	5,1	3,7	5,1	2,4	7,7	5,0	.	5,0	5,0	5,0
CYFRA 21-1	2,2	2,4	1,7	0,4	7,5	4,1	7,8	1,7	0,6	34,3	1,8	1,3	1,8	0,8	2,7	2,1	.	2,1	2,1	2,1
TPA	61,1	53,9	52,0	11,0	174,0	87,9	98,3	54,0	13,0	409,0	47,0	12,7	47,0	38,0	56,0	86,0	.	86,0	86,0	86,0
TPS	77,0	58,6	74,0	11,0	174,0	115,6	123,7	99,0	10,0	550,0	25,0	12,7	25,0	16,0	34,0	117,0	.	117,0	117,0	117,0
TK	7,6	7,3	5,5	2,2	25,1	6,3	3,4	5,7	1,8	13,9	3,2	0,5	3,2	2,8	3,5	17,2	.	17,2	17,2	17,2
MonoTotal	131,5	103,3	108,6	10,4	289,4	217,6	315,5	141,4	36,2	1.435,0	110,9	36,8	110,9	84,9	136,9	219,1	.	219,1	219,1	219,1
SCC	0,8	0,6	0,6	0,1	2,0	0,5	0,4	0,3	0,2	1,4	0,5	0,4	0,5	0,2	0,7	0,8	.	0,8	0,8	0,8
Chromogranin A	92,6	127,4	48,9	27,9	405,9	89,6	103,6	50,3	16,6	358,7	45,9	16,6	45,9	34,1	57,6	71,6	.	71,6	71,6	71,6
IGF-I	406,1	65,6	414,8	316,6	499,2	312,4	98,7	287,1	141,2	459,1	153,9	9,7	153,9	147,0	160,7	177,7	.	177,7	177,7	177,7
TIMP-1	112,2	36,2	104,4	71,0	172,7	137,5	41,3	128,5	90,9	239,7	102,8	61,7	102,8	59,1	146,4	159,8	.	159,8	159,8	159,8
TIMP-2	68,7	20,0	68,3	47,5	102,5	70,5	17,9	68,9	46,0	103,6	84,0	4,8	84,0	80,6	87,4	122,9	.	122,9	122,9	122,9
MMP-1	255,7	215,8	194,9	35,7	574,6	618,3	410,8	715,1	84,6	1.522,2	1.206,3	1.593,8	1.206,3	79,3	2.333,3	776,5	.	776,5	776,5	776,5
MMP-2	186,5	38,6	185,3	115,3	238,1	167,5	38,2	158,6	115,3	278,4	171,7	16,8	171,7	159,8	183,6	290,1	.	290,1	290,1	290,1
MMP-7	5,9	3,1	5,0	2,5	11,4	5,3	1,7	5,2	2,9	8,4	6,1	0,2	6,1	5,9	6,3	7,7	.	7,7	7,7	7,7
MMP-9	101,1	45,1	95,5	33,3	154,7	250,0	200,5	175,2	56,3	848,5	101,4	23,8	101,4	84,6	118,3	259,7	.	259,7	259,7	259,7
MCP-1	278,2	274,9	206,0	94,3	941,9	346,7	277,5	244,1	46,1	956,2	126,9	46,9	126,9	93,7	160,0	105,8	.	105,8	105,8	105,8
IL-6	69,7	143,5	4,2	3,2	415,0	57,5	163,1	3,6	3,2	667,5	3,6	0,5	3,6	3,2	4,0	5,2	.	5,2	5,2	5,2
IL-8	38,1	70,7	7,7	3,5	210,5	252,3	955,5	13,8	3,2	4.078,8	4,4	0,2	4,4	4,3	4,5	283,4	.	283,4	283,4	283,4
VEGF	327,9	270,3	213,5	85,0	847,4	340,5	417,1	154,6	39,6	1.551,2	213,9	180,4	213,9	86,3	341,5	245,8	.	245,8	245,8	245,8
ICAM-1	112,5	45,7	111,0	55,1	186,1	132,3	48,6	116,4	62,8	207,7	91,5	9,9	91,5	84,5	98,5	164,3	.	164,3	164,3	164,3
VCAM-1	1.139,8	437,3	1.123,1	263,2	1.627,6	989,0	278,5	917,9	533,6	1.394,9	1.267,4	199,5	1.267,4	1.126,3	1.408,5	1.526,7	.	1.526,7	1.526,7	1.526,7
PAI-1	37.986,0	25.306,4	31.190,8	10.386,1	76.831,2	52.237,2	30.676,1	42.539,8	23.841,8	126.892,4	36.764,4	33.921,3	36.764,4	12.778,4	60.750,4	29.891,6	.	29.891,6	29.891,6	29.891,6

Table 31. Presurgery tumor marker levels in relation to tumor size (T) in patients with adenocarcinoma (n= 34).

	Sensitivity 95% specificity (%)	95% CI (%)		AUC	Cut off	PPV (%)	NPV (%)	RR
CEA	34,1	21,7	46,5	0,607	3,7	96,7	24,3	1,27
CYFRA 21-1	54,1	38,5	69,8	0,800	2,0	97,9	29,1	1,38
TPA	39,3	27,9	55,5	0,732	69,0	97,1	26,1	1,31
TPS	30,6	18,8	42,4	0,665	121,0	96,3	21,3	1,22
TK	11,8	4,5	19,1	0,459	13,8	76,9	17,6	1,12
MonoTotal	32,9	20,7	45,1	0,730	200,7	96,6	21,9	1,23
SCC	16,5	8,2	24,8	0,655	1,8	93,3	18,4	1,08
Chromogranin A	8,3	2,2	14,5	0,614	225,9	87,5	17,2	1,05
IGF-I	5,2	1,5	10,3	0,437	565,3	75,0	16,7	0,90
TIMP-1	13,1	5,4	20,8	0,668	183,3	91,7	18,9	1,13
TIMP-2[a]	11,9	4,5	19,3	0,531	48,6	75,0	17,3	0,90
MMP-1	22,6	12,5	32,8	0,497	917,3	95,0	20,7	1,19
MMP-2	14,1	6,1	22,1	0,522	222,5	91,7	17,8	1,12
MMP-7	15,3	7,0	23,6	0,619	8,3	92,9	18,2	1,13
MMP-9	23,8	13,4	34,2	0,612	244,3	95,2	21,0	1,20
MCP-1	2,4	1,7	5,6	0,452	1.545,9	66,7	16,2	0,79
IL-6	35,3	22,7	47,9	0,627	9,8	96,8	20,3	1,24
IL-8	12,9	5,3	20,6	0,465	143,1	91,7	15,9	1,11
VEGF	20,0	10,5	29,5	0,561	404,9	94,4	19,0	1,16
ICAM-1	3,6	1,5	7,6	0,559	255,8	75,0	17,3	0,90
VCAM-1	11,9	4,5	19,3	0,395	1.502,4	83,3	17,8	1,11
PAI-1	8,3	2,2	14,5	0,485	79.177,1	87,5	18,1	1,06

[a] Negative association

Table 32. Presurgery tumor marker sensitivity at 95% specificity comparing benign control group (n= 20) and NSCLC (n=93).

	Adenocarcinoma (n=34)			Squamous cell carcinoma (n=59)		
	Sensitivity 95% specificity (%)	AUC	Cut off	Sensitivity 95% specificity (%)	AUC	Cut off
CEA	46,7	0,680	3,7	27,3	0,564	3,8
CYFRA 21-1	36,7	0,720	2,0	63,6	0,840	2,0
TPA	33,3	0,660	69,0	42,6	0,769	69.5
TPS	30,0	0,590	126,0	30,9	0,703	121,0
TK	10,0	0,390	13,8	12,7	0,499	13,9
MonoTotal	26,7	0,650	206,0	36,4	0,780	200,7
SCC	3,3	0,545	1,8	23,6	0,720	1,9
Chromogranin A	13,8	0,650	227,0	5,5	0,594	225,9
IGF-I	1,5	0,400	555,1	8,1	0,460	565,3
TIMP-1	6,9	0,590	183,5	16,4	0,710	183,3
TIMP-2[a]	13,8	0,546	48,5	10,9	0,520	48,6
MMP-1	17,2	0,480	917,3	25,5	0,508	986,6
MMP-2	13,3	0,500	227,0	14,5	0,534	222,5
MMP-7	10,0	0,580	8,4	18,2	0,534	8,3
MMP-9	27,6	0,610	244,3	21,8	0,610	267,7
MCP-1	1,7	0,440	1.545,9	3,6	0,460	1.545,9
IL-6	23,3	0,530	16,7	41,8	0,677	9,8
IL-8	10,0	0,440	170.84	14,5	0,477	143,1
VEGF	23,3	0,580	457,2	18,2	0,548	404.93
ICAM-1	1,5	0,480	356,7	5,5	0,601	255,8
VCAM-1	10,3	0,430	1.514,5	12,7	0,376	1.502,4
PAI-1	6,9	0,470	80.774,5	9,1	0,490	79.177,1

[a] Negative association

Table 33. Presurgery tumor marker sensitivity at 95% specificity comparing benign group and NSCLC histologic subgroups.

	NSCLC (Remission) (n=117 samples)					NSCLC (Progression) (n=21 samples)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA*	2,2	1,5	1,7	0,5	10,6	13,6	25,4	5,2	0,6	110,8	0,0004
CYFRA 21-1*	1,0	1,0	0,8	0,0	7,0	33,8	96,3	2,2	0,3	404,0	0,0001
TPA*	29,0	21,5	25,0	6,3	105,0	135,2	164,2	65,0	10,0	656,0	<0,0001
TPS*	58,9	56,5	43,0	10,0	421,0	300,0	492,3	63,5	19,0	1.718,0	0,0180
TK*	6,8	3,7	6,0	2,3	23,1	23,2	40,8	12,5	2,4	193,5	0,0004
MonoTotal*	77,1	61,1	63,7	0,0	383,3	399,3	656,2	141,8	39,4	2.632,0	0,0001
SCC*	0,5	0,4	0,4	0,0	1,9	1,6	2,7	0,6	0,0	11,9	0,0262
Chromogranin A*	63,1	78,6	42,1	14,3	571,2	160,9	223,5	67,2	25,4	748,1	0,0057
IGF-I	291,3	87,1	300,5	112,1	489,1	305,1	74,2	303,3	183,3	421,8	nss
TIMP-1*	120,1	35,0	114,6	67,7	282,6	145,5	45,7	131,0	85,6	271,0	0,0054
TIMP-2	78,8	21,9	75,7	37,6	149,0	78,9	24,8	72,2	47,0	132,0	nss
MMP-1*	449,1	548,5	265,6	35,7	3.944,0	801,7	814,8	578,0	41,8	2.827,6	0,0180
MMP-2	212,4	45,4	203,0	137,0	353,2	213,4	51,7	204,0	127,1	342,1	nss
MMP-7*	5,6	2,2	5,3	2,4	15,2	7,7	4,2	6,6	2,8	19,9	0,0122
MMP-9	136,4	66,2	122,7	28,0	379,4	168,1	120,1	141,2	18,0	427,6	nss
MCP-1*	329,4	322,3	196,6	67,2	2.000,0	654,2	687,2	397,2	132,6	2.661,5	0,0059
IL-6*	73,1	227,6	3,6	3,2	1.600,7	117,8	303,1	15,5	3,2	1.223,7	0,0100
IL-8	122,2	557,9	11,0	3,2	4.398,1	72,5	126,3	18,0	3,4	451,3	nss
VEGF*	269,6	402,4	151,3	16,0	2.915,2	472,7	558,1	291,8	72,6	2.123,0	0,0062
ICAM*	129,6	53,2	123,7	54,0	322,0	164,0	93,1	142,7	43,2	462,9	0,0357
VCAM	1.184,1	296,2	1.126,9	244,9	2.075,9	1.059,3	372,7	1.012,0	263,2	1.880,9	nss
PAI-1	43.629,3	24.479,9	37.550,3	10.340,2	125.070,4	50.574,3	23.763,0	46.368,7	12.428,2	91.233,1	nss

Table 34. Tumor marker levels in relation to remission / progression status during follow up.

	Squamous (n=13)					Adenocarcinoma (n=8)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA*	4,0	4,3	2,5	0,6	15,2	31,3	38,0	14,5	6,1	110,8	0,0008
CYFRA 21-1	53,2	120,0	3,7	0,3	404,0	2,3	2,0	1,5	0,7	6,0	nss
TPA	171,8	196,9	92,5	13,0	656,0	80,3	80,7	47,5	10,0	239,0	nss
TPS	397,3	607,4	116,5	21,0	1.718,0	154,1	193,7	62,5	19,0	480,0	nss
TK	26,0	51,1	12,5	2,4	193,5	18,8	15,9	11,2	5,9	48,6	nss
MonoTotal	518,9	805,5	191,5	43,6	2.632,0	205,1	220,4	98,5	39,4	683,6	nss
SCC*	2,3	3,2	1,0	0,4	11,9	0,3	0,2	0,3	0,0	0,7	0,0006
Chromogranin A	124,6	160,7	67,6	25,4	633,8	228,1	313,6	46,9	34,3	748,1	nss
IGF-I	263,9	56,9	283,8	183,3	304,7	360,1	61,1	358,9	299,7	421,8	nss
TIMP-1	154,3	54,0	139,7	85,6	271,0	131,3	24,5	120,1	106,4	177,0	nss
TIMP-2	78,0	27,6	69,7	47,0	132,0	80,5	21,1	75,1	57,5	115,8	nss
MMP-1	931,8	846,5	751,3	41,8	2.827,6	590,2	765,6	295,9	151,2	2.441,6	nss
MMP-2	210,1	58,6	194,0	127,1	342,1	218,7	41,4	224,3	150,5	274,6	nss
MMP-7	8,3	4,9	6,6	3,8	19,9	6,7	3,0	6,1	2,8	12,3	nss
MMP-9	154,2	107,2	140,1	18,0	427,6	190,7	143,5	144,2	44,7	418,6	nss
MCP-1	671,1	790,9	377,6	132,6	2.661,5	626,7	524,9	546,2	150,9	1.756,7	nss
IL-6	171,5	379,8	19,2	3,2	1.223,7	30,6	35,7	13,1	3,2	90,9	nss
IL-8	74,8	139,0	21,7	5,1	451,3	68,7	111,3	13,1	3,4	328,0	nss
VEGF	517,1	707,2	230,0	72,6	2.123,0	400,7	151,5	380,5	138,2	584,2	nss
ICAM-1	174,4	108,5	144,7	61,2	462,9	147,2	63,9	135,7	43,2	273,6	nss
VCAM-1	944,8	314,7	926,1	263,2	1.481,2	1.245,4	403,9	1.181,0	764,0	1.880,9	nss
PAI-1	47.306,5	24.671,2	46.070,9	12.428,2	91.233,1	55.884,6	22.757,1	56.650,2	23.188,8	88.180,0	nss

Table 35. Tumor marker levels in relation to histology in moment of progression during follow up.

	Sensitivity 95% specificity	95% CI		AUC	Cut off	PPV (%)	NPV (%)	RR
CEA	50,0	17,3	82,7	0,7	4,8	64,7	90,9	7,93
CYFRA 21-1	52,0	20,0	85,3	0,8	2,6	64,7	90,1	9,19
TPA	50,0	17,3	82,7	0,8	77,0	62,5	89,9	8,35
TPS	44,0	13,6	75,2	0,7	153,5	57,1	88,4	7,26
TK	52,0	20,0	85,3	0,7	12,7	60,0	88,9	8,40
MonoTotal	52,0	20,0	85,3	0,8	161,0	64,7	90,2	8,33
SCC	19,0	0,0	33,7	0,7	1,6	44,4	85,6	4,73
Chromogranin A	20,0	0,0	35,5	0,7	169,3	40,0	87,2	2,77
IGF-I	14,3	0,0	33,7	0,5	413,4	33,3	84,6	2,16
TIMP-1	19,0	0,4	41,7	0,7	180,0	37,5	86,6	3,36
TIMP-2[a]	14,3	0,0	25,1	0,5	53,5	25,0	85,3	1,88
MMP-1	14,3	0,0	33,7	0,7	1.481,5	33,3	85,9	2,64
MMP-2	4,8	0,0	15,6	0,5	295,2	14,3	84,5	1,19
MMP-7	14,3	0,0	33,7	0,7	10,5	33,3	85,8	2,62
MMP-9	19,0	0,0	33,7	0,5	258,4	40,0	86,6	2,64
MCP-1	23,8	3,3	49,4	0,7	876,6	45,5	87,2	3,33
IL-6	9,5	0,0	25,1	0,7	391,6	25,0	85,2	1,87
IL-8	14,3	0,0	33,7	0,6	252,8	33,3	85,8	2,97
VEGF	9,5	0,0	25,1	0,7	912,4	25,0	85,2	1,86
ICAM-1	14,3	0,0	33,7	0,6	251,4	33,3	85,9	3,00
VCAM-1	8,7	0,0	25,1	0,4	1.767,5	28,6	85,4	1,88
PAI-1	4,8	0,0	15,6	0,6	90.021,5	14,3	84,6	1,02

[a] Negative association

Table 36. Tumor marker sensitivity at 95% specificity in NSCLC patients during follow up monitoring.

Correlation		Control group	NSCLC group	NSCLC group Pre-surgery and Follow up				
				Pre-surgery	6 month	12 month	18 month	24 month
CYFRA / MT	r value	0,78	0,76	0,80	0,76	0,66	0,58	0,60
	p value	0,0003	<0,0001	<0,0001	<0,0001	<0,0001	0,0018	0,0018
CYFRA / TPA	r value	0,51	0,73	0,82	0,46	0,62	0,60	0,64
	p value	0,0354	<0,0001	<0,0001	0,0008	<0,0001	0,0014	0,0025
TIMP-2 / MMP-2	r value	0,66	0,68	0,67	0,87	0,84	-----	-----
	p value	0,0040	<0,0001	<0,0001	<0,0001	<0,0001	-----	-----
TPS / MT	r value	-----	0,73	0,76	0,50	0,61	0,62	0,58
	p value	-----	<0,0001	<0,0001	0,0002	<0,0001	0,0008	0,0069
TPA / MT	r value	-----	0,78	0,84	0,48	0,59	0,65	0,62
	p value	-----	<0,0001	<0,0001	0,0004	0,0001	0,0003	0,0037
TPA / TPS	r value	-----	0,68	0,68	0,42	0,75	0,63	-----
	p value	-----	<0,0001	<0,0001	0,0019	<0,0001	0,0006	-----
CYFRA / TPS	r value	-----	0,54	0,57	-----	0,60	-----	-----
	p value	-----	<0,0001	<0,0001	-----	<0,0001	-----	-----
TIMP-1 / MMP-1	r value	-----	0,50	-----	-----	0,49	-----	-----
	p value	-----	<0,0001	-----	-----	0,0021	-----	-----
PAI-1 / TIMP-1	r value	-----	0,40	-----	0,63	0,47	-----	-----
	p value	-----	<0,0001	-----	<0,0001	0,0036	-----	-----
IL-6 / IL-8	r value	-----	0,40	-----	0,57	0,53	-----	-----
	p value	-----	<0,0001	-----	<0,0001	0,0008	-----	-----
IL-6 / VEGF	r value	-----	0,47	-----	-----	0,53	-----	0,65
	p value	-----	<0,0001	-----	-----	0,0008	-----	0,0006
IL-8 / MCP-1	r value	0,72	-----	-----	-----	-----	-----	-----
	p value	0,0011	-----	-----	-----	-----	-----	-----
TPS / PAI-1	r value	0,70	-----	-----	-----	-----	-----	-----
	p value	0,0018	-----	-----	-----	-----	-----	-----
PAI-1 / VEGF	r value	0,68	-----	-----	-----	-----	-----	-----
	p value	0,0025	-----	-----	-----	-----	-----	-----
MT / MMP-2	r value	0,66	-----	-----	-----	-----	-----	-----
	p value	0,0036	-----	-----	-----	-----	-----	-----
TPA / VCAM-1	r value	0,63	-----	-----	-----	-----	-----	-----
	p value	0,0050	-----	-----	-----	-----	-----	-----
CYFRA / MMP-2	r value	0,56	-----	-----	-----	-----	-----	-----
	p value	0,0235	-----	-----	-----	-----	-----	-----
TK / MMP-7	r value	0,59	-----	-----	-----	-----	-----	-----
	p value	0,0136	-----	-----	-----	-----	-----	-----
TPS / MMP-7	r value	0,52	-----	-----	-----	-----	-----	-----
	p value	0,0374	-----	-----	-----	-----	-----	-----
TK / IL-6	r value	0,50	-----	-----	-----	-----	-----	-----
	p value	0,0415	-----	-----	-----	-----	-----	-----

Table 37. Correlation between biomarkers levels in control group, NSCLC group and during follow up (only significant correlations).

			CYTOKERATINS								METALOPROTEINASE REGULATORS						INFLAMMATION				ADHESION		
		CEA	CYFRA 21-1	TPA	TPS	MonoTotal	TK	**SCC**	**Chromogranin A**	**IGF-I**	TIMP-1	TIMP-2	MMP-1	MMP-2	MMP-7	**MMP9-9**	MCP-1	IL-6	IL-8	VEGF	**ICAM-1**	VCAM-1	PAI-1
	CEA																						
CYTOKERATINS	CYFRA 21-1			**X**	X	**X**								O									
	TPA		**X**		X	X																O	
	TPS		X	X		X									O								O
	MonoTotal		**X**	X	X									O									
	TK														O			O					
	SCC																						
	Chromogranin A																						
	IGF-I																						
METALOPROTEINASE REGULATORS	TIMP-1												X										X
	TIMP-2													**X**									
	MMP-1										X												
	MMP-2		O			O						**X**											
	MMP-7				O		O																
	MMP9																						
INFLAMMATION	MCP-1																		O				
	IL-6						O												X	X			
	IL-8																O	X					
	VEGF																	X					O
ADHESION	**ICAM-1**																						
	VCAM-1			O																			
	PAI-1				O						X									O			

X Correlation in control and NSCLC

X Correlation in NSCLC

O Correlation in control group

Table 38. Summary of biomarkers correlation.

	Remission in Last Control (n=45)					Progression in Last Control (n=43)					
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum	p value
CEA	4,3	6,8	2,5	0,8	41,3	42,6	174,0	2,2	0,8	1.072,0	nss
CYFRA 21-1*	3,0	4,9	1,7	0,4	31,1	8,3	13,8	2,7	0,8	64,6	0,0109
TPA*	55,5	39,7	55,0	10,0	190,0	106,2	89,3	68,0	10,0	409,0	0,0079
TPS	84,4	67,3	64,0	10,0	346,0	128,1	139,8	100,5	10,0	768,0	nss
TK	6,6	3,5	5,4	1,8	17,1	10,4	10,7	6,5	2,5	50,2	nss
MonoTotal*	190,5	333,1	126,7	10,4	2.167,8	337,0	396,2	173,0	23,6	1.861,8	0,0072
SCC	2,1	6,2	0,7	0,1	38,2	1,4	2,1	0,9	0,1	12,2	nss
Chromogranin A	67,9	74,8	41,7	16,6	358,7	78,2	106,5	52,5	10,6	563,7	nss
IGF-I	349,2	120,9	328,2	147,0	686,7	336,8	125,3	339,4	141,2	670,9	nss
TIMP-1*	127,8	38,1	119,0	71,0	227,3	144,0	40,8	143,5	59,1	267,8	0,0429
TIMP-2	73,3	19,7	70,1	40,1	123,9	71,6	20,9	65,6	40,5	122,9	nss
MMP-1*	537,9	680,1	259,1	35,7	3.516,6	853,9	868,7	569,6	35,7	3.629,8	0,0381
MMP-2	173,0	38,7	173,4	106,5	278,4	181,9	49,1	185,7	113,0	296,1	nss
MMP-7	5,6	2,1	5,0	2,5	12,2	6,0	2,8	5,3	2,5	16,8	nss
MMP-9	195,2	167,3	154,7	29,3	848,5	183,9	146,2	142,3	33,3	739,0	nss
MCP-1	273,8	211,3	200,7	39,5	867,1	340,9	376,8	211,4	64,8	2.188,1	nss
IL-6*	22,6	67,2	3,8	3,2	415,0	74,0	171,8	7,6	3,2	765,0	0,0151
IL-8*	82,1	320,4	8,2	3,2	2.000,0	122,5	321,7	18,9	3,2	1.513,2	0,0204
VEGF	222,3	212,6	157,3	16,0	847,4	305,1	334,4	162,1	16,0	1.595,5	nss
ICAM-1	123,1	41,3	113,6	61,2	249,2	139,3	66,6	130,9	54,7	327,2	nss
VCAM-1	1.060,4	313,2	1.050,6	263,2	1.694,9	1.010,0	340,5	926,3	244,9	1.634,0	nss
PAI-1	45.124,2	18.953,1	42.997,5	12.000,2	90.351,1	40.792,5	23.270,1	35.647,0	10.386,1	124.547,7	nss

Table 39. Presurgery marker levels in patients with NSCLC in relation to clinical status in last control.

	Cut off	p value Univariate Kaplan Meier	p value Univariate Cox regression
CEA*	4,8	nss	0.0021
CYFRA 21-1	2,6	nss	nss
TPA*	77,0	0,0081	0.0007
TPS*	153,5	nss	0.0228
TK	12,7	nss	nss
MonoTotal*	200,0	0,0113	<0.0001
SCC	1,6	nss	nss
Chromogranin A	169,3	nss	nss
IGF-I	413,4	nss	nss
TIMP-1	180,0	nss	nss
TIMP-2	126,9	nss	nss
MMP-1*	1.481,5	0,0225	nss
MMP-2*	295,2	0,0022	0.0201
MMP-7*	10,5	0,0340	0.0427
MMP-9	258,4	nss	nss
MCP-1	876,6	nss	nss
IL-6	391,6	nss	nss
IL-8	252,8	nss	nss
VEGF	912,4	nss	nss
ICAM-1	251,4	nss	nss
VCAM-1	1.767,5	nss	nss
PAI-1	90.021,5	nss	nss
T*		0,0002	0.0113
N		nss	nss
Stage*		0,0003	0,0002
Histology		nss	nss

Table 40. Univariate DFS analysis

	p value	Hazard ratio	95,0% CI	
Stage	0,0006			
Stage I		1,00		
Stage II	0,4050	1,47	0,59	3,68
Stage III	0,0001	4,89	2,25	10,59
MonoTotal	0,0203	0,43	0,21	0,88
MMP1	0,0098	0,31	0,13	0,75

	p value	Hazard ratio	95,0% CI	
Stage	0,0004			
Stage I		1,00		
Stage II	0,2380	1,75	0,69	4,43
Stage III	0,0001	4,86	2,21	10,70
TPA	0,0229	2,36	1,13	4,94
MMP1	0,0188	2,93	1,20	7,20

	p value	Hazard ratio	95,0% CI	
Stage	0,0007			
Stage I		1,00		
Stage II	0,2562	1,71	0,68	4,29
Stage III	0,0002	4,15	1,98	8,73
MonoTotal	0,0222	2,25	1,12	4,51
MMP7	0,0210	4,50	1,25	16,17

	p value	Hazard ratio	95,0% CI	
Stage	0,0010			
Stage I		1,00		
Stage II	0,1358	2,04	0,80	5,18
Stage III	0,0002	4,20	1,97	8,94
TPA	0,0123	2,53	1,22	5,22
MMP7	0,0167	4,83	1,33	17,51

Table 41. Multivariate DFS analysis (Cox multivariate model using cut-offs).

	Cut off	p value Univariate Kaplan Meier	p value Univariate Cox regression
CEA*	4,8	0,0457	0.0009
CYFRA 21-1	2,6	nss	nss
TPA*	77,0	0,0020	0.0044
TPS*	153,5	0,0116	0.0315
TK	12,7	nss	nss
MonoTotal*	200,0	0,0018	<0.0001
SCC	1,6	nss	nss
Chromogranin A*	169,3	nss	0.0070
IGF-I	413,4	nss	nss
TIMP-1	180,0	nss	nss
TIMP-2	126,9	nss	nss
MMP-1	1.481,5	nss	nss
MMP-2	295,2	nss	nss
MMP-7	10,5	nss	0.0177
MMP-9	258,4	nss	nss
MCP-1*	876,6	0,0042	nss
IL-6	391,6	nss	nss
IL-8	252,8	nss	nss
VEGF	912,4	nss	nss
ICAM-1	251,4	nss	nss
VCAM-1	1.767,5	nss	nss
PAI-1	90.021,5	nss	nss
T*		0,0013	0.0195
N*		0,019	0.0322
Stage*		<0,0001	<0,0001
Histology		nss	nss

Table 42. Univariate OS analysis.

	p value	Hazard ratio	95,0% CI	
Stage	<0,0001			
Stage I		1,00		
Stage II	0,0222	3,59	1,20	10,76
Stage III	<0,0001	10,69	3,82	29,97
Chromogranin	0,0283	3,93	1,16	13,33
MMP7	0,0330	4,82	1,14	20,46
MCP1	0,0437	3,23	1,03	10,11
MonoTotal	0,0080	2,91	1,32	6,39

	p value	Hazard ratio	95,0% CI	
Stage	<0,0001			
Stage I		1,00		
Stage II	0,0063	4,79	1,56	14,73
Stage III	<0,0001	11,29	4,00	31,89
Chromogranin	0,0626	3,12	0,94	10,36
MMP7	0,0202	5,43	1,30	22,68
MCP1	0,0945	2,66	0,81	8,68
TPA	0,0072	3,07	1,36	6,94

Table 43. Multivariate OS analysis (Cox multivariate model using cut-offs).

	Adenocarcinoma		Squamous	
	p value DFS	p value OS	p value DFS	p value OS
CEA*	nss	0,0146	nss	nss
CYFRA 21-1*	0,0378	nss	nss	nss
TPA*	0,0148	nss	0,0231	0,0124
TPS	nss	nss	nss	nss
TK	nss	nss	nss	nss
MonoTotal*	0,0317	nss	<0,0001	<0,0001
SCC	nss	nss	nss	nss
Chromogranin A*	nss	nss	nss	0,0086
IGF-I	nss	nss	nss	nss
TIMP-1	nss	nss	nss	nss
TIMP-2*	0,025	0,0029	nss	nss
MMP-1*	nss	0,0131	nss	nss
MMP-2*	0,0013	0,0011	nss	nss
MMP-7*	0,0164	nss	nss	0,0144
MMP-9	nss	nss	nss	nss
MCP-1	nss	nss	nss	nss
IL-6	nss	nss	nss	nss
IL-8	nss	nss	nss	nss
VEGF	nss	nss	nss	nss
ICAM-1	nss	nss	nss	nss
VCAM-1	nss	nss	nss	nss
PAI-1	nss	nss	nss	nss

Table 44. Univariate DFS/OS analysis according to the histologic subtypes of NSCLC group.

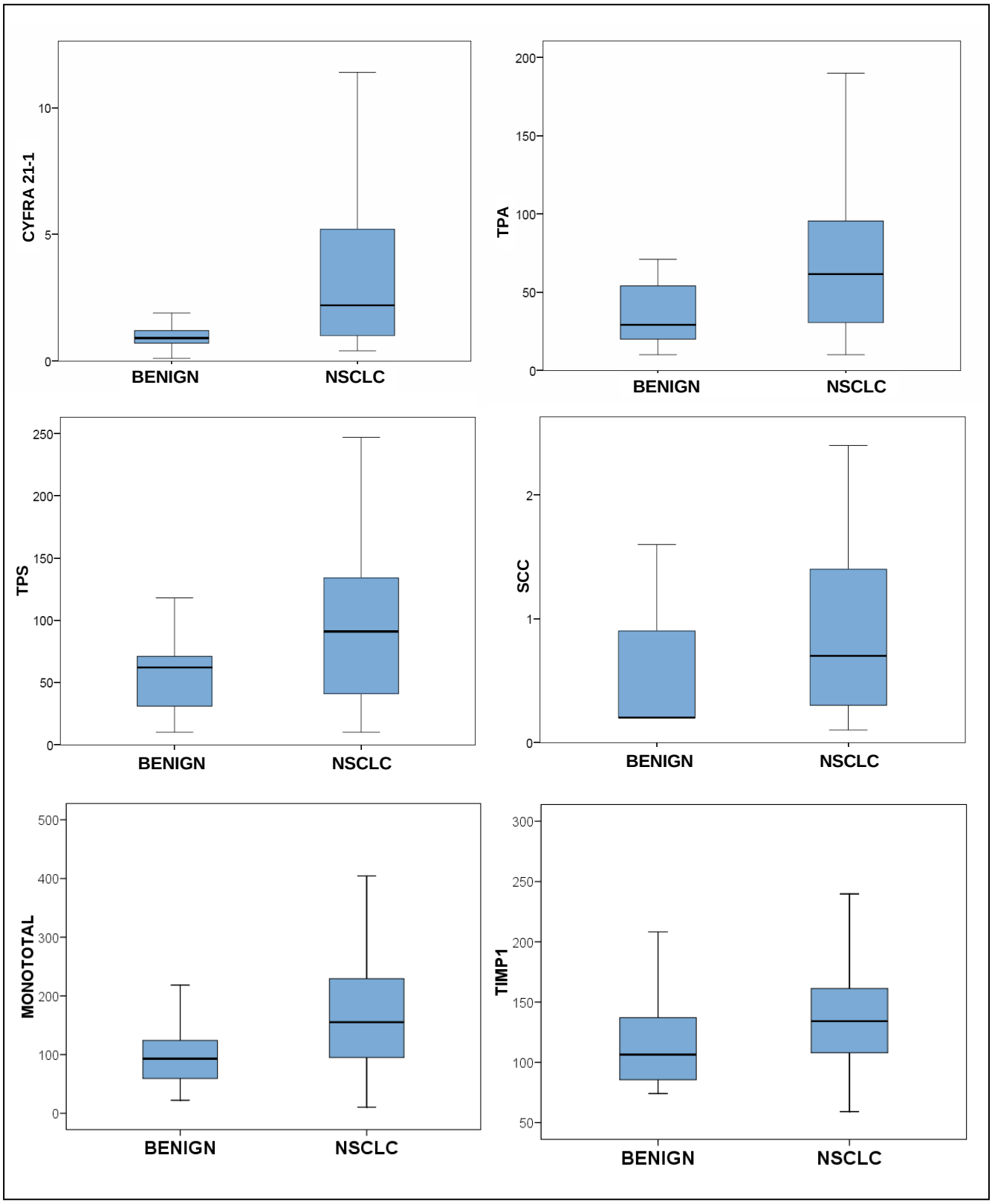

Figure 11. Presurgery tumor marker levels in control group vs NSCLC patients (presented box-plots of significantly different markers)

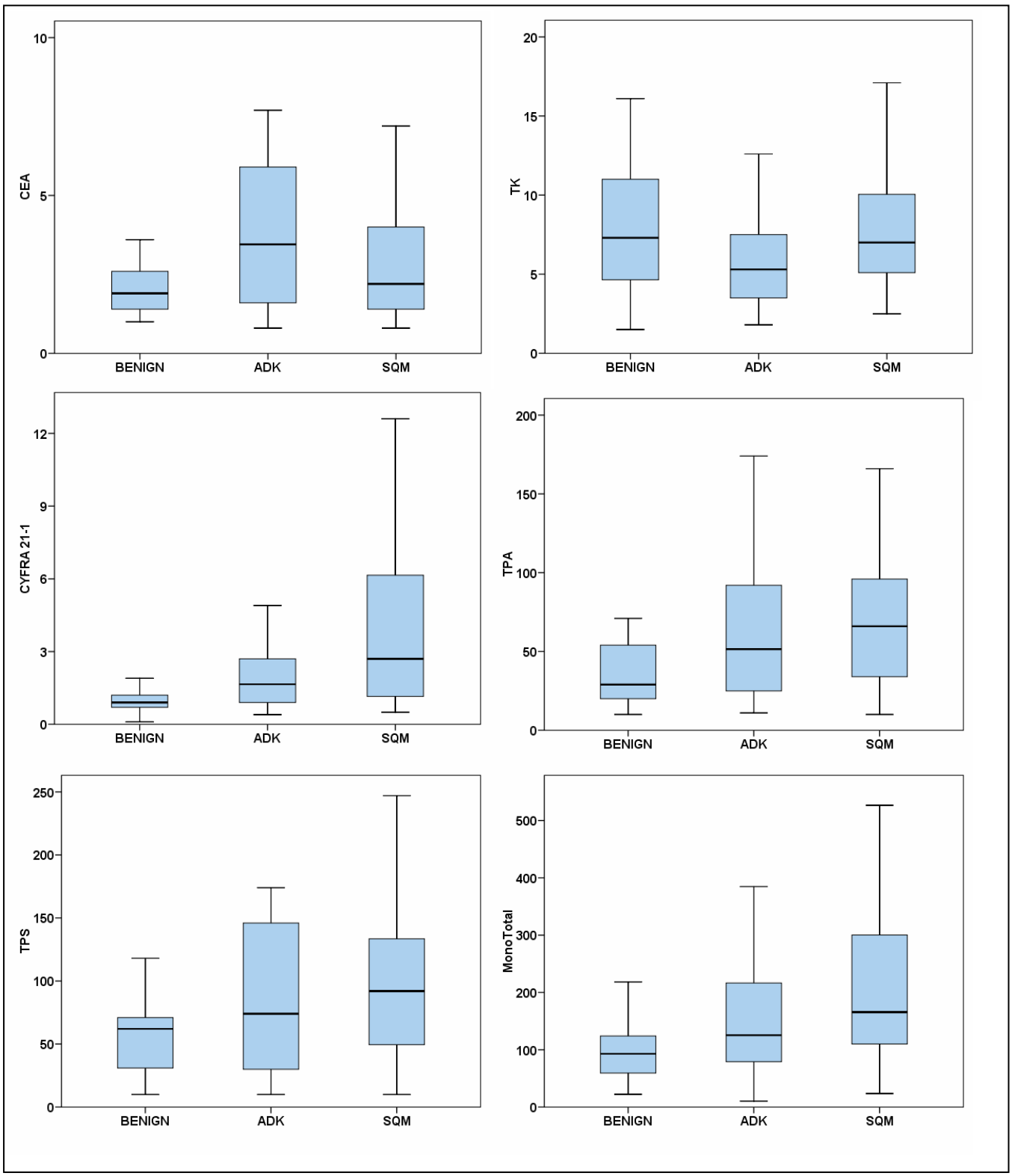

Figure 12a. Presurgery tumor marker levels in control group vs NSCLC histology subgroups (presented box-plots of significantly different markers)

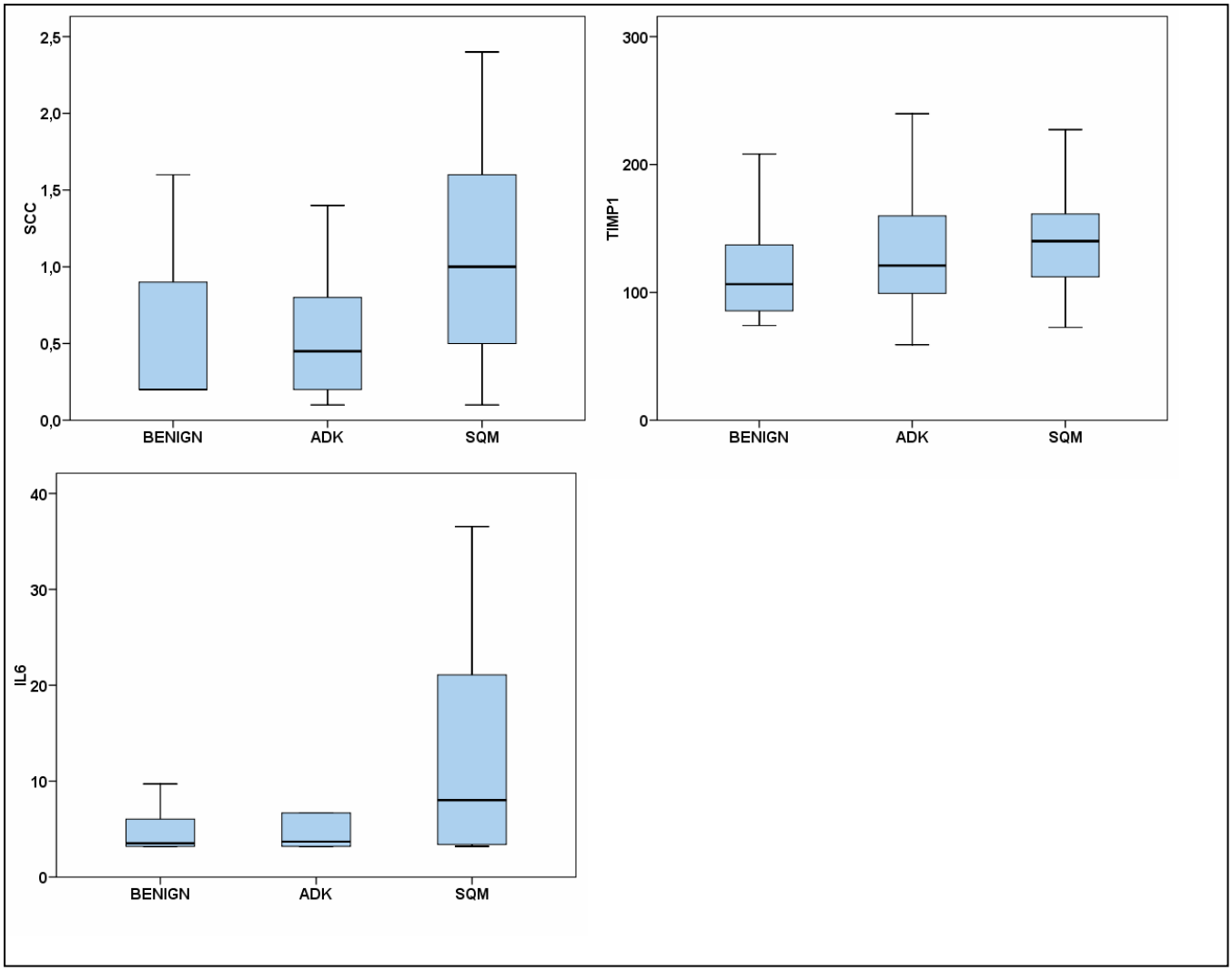

Figure 12b. Presurgery tumor marker levels in control group vs NSCLC histology subgroups (presented box-plots of significantly different markers)

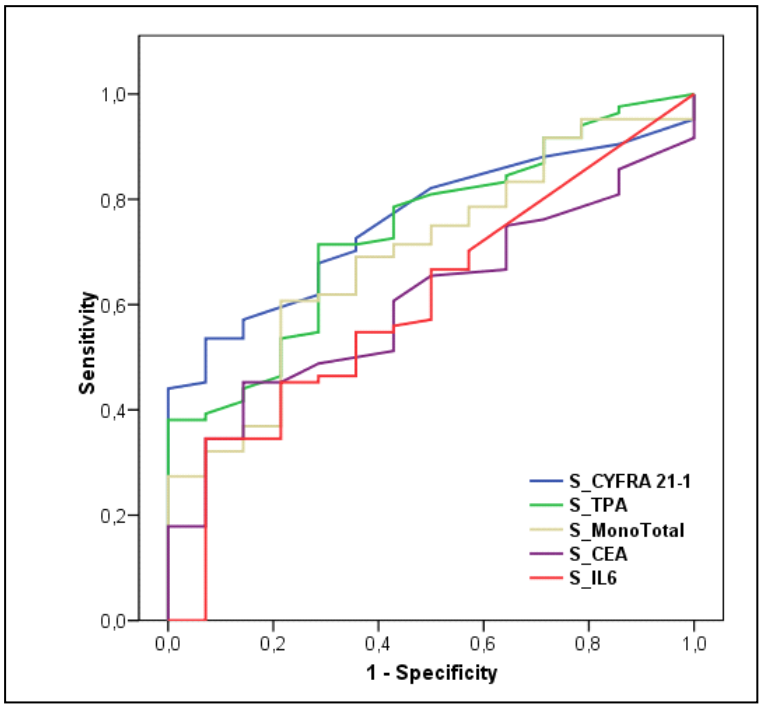

Figure 13. ROC curves for markers with the highest sensitivity comparing patients with benign lung disease and NSCLC.

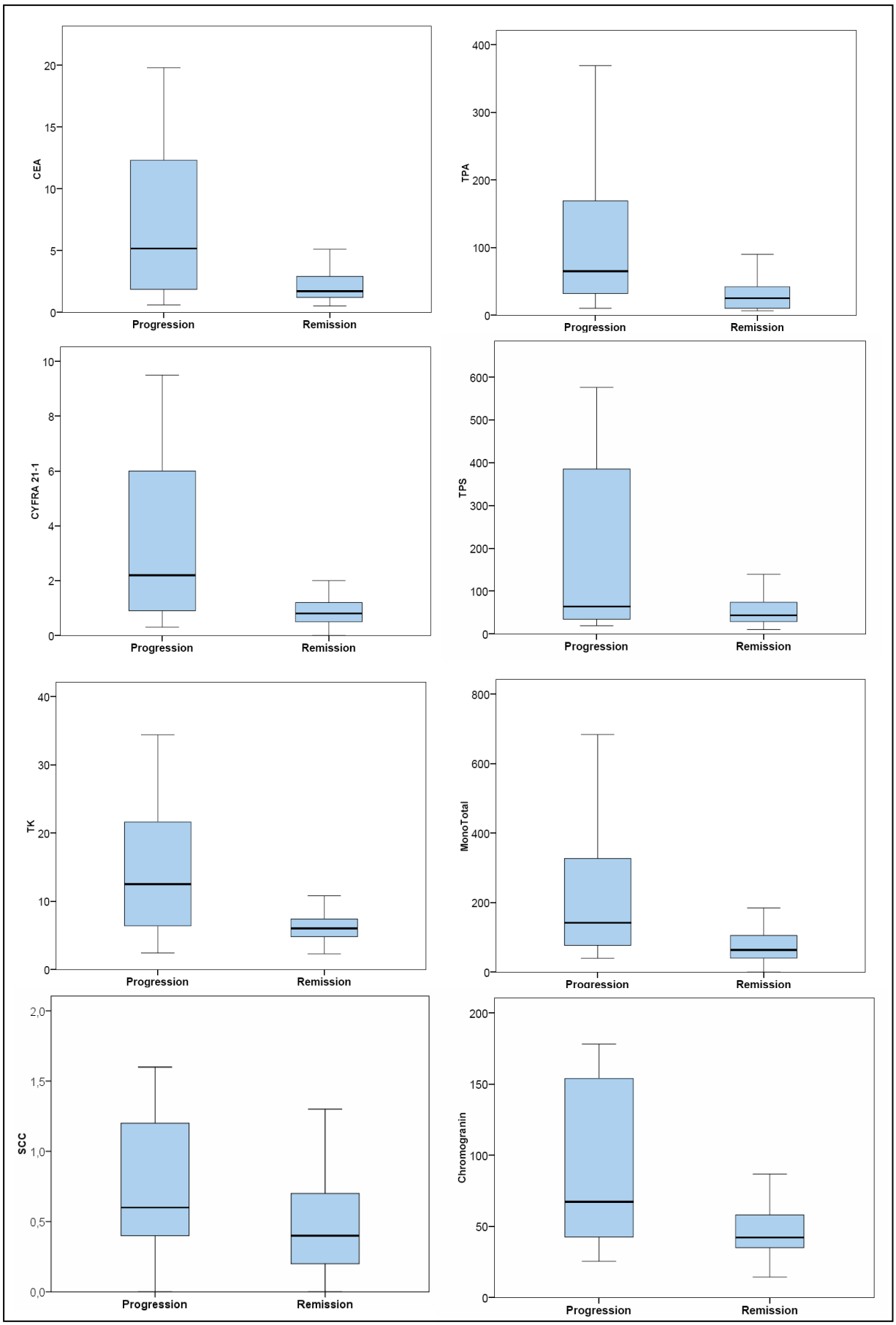

Figure 14a. Tumor marker levels related to remission and progression during follow-up (box-plots showing significant markers)

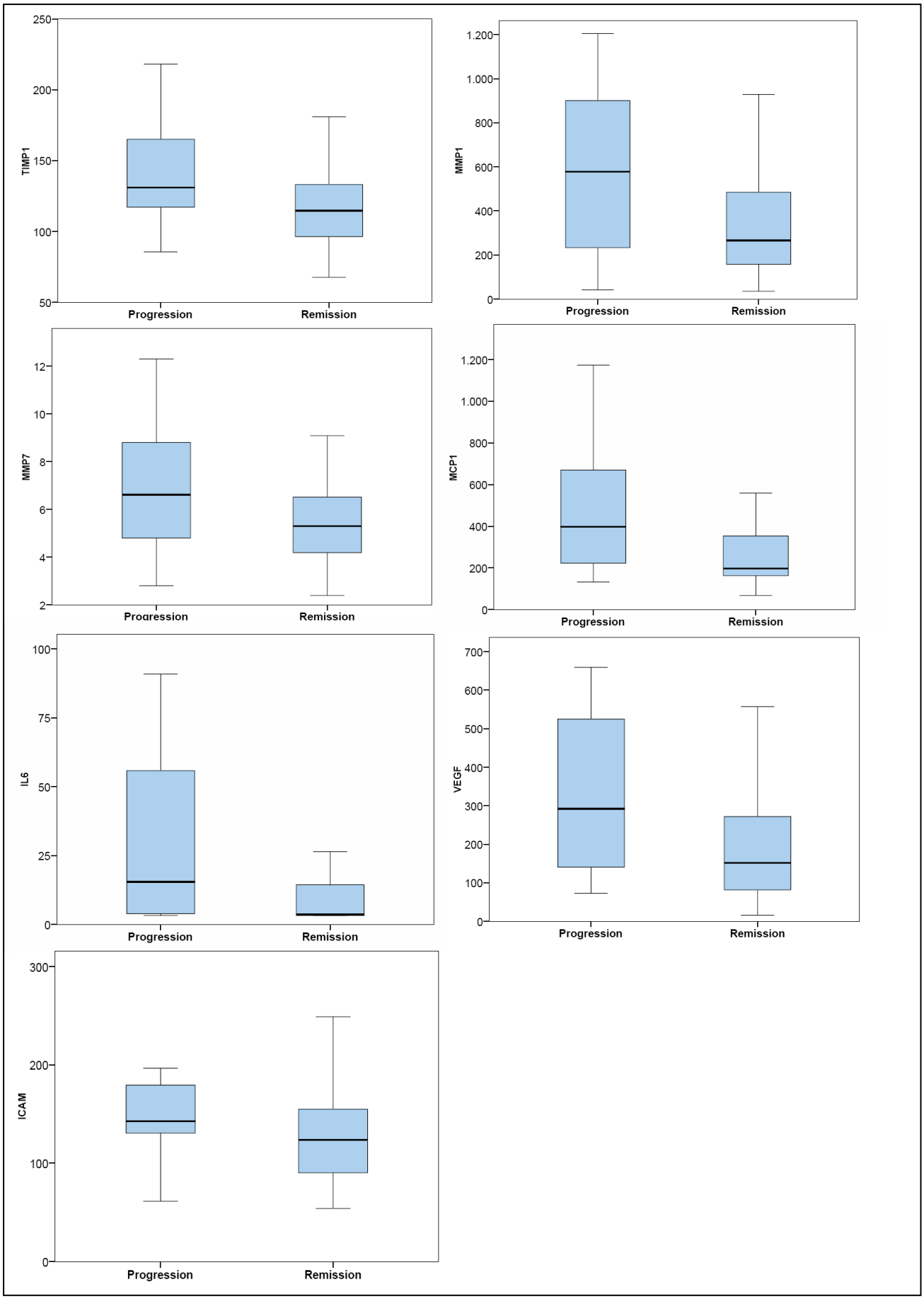

Figure 14b. Tumor marker levels related to remission and progression during follow-up (Box-plots showing significant markers).

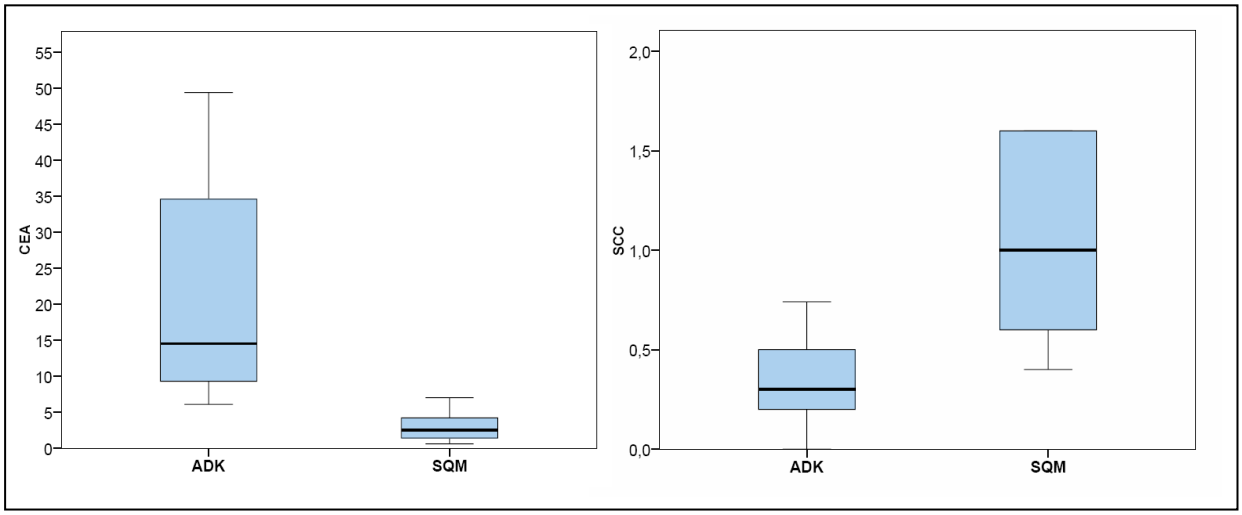

Figure 15. Tumor marker levels during progression in relation to histology (significant markers)

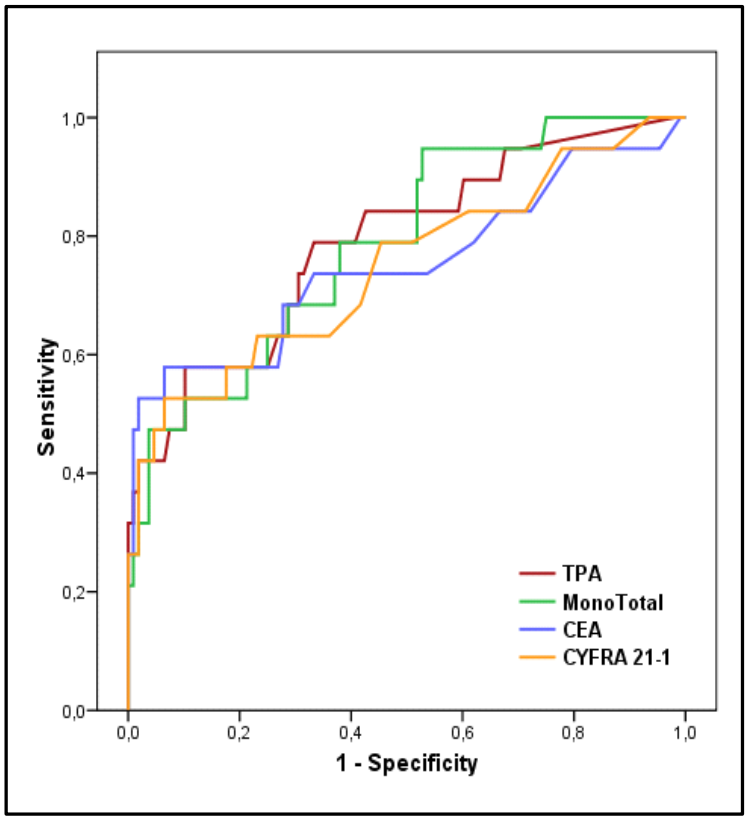

Figure 16. ROC curves for markers with the highest sensitivity – comparing remission vs. progression during follow-up of NSCLC patients.

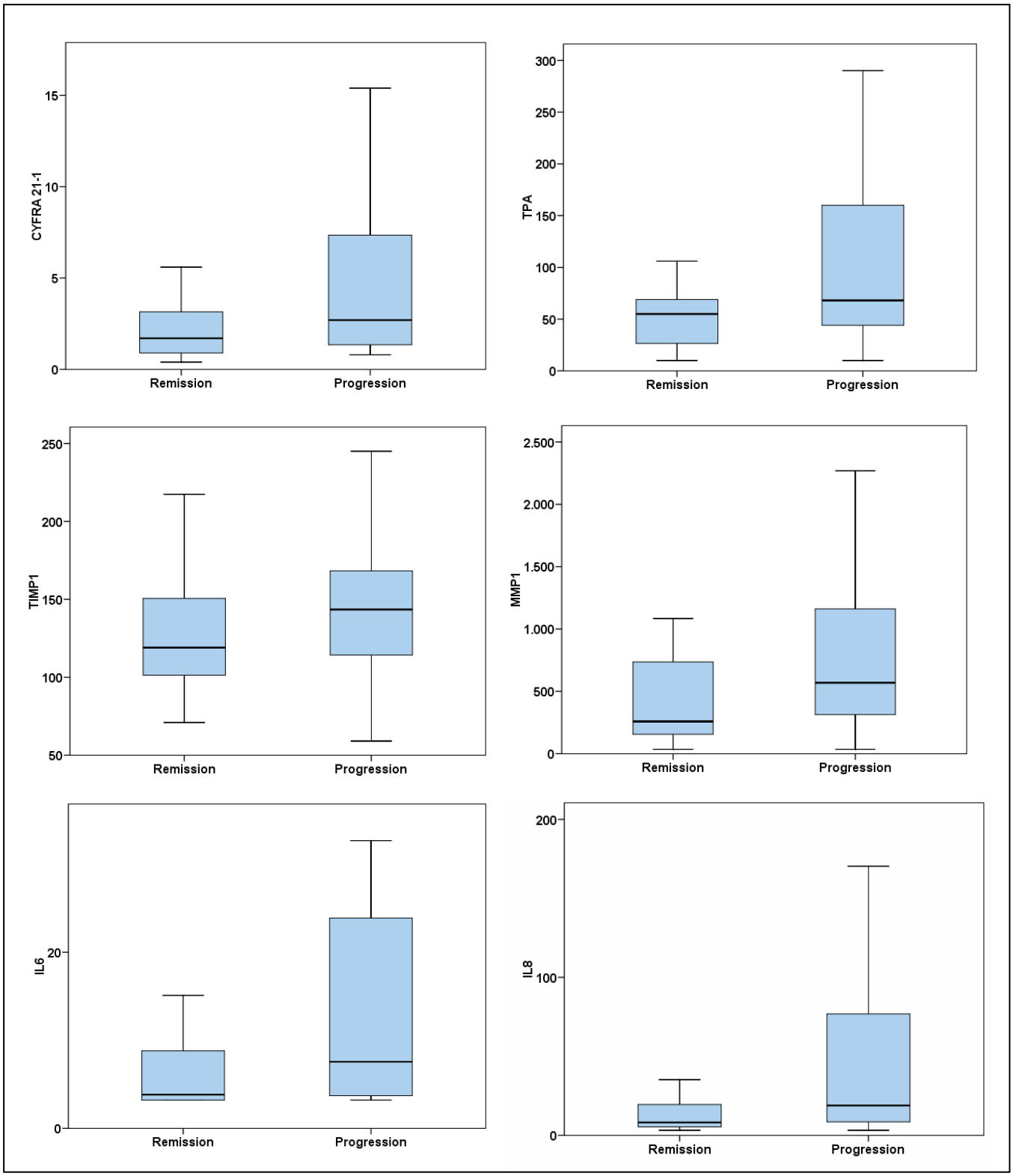

Figure 17. Presurgery marker levels in patients with NSCLC in relation to status in last control.

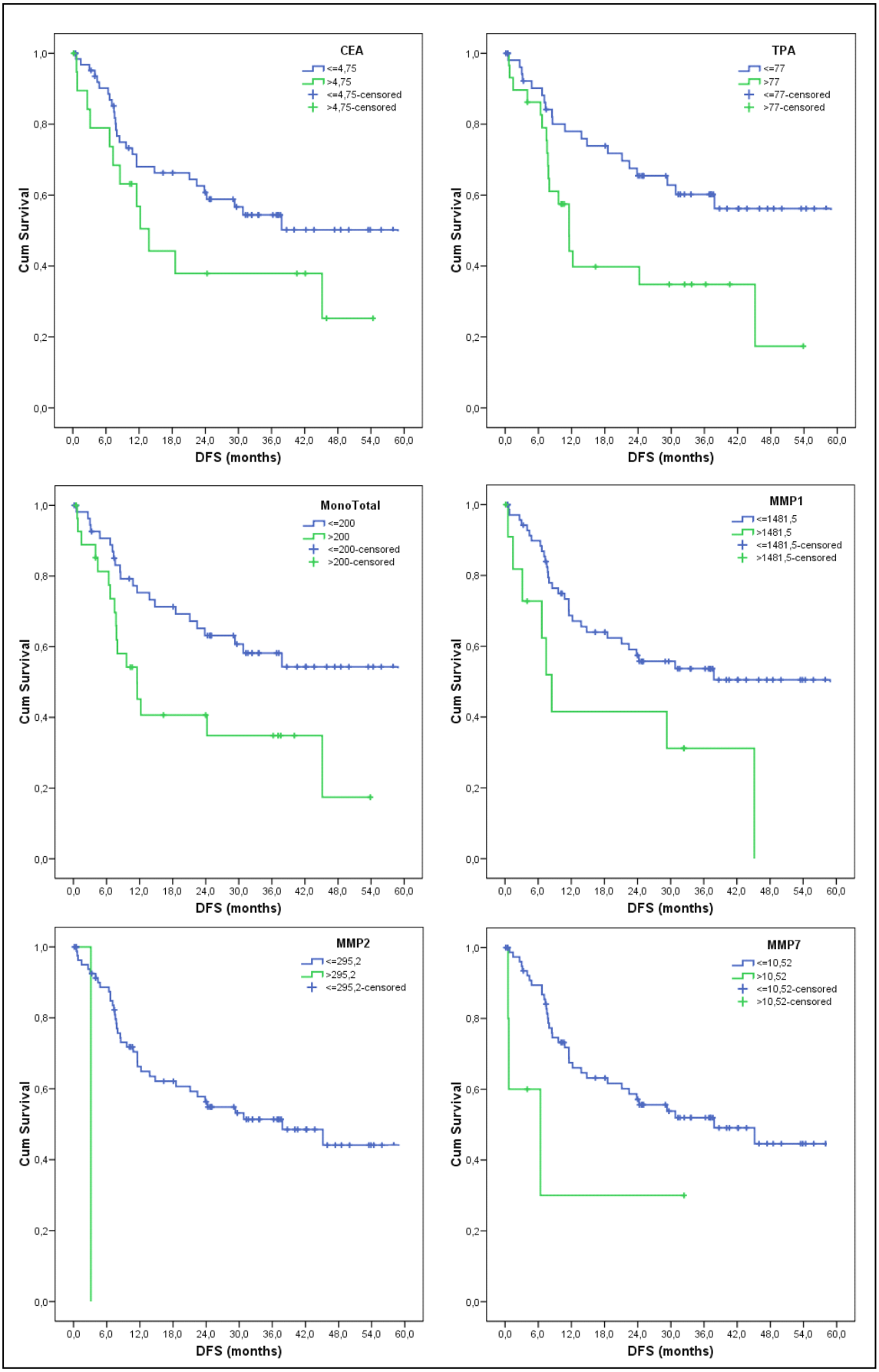

Figure 18a. Disease free survival rate in NSCLC patients according to the preoperative serum biomarker levels (only prognostic significant markers are shown).

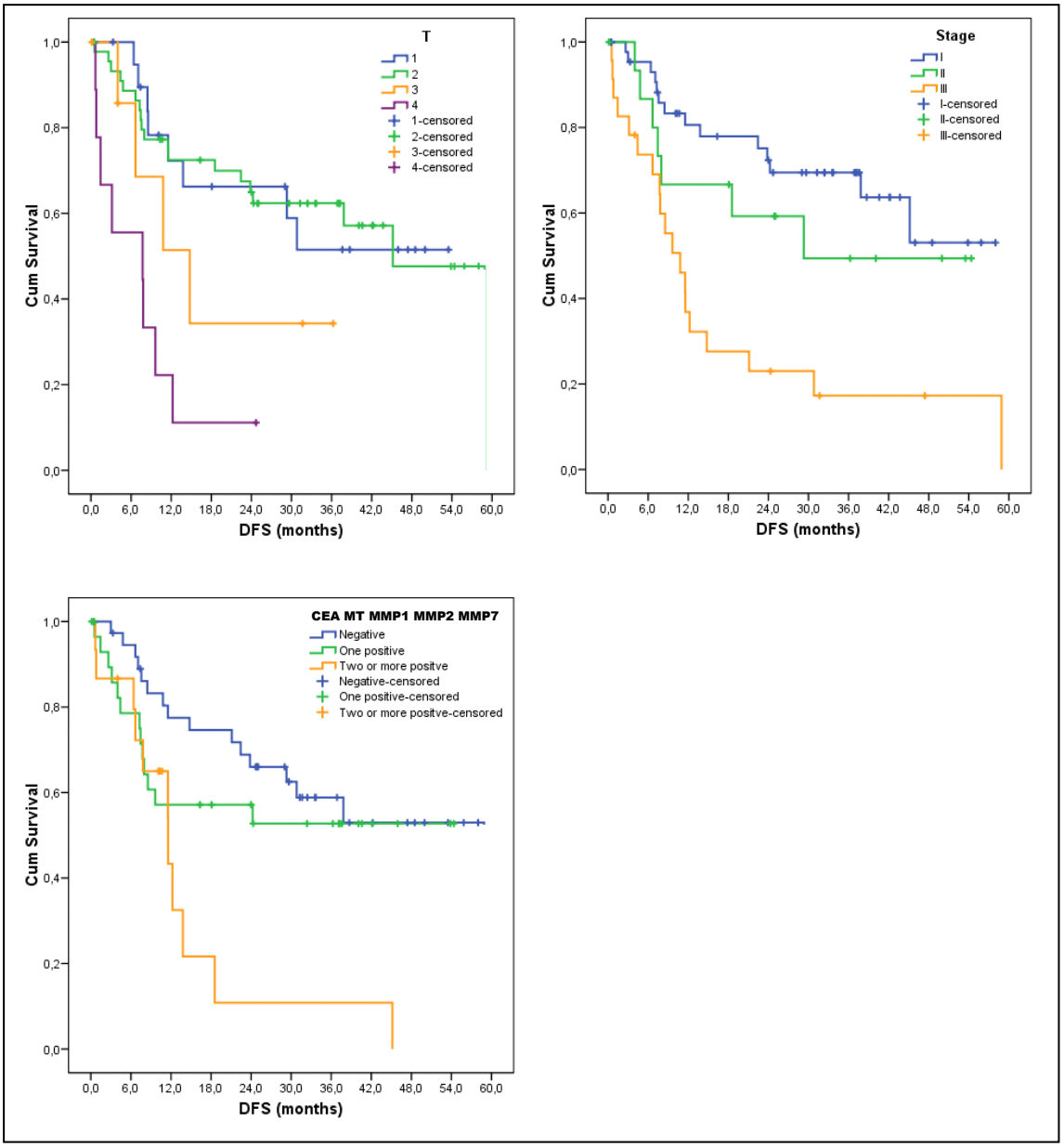

Figure 18b. Disease free survival rate in NSCLC patients according to the preoperative serum biomarker levels, stage and T status.

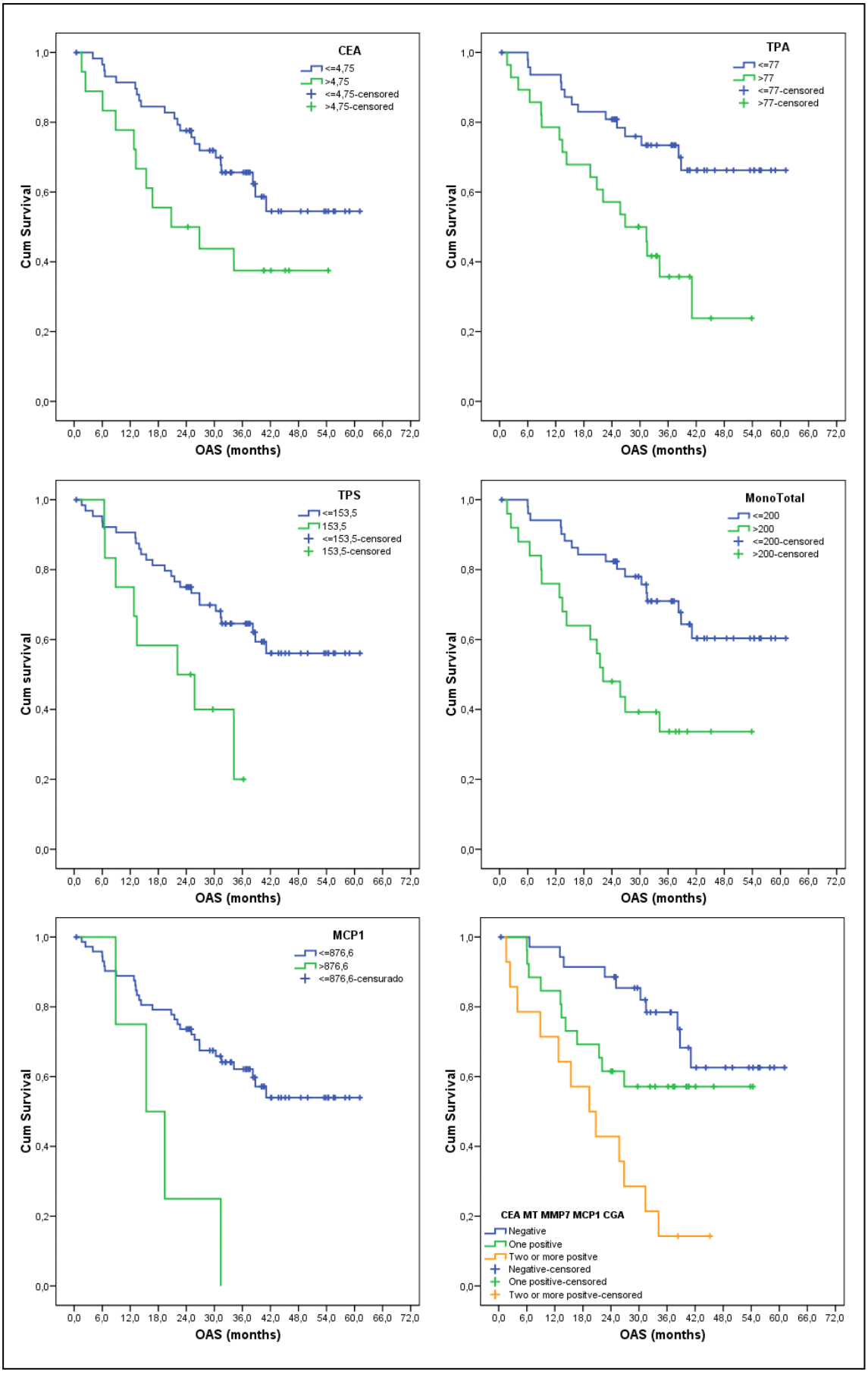

Figure 19a. Overal survival rate in NSCLC patients according to the preoperative serum biomarker levels (only prognostic significant markers are shown).

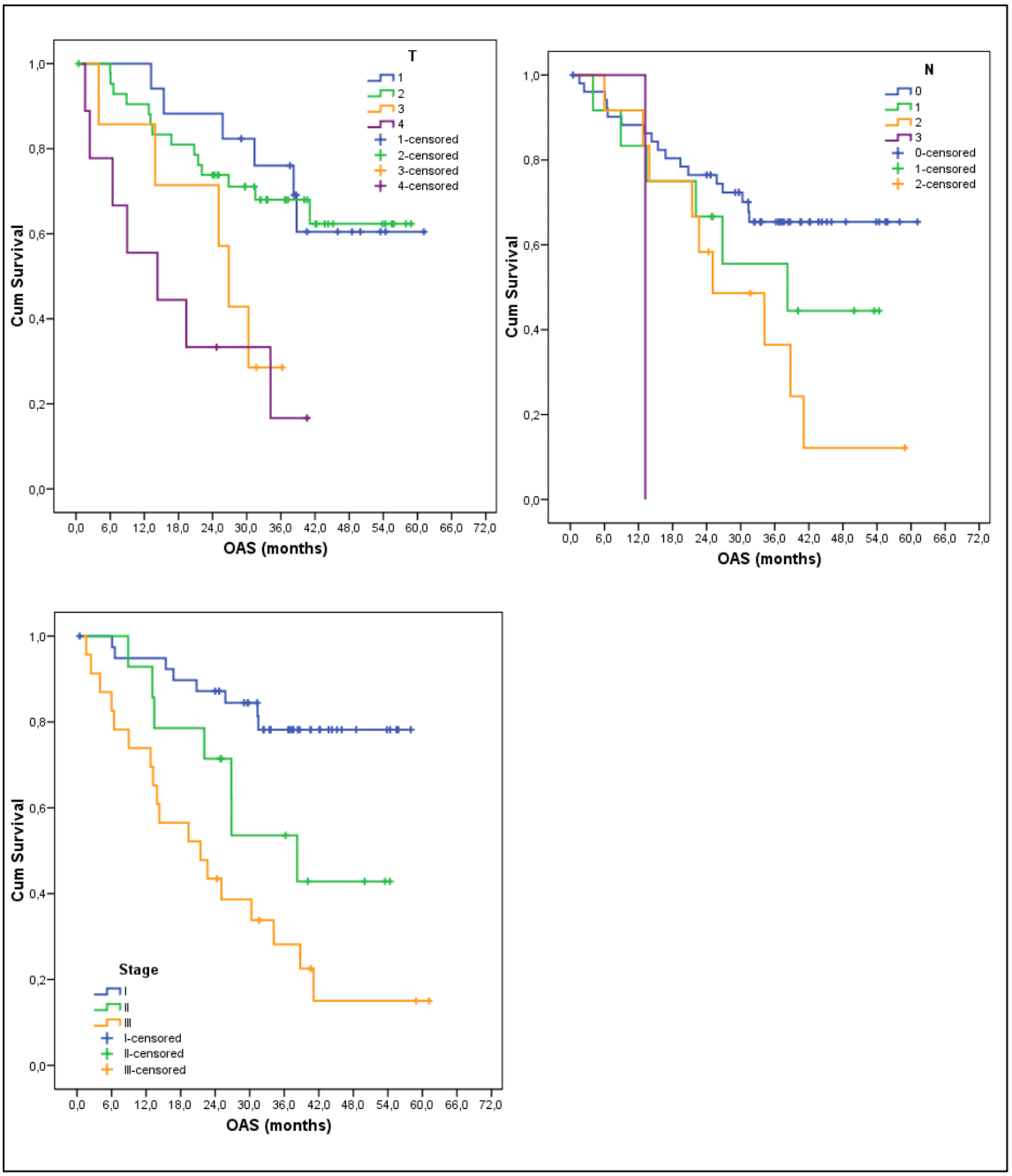

Figure 19b . Overal survival rate in NSCLC patients according to the stage, T status and N status.

7. CITATIONS OF AUTHOR

7.1 ARTICLES

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