Aus der Universitätsklinik und Poliklinik für Strahlentherapie der Medizinischen Fakultät
der Martin-Luther-Universität Halle-Wittenberg (lt. Uni-Amtsblatt Nr. 10.07.2011)

(Direktor: Prof. Dr. med. habil. Dirk Vordermark)

Prognostische und prädiktive Bedeutung von Osteopontin und anderen hypoxie-assoziierten Plasmaproteinen in der Radiotherapie des lokal-fortgeschrittenen und metastasierten Bronchialkarzinoms

Dissertation
zur Erlangung des akademischen Grades
Doktor der Medizin (Dr. med.)

vorgelegt
der Medizinischen Fakultät
der Martin-Luther-Universität Halle-Wittenberg

von Christian Emil Arthur Ostheimer
geboren am 04.12.1984 in München

Gutachter: Herr Prof. Dr. med. Dirk Vordermark (Halle)
Herr PD Dr. med. Bernd Schmidt (Halle)
Herr Prof. Dr. med. Daniel Zips (Tübingen)

Eröffnung des Promotionsverfahrens: 07.07.2015
Verteidigung der Promotions 16.12.2015

From the Department of Radiation Oncology of the Medical Faculty of the Martin Luther University Halle-Wittenberg

(Director: Prof. Dr. med. habil. Dirk Vordermark)

Prognostic and predictive significance of osteopontin and other hypoxia-related plasma proteins in the radiotherapy of locally advanced and metastatic bronchial carcinoma

Dissertation
to obtain the academic degree
doctor medicinae (Dr. med.)

submitted
to the Medical Faculty
of the Martin Luther University Halle-Wittenberg

by Christian Emil Arthur Ostheimer
born 04.12.1984 in Munich

Reviewer: Prof. Dr. med. Dirk Vordermark (Halle)
Prof. Dr. med. Bernd Schmidt (Halle)
Prof. Dr. med. Daniel Zips (Halle)

Date of opening of the doctorate: 07.07.2015
Date of oral examination of the doctorate: 16.12.2015

This work is dedicated in memory of my grandmother, Ms. Erna-Maria Ostheimer

Summary

Response to radiation is impaired in hypoxic tumors compared to normoxic tissues, limiting treatment outcome of patients undergoing radiotherapy. It is crucial to identify patients with significant tumor hypoxia before treatment and to select them for hypoxia-specific therapies to overcome hypoxic radiation resistance and improve prognosis. I, therefore, evaluated the hypoxia-related proteins osteopontin (OPN), carbonic anhydrase IX (CAIX) and vascular endothelial growth factor (VEGF) for their prognostic impact in the radiotherapy of lung cancer.

From 2008 to 2011 a total of 97 patients with locally advanced or metastatic bronchial carcinoma (non small-cell lung cancer, NSCLC, and small-cell lung cancer, SCLC) were prospectively investigated. All patients were treated with primary curative- or palliative-intent radiotherapy ± chemotherapy. OPN plasma samples were obtained before (t0), at the end (t1) and four weeks after radiotherapy (t2). Pre-treatment (baseline) plasma levels of CAIX and VEGF were determined. Plasma concentrations were detected by enzyme-linked-immuno-sorbent-assay (ELISA). Absolute baseline plasma protein levels were tested for correlation, their association with clinicopathological patient characteristics and their impact on prognosis. OPN plasma level changes over time were monitored and correlated with therapy response and prognosis.

In all patient subgroups, median OPN plasma levels decreased during and after radiotherapy (n.s.). A positive correlation between OPN plasma levels detected at the different time points was noted but baseline OPN, VEGF and CAIX did not correlate. Baseline OPN plasma levels were associated with age (p=.03), gender (p=.03), weight loss (p=.001), lung function (FeV1, p=.002), T-stage (p=.02) and GTV (p=.01). Patients with distant metastases had considerably increased OPN plasma levels at all time points (p=.001); VEGF was significantly elevated in patients with larger GTV (p=.002) and low hemoglobin blood concentration (p=.04) and CAIX was related to N-stage (p=.04).

Therapy response was associated with OPN t1 plasma levels (p=.002) and their changes during radiotherapy (p=.04). OS was significantly reduced in patients with high OPN t0, t1 and t2 (p=.04, .004 and .02) plasma levels. OPN t0 (p=.02), t2 and OPN plasma level changes after radiotherapy (p=.002) remained independent predictors for OS in multivariate analysis. Biomarker combination resulted in an augmented prognostic effect with the triple marker combination VEGF-CAIX-OPN most significantly impacting OS. OPN t1, t2 plasma levels and their changes after radiotherapy significantly predicted PFS (p=.02 and .001) and baseline VEGF plasma levels remained independent predictors for both OS (p=.004) and PFS (p=.009).

My results suggest that elevated pre-treatment plasma levels of OPN, VEGF and CAIX indicate advanced tumor disease and that radiotherapy only marginally influences OPN plasma levels over time despite some evidence of a relation between OPN plasma levels and therapy response.

Ostheimer, Christian: Prognostic and predictive significance of osteopontin and other hypoxia-related plasma proteins in the radiotherapy of locally advanced and metastatic bronchial carcinoma, Halle (Saale), Univ., Med. Fac., Diss., 79 pages, 2015

Table of contents

		page
1. Introduction		1
1.1	Tumor hypoxia: Basic principles and clinical implications	1
1.2	Detection of clinically significant tumor hypoxia: hypoxic imaging and invasive oxygen electrode measurements	2
1.3	Extrinsic hypoxia markers	3
1.4.	Intrinsic hypoxia-related proteins	4
1.4.1	Physiological function of osteopontin and its role in malignancy	6
1.4.2	Osteopontin in tumor hypoxia	7
1.4.3	Detection and targeting of osteopontin and hypoxia-related proteins	8
2. Purpose		9
3. Material and Methods		16
3.1	Patient collective, inclusion criteria, indication for radiotherapy and follow-up	10
3.2	Patient collective and subgroups	11
3.2.1	Entire patient collective (n=97)	11
3.2.2	Curative-intent NSCLC (M0-stage) cohort (n=61)	11
3.2.3	Palliative-intent NSCLC (M1-Stage) cohort (n=20)	12
3.2.4	SCLC cohort (n=16)	12
3.3	Curative- and palliative-intent radiotherapy and radiochemotherapy	12
3.4	Plasma sample acquisition, storage and processing	14
3.4.1	Specifications of Human OPN ELISA Assay	14
3.4.2	Specifications of Quantikine Human VEGF ELISA	14
3.4.3	Specifications of Human CA IX Quantikine ELISA	15
3.5	Statistical analysis and endpoints	15
3.6	Ethics vote and informed consent	17
4. Results		17
4.1	Plasma marker concentration, changes and interrelation in all patients and subgroups	17
4.2	Patient and treatment characteristics and their association with pre-treatment plasma protein levels in the entire patient collective and curative-intent NSCLC M0 patients	19
4.3	Univariate analysis of plasma biomarker levels and their changes in the entire patient collective and in the curative-intent NSCLC M0 patient cohort	20
4.3.1	Initial tumor control and response to radiotherapy	20

4.3.2 Overall survival (OS) 24
4.3.3 Progression-free survival (PFS) 25
4.3.4 Time to progression (TTP) 28
4.3.5 Metastasis-free survival (MFS) 30
4.3.6 Freedom from local relapse (FFLR) 30
4.4 Univariate analysis of the association of clinical patient characteristics with prognosis in the entire and curative-intent NSCLC M0 patient cohort 30
4.5 Multivariate and combined analysis of plasma biomarker levels and their changes in the curative-intent NSCLC M0 patient cohort 32
4.5.1 Absolute plasma OPN levels in a multivariate prognostic model 32
4.5.2 Relative OPN plasma level changes in a multivariate prognostic model 34
4.5.3 Absolute baseline CAIX and VEGF plasma levels in a multivariate prognostic model 36
4.5.4 Combination of OPN, CAIX and VEGF and their impact on OS and PFS 38
4.5.5 Combined analysis of baseline OPN, CAIX and VEGF plasma levels in a multivariate prognostic model 40

5. Discussion 43

5.1 Influence of radiotherapy on the OPN plasma level course over time 43
5.2 Baseline biomarker plasma levels and their interrelation 44
5.3 Pre-therapeutic plasma biomarker levels as indictors of advanced disease and biologically aggressive tumor behavior 45
5.4 The predictive power of the biomarkers OPN, VEGF and CAIX in the radiotherapy of NSCLC 47
5.5 The prognostic value of serial plasma OPN detection in the curative-intent radiotherapy of NSCLC 49
5.6 The prognostic role of serial OPN detection in tumor metastasis 51
5.7 The use of a co-detection of the potential hypoxia-related proteins OPN, CAIX and VEGF for a plasma hypoxia score in the curative-intended radiotherapy of NSCLC 52
5.8 Methodological limitations of this work 54

6. Conclusions 56

References 58

Theses 79

Table of abbreviations and symbols

CAIX	carbonic anhydrase IX
CD44	cluster of differentiation 44
CI	confidence interval
CT	computed tomography
DFS	disease-free survival
DNA	desoxyribonucleic acid
ECOG	Eastern Cooperative Oncology Group
ELISA	enzyme-linked-immuno-sorbent-assay
FFLR	freedom from local relapse
FeV1	forced expiratory one second volume (% of normal value)
F-MISO	fluoromisonidazole
Fx	fraction
GLUT-1	glucose transporter 1
GTV	gross tumor volume (ml)
G	tumor grading (differentiation G1 to G4)
Gy	Gray
Hb	hemoglobin
HIF-1α	hypoxia inducible factor 1α
IgG	immunoglobuline G
Ltd.	Limited
MFS	metastasis-free survival
mg	milligram
ml	milliliter
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
MMP	matrix metalloproteinases
m^2	square meter
n	patient number
ng	nanogram
nm	nanometer
NSCLC	non small-cell lung cancer
OER	oxygen-enhancement ratio
OPN	osteopontin

OS	overall survival
PET	positron emission tomography
PFS	progression-free survival
p	p-value (significance level)
pg	picogram
pH	hydrogen potential
pO_2	partial oxygen pressure (tissue)
PTV	planning target volume (ml)
r	correlation coefficient (Pearson)
rr	relative risk
RGD	tripeptide arginyl-glycyl-aspartic acid (Arg-Gly-Asp)
ROS	reactive oxygen species
rpm	rates per minute
SCLC	small-cell lung cancer
SD	single dose (Gray)
shRNA	small hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SIBLING	small integrin-binding ligand N-linked glycoprotein
SPECT	single-photon computed tomography
TD	total dose (Gray)
TNM	tumor node metastasis
TTP	time to progression
TPZ	tirapazamine
UICC	Union internationale contre le cancer
uPa	urokinase plasminogen activator
USA	United States of America
VEGF	vascular endothelial growth factor

1. Introduction:

1.1 Tumor hypoxia: Basic principles and clinical implications

In many human cancers, particularly lung cancer, cure rates remain low throughout stages [1,2] and often, treatment resistance accounts for poor prognosis and therapeutic outcome. In radiation oncology, a critical factor for treatment resistance is tumor hypoxia which constitutes an important aspect of the tumor microenvironment [3-5].

The discovery of the influence of oxygenation on cellular response to radiation can be traced back to the observations of Holthusen, Schwarz and Mottram et al. who, more than 80 years ago, first described differing radiation sensitivity under anoxic and normoxic conditions. The specific relevance of tumor oxygenation for radiotherapy is outlined by Thomlinson and Gray [6]. The so-called “oxygen effect” belongs to the fundamental principles in radiation biology and is defined by the oxygen-enhancement-ratio (OER) which states that anoxic cells need a 2- to 3-fold higher radiation dose compared to normoxic cells in order to achieve the same biological effect (i.e. cell death) [7]. In other words, the OER predicates a 2- to 3-fold increased radio-sensitivity of well-oxygenated cells in comparison to anoxic cells. The responsible mechanism for increased radiation damage in the presence of oxygen is the formation of reactive oxygen species (ROS) which cause additional and permanent DNA damage, requiring lower radiation doses.

Solid tumors however, often feature extensive hypoxia [5,8-10] which is related to an imbalance between oxygen demand and supply. While oxygen consumption is significantly increased due to rapid tumor cell proliferation, oxygen delivery is inefficient owing to abnormal and dysfunctional microvasculature and diminished microcirculation (i.e. “perfusion-related hypoxia”) [11-13]. In addition to the fluctuating and unstable blood flow, deteriorated diffusion geometry with an increased diffusion distance limits oxygenation, particularly at the proliferative edge (i.e. “invasive front”) [14-16] of malignant tumors (i.e. “diffusion related hypoxia”) [12,17,18]. Another aspect contributing to tumor hypoxia is cancer- or treatment-induced anemia which is related to a reduced oxygen-carrying capacity due to hemoglobin depletion [19,20] which in turn adversely impacts prognosis in patients undergoing cancer treatment [4,21,22,24,25]. This underlines that tumor hypoxia cannot be regarded as a monocausal and constant phenomenon but rather is of dynamic nature with acute and chronic aspects [26,27] and both inter- and intra-tumor variation [28,29]. By inducing fundamental genomic and proteomic changes [12,30], hypoxia significantly alters the proliferative and metabolic behavior of tumor cells, that is compromising cell differentiation, DNA repair and apoptosis [3,31-33] and increasing

angiogenesis, mutability and tumor cell viability [3,12,19,20,26,27,31-39]. Ultimately, these alterations allow improved tumor cell adaptation to the hostile, hypoxic environment where hypoxia can be regarded as a “selection factor” enhancing clonal selection of well-adapted tumor cells and driving the tumor to a more aggressive, invasive, metastatic and highly malignant phenotype [40-46], featuring resistance to chemoradiotherapy [5,12,18,47-52].
On a clinical perspective, this is of considerable importance, particularly in the radiotherapy of cancer [24,53-56]: Nordsmark et al. provided solid evidence that pre-treatment hypoxia (pO_2 electrode readings) is associated with inferior overall survival, response to radiation and tumor control in head-and-neck cancer patients including a subgroup of patients treated at the department of Radiation Oncology Halle [25,57,58].

1.2 Detection of clinically significant tumor hypoxia: hypoxic imaging and invasive oxygen electrode measurements

Being a critical factor of radiation resistance, tumor hypoxia is regarded as a major therapeutic target in radiation oncology. At first however, reliable and feasible methods for the detection and quantification of clinically significant tumor hypoxia have to be found, evaluated and implemented into the clinical routine.
In the literature, different attempts to describe tumor oxygenation and to quantify tumor hypoxia have been investigated [59,60]. Traditionally, polarographic needle electrodes such as the “Sigma Eppendorf electrode system” [61], which are invasively placed into tumors, have been used to directly measure tissue oxygen levels in the experimental setting [28,62-64]. Clinically, there is evidence that pre-treatment oxygen levels, detected by microelectrode measurements, are of prognostic and predictive quality in different human cancer entities treated by radiotherapy [12,23,65]: In head and neck cancer or soft tissue sarcoma patients for instance, a low intra-tumoral pO_2 correlated with inferior survival after radiotherapy [53,57]. Despite the proven validity of this method its routine clinical application is limited by methodological (resolution, oxygen consumption by microelectrodes limiting continuous oxygen measurements within tumors over time, inter-observer variability, equipment costs, validation with other methods of measuring hypoxia [66-70] and biological (heterogeneity of oxygenation within tumors, i.e. location, distribution, level, duration and onset of hypoxia [17,64,71]) drawbacks [72,73]. In particular, the invasiveness and restriction of this method to easily accessible tumors as well as the transient nature of hypoxia underline the necessity for dynamic monitoring of tumor oxygenation throughout radiotherapy [12,69,74-76].

Consequently, imaging technologies such as magnetic resonance imaging (MRI) [19,77-80], single-photon computed tomography (SPECT) [81] and positron emission tomography (PET) [82-84] using different hypoxic tracers such as 18-fluoromisonidazole (^{18}F-MISO) [19,85] have been described as more feasible non-invasive approaches [85-87] to visualize tumor hypoxia, particularly accounting for its dynamic nature. High reproducibility and a good correlation with tumor oxygenation was reported for F-MISO-PET [88-90]. Hypoxia imaging with ^{18}F-MISO-PET was suggested as a useful and feasible approach of guided dose escalation in the intensity-modulated radiotherapy of head and neck cancer with the goal of safely improving tumor control probability [91]. In the same tumor entity, F-MISO-PET imaging efficiently labeled hypoxic cells, successfully predicted the risk for tumor recurrence after radiotherapy [92] and was able to identify head and neck cancer patients who benefitted from additional treatment with the hypoxic cytotoxin tirapazamine [93]. The potential use of hypoxic PET-imaging in radiotherapy is experimentally documented for different cancer entities [94,95] including malignant glioma [96], sarcoma [97] and non small-cell lung cancer (NSCLC) where ^{18}F-MISO uptake correlates with outcome after radiotherapy [98]. To increase their sensitivity and reliability, further evaluation and valididation of hypoxic imaging is needed [65], preferably by correlation or combination with other approaches of mapping tumor hypoxia such as immunohistochemistry [99], polarographic needle electrodes [100] or endogenous and exogenous hypoxia markers [99,101-107]. Ultimately, biokinetics and application of hypoxic tracers has to be improved before hypoxic imaging techniques may be implemented into the clinical routine [85].

1.3 Extrinsic hypoxia markers

While invasive approaches rather seem not suitable for the routine detection of tumor hypoxia (primarily due to their limited reach and invasive nature), so-called “extrinsic hypoxia markers” have amended the strategies of experimentally assessing tumor oxygenation in cancer patients before initiation of treatment in order to select them for hypoxia-specific targeted therapies which may be available in future.

Exogenous hypoxia markers including EF5 or pimonidazole, which is the most thoroughly investigated, are non-physiologic substances which have to be brought into the human body (by injection for example) where they accumulate under hypoxic conditions by chemical reduction (“bio-reducible markers”) [65,108]. Besides the high spatial resolution, the distinct advantage of this approach is its ability to delineate real hypoxia from anoxia

(where exogenous hypoxia markers are not metabolized and thus do not accumulate). This previously has been demonstrated clinically and prognostically in patients with head and neck cancer [12,109].

To some extent however, extrinsic hypoxia markers exhibit limited range and are more reflective of chronic rather than acute hypoxia, making them susceptible for partially missing oxygen tensions which might be of therapeutic relevance [76].

Generally, they do not incorporate necrotic areas which makes the correlation and validation of extrinsic hypoxia markers with invasive methods of measuring tumor oxygenation difficult due to the missing congruency between these two methods [110,111].

Analogue to invasive or imaging techniques, exogenously administered hypoxia markers suffer from depicting tumor oxygenation at a specific time point which neglects the dynamic and changing nature of tumor oxygenation [68,112].

Apart from the necessity of exogenous (possibly repeated) marker injection, consecutive biopsy is required for evaluation of hypoxic regions, for instance by immunohistochemistry [12]. However, imaging methods such as hypoxic PET could, combined with extrinsic hypoxia markers, complement and increase the validity of this approach [102,113].

1.4 Intrinsic hypoxia-related proteins

Unlike exogenous hypoxia markers, endogenous hypoxia-related proteins are naturally present in the human body where they are directly or indirectly induced by hypoxia which is why they are referred to as “intrinsic hypoxia markers”. For their potential relation to (tumor) hypoxia, these proteins have been suggested as surrogates of tumor oxygenation and possibly clinical radiation resistance [4,110]. Hypoxia inducible factor 1α (HIF-1α), glucose transporter 1 (GLUT-1), carbonix anhydrase IX (CAIX), vascular endothelial growth factor (VEGF) and osteopontin (OPN) constitute the best characterized hypoxia-related molecules [65,68]. HIF-1α is the only protein which is directly stimulated by hypoxia [40,114-116] and its overexpression has been associated with poor prognosis, advanced disease, an aggressive cancer phenotype and inferior response to cancer therapy in different malignancies [117-122]. HIF-1α has also been linked to tumor hypoxia-related radiotherapy failure in head-and-neck cancer [123] an in NSCLC, there is evidence which suggests that the prognostic effect of HIF-1α may be augmented by a co-expression or co-detection with other markers [124]. CAIX, GLUT-1 and VEGF are three downstream effectors of HIF-1α [17,110,118,125,126] and have been linked to hypoxia [12,69,110,127-

129] and their co-expression displayed a significant association with prognosis in different cancer types including NSCLC [130].

The biological mechanism of hypoxic VEGF induction is that of a decrease in tissue oxygenation which triggers VEGF-mediated angiogenesis to improve perfusion and in consequence, restore a sufficient tissue partial oxygen pressure. A strong stimulation of VEGF by hypoxia was demonstrated in-vitro and in-vivo [131,132] and the prognostic value of VEGF overexpression and increased plasma levels has been documented for different types of human malignancies [133-135], including NSCLC [136-138].

Recently, VEGF mRNA was reported to significantly decrease after surgery and to be associated with overall- and disease-free survival in NSCLC patients, underlining the prognostic and predictive potential of this biomarker [139].

The relationship between hypoxia and CAIX, which seems to be localized primarily in hypoxic and necrotic tumor areas, has been well documented and tumor hypoxia suggested as a driving force for CAIX expression which is related to poor prognosis and resistance to radiotherapy in different types of cancer [73,140-142]. Interestingly, CAIX has been shown to most accurately predict clinically significant tumor hypoxia in NSCLC [143] where it is critically involved in the cellular response to hypoxia [144]. Here, it enables tumor cells to adapt to toxic, anoxic conditions and stimulates their migratory and invasive potential [145]. In the consequence, hypoxia-induced up-regulation of CAIX has been demonstrated to enhance tumor malignancy and increase resistance to cancer therapy which translates into poor prognosis and outcome in many human cancers, including lung- and head-and-neck cancer [140,142,144-151]. Clinically interesting is that the co-expression of CAIX and HIF1α seems to be predictive for radioresistance in the radiotherapy of cancer [152,153]. Yet, some studies were not able to confirm these observations, suggesting a differential influence of the tumor-biological entity on the prognostic significance of CAIX expression [70,154,155].

Despite the robust relation of the aforementioned markers to hypoxia in-vitro [69,110], their hypoxic induction in-vivo remains complex and is not free from confounding elements of the tumor microenvironment such as Ph [116,156-159]. Thus, in-vitro and in-vivo study results on the relationship of intrinsic hypoxia markers with tumor hypoxia are conflicting [160]: While some studies report HIF-1α levels to correlate with invasively (needle electrodes) detected pO_2 [69,73,110,161,162], other groups report no or only weak correlations with tumor oxygenation [163-167]. Nevertheless, endogenously secreted

hypoxia-related proteins may be a promising, less invasive, feasible and untoxic approach to quantify tumor oxygenation [17,68,130,168,169].

1.4.1 Physiological function of osteopontin and its role in malignancy

OPN is an acidic extracellular matrix (44-75kDa) glycoprotein [171,171] and belongs to the SIBLING protein family [172]. Originally discovered in 1989 in bone tissue where it is involved in matrix turnover and bone remodeling [173-179], OPN is physiologically expressed in various human tissues [180,181]. OPN undergoes post-transcriptional modification by alternate splicing [182,183], yielding three splice variants, functionally different in both physiologic conditions and in malignancy [184-186]. OPN exhibits various active domains including a binding site for CD44 surface receptors [187,188] and a RGD motif [171,172] which is the major interaction site for $\alpha_v\beta_3$-integrin receptors [189].

On the physiological level, OPN facilitates cell migration, motility, adhesion and chemotaxis in immune or inflammatory processes [190.192] and mediates degradation of the extra-cellular matrix [193-196]. It is also linked to angiogenesis [197,198] and participates in vascular remodeling [199-202].

In most malignant tumors, OPN is widely overexpressed [203-207] and protein secretion is significantly increased [208,209] which is related to enhanced tumor cell migration, proliferation, invasion and metastatic spread [189,210-213]. The latter is mediated by its αβ-integrin and CD44 receptors [211,214-216] which induce matrix metalloproteinases and urokinase-plasminogen-activator in various cancer types [189,217-221], ultimately resulting in the degradation of extra-cellular matrix.

Numerous in-vitro studies support the role of OPN in the enhancement of metastatic potential in different human cancer cell lines [222-224], including lung cancer [225] which is underlined by clinical investigations demonstrating a strong association of OPN with tumor metastasis [211,226,227].

Increased cell survival, escape from apoptosis and growth promotion mark some of the major tumorigenic effects of increased OPN expression during cancer progression which has prognostic implications [197,198,210,228,229]. OPN also plays a critical role in the tumor microenvironment (“tumor-host-interface”) where it seems to exert differential functions depending on the source of OPN production such as in tumor-associated macrophages [211.217,230,231].

Besides other cancer entities [209,211], in lung cancer, OPN is considerably overexpressed compared to normal lung tissue and healthy controls [186,208,232,233].

In (lung) cancer, both increased OPN expression and elevated plasma levels are significantly associated with parameters of advanced disease stage, prognosis [206,233-236] response to chemotherapy [237-240] and outcome after surgical treatment [1].

1.4.2 Osteopontin in tumor hypoxia

Apart from the pivotal role of OPN in human malignancy and besides its critical impact on prognosis of cancer patients, this protein is also potentially valuable in radiotherapy of cancer for its possible association with (tumor) hypoxia [4,241-243]. Numerous studies suggested that both OPN expression in tumor tissue and OPN protein secretion into plasma are related to hypoxia.

Evidence from different cancer entities including malignant glioma, sarcoma and lung cancer shows that hypoxia can induce OPN mRNA expression in tumor tissue as well as its secretion into plasma [25,244-249]. In cancer of the head-and-neck for instance, hypoxia increased OPN secretion into plasma where high OPN levels predicted poor survival and freedom from relapse [246] which underlines the clinical importance of the potential relation between elevated OPN (plasma/tumor) and hypoxia. For the same cancer entity, Bache et al. demonstrated that immunohistochemical OPN tumor staining correlated with tumor pO_2, detected by oxygen electrodes. Furthermore, high OPN expression in tumor tissue was correlated with low hemoglobin and high HIF-1α values [250,251]. The same correlation with tumor pO_2 could be determined for OPN plasma levels [241,242,246,251]. These results later were confirmed by Le et al. who reported a significant correlation of OPN tumor staining and tumor pO_2 [247]. Overgaard et al. amended these findings in that he showed that high OPN plasma levels predicted poor disease-free survival and that only patients with high OPN plasma levels benefitted from the hypoxic radiosensitizer nimorazole [252,253]. Similarly, the application of the hypoxic cytotoxin tirapazamine (TPZ) significantly decreased OPN mRNA in nasopharyngeal cancer, thereby additively reducing tumor cell survival in-vitro [254.] In head and neck cancer however, the potential of TPZ is rather unclear [255].

The use of OPN plasma levels as a potential surrogate of clinically significant tumor hypoxia in NSCLC has been demonstrated by Le et al. In their study, they showed that pO_2 was significantly reduced in resectable NSCLC compared to healthy lung tissue and that high plasma OPN levels correlated with low tumor pO_2. Notably, patients with low OPN had a significantly reduced risk for tumor recurrence after therapy (resection) implying better oxygenated tumors in these patients [161].

These findings are complemented by the results of Li, Blasberg and Mack et al. who reported both significantly increased OPN expression and elevated OPN plasma levels in NSCLC patients (compared to healthy controls) being related to reduced therapy response and overall survival after chemotherapy or surgery of NSCLC [228,239,256].

1.4.3 Detection and targeting of osteopontin and hypoxia-related proteins

The quantification of the expression of endogenous hypoxia-related proteins usually requires tumor tissue and utilizes approaches such as immunohistochemistry.

One clear advantage of circulating intrinsic hypoxia-related molecules including OPN, CAIX and VEGF is their easy detection in body fluids, primarily patient plasma or serum of cancer patients [257-259]. Evidence shows that the detection of biomarker levels in patient plasma is more reliable and should be preferred [260,261].

The established measurement platform is that of enzyme-linked immuno-sorbent assay (ELISA) which is commercially available and routinely practicable in the clinical setting [262]. However, results on plasma marker concentration are critically dependent on the ELISA system used and are generally not conferrable from one ELISA to another despite of a correlation of plasma concentrations between ELISAs [263,264]. The latter fact might contribute to the difficulty of demarcating clear plasma protein level cut-off values which are still under investigation. Indisputable however is that both protein expression and plasma protein levels of the aforementioned hypoxia-related molecules are significantly and prognostically relevant increased in cancer patients. New developments and improvements of ELISA techniques, such as the use of a dual-antibody detection system, continuously increase the sensitivity of marker detection [265].

After decades of research in exploiting hypoxic radiation resistance including attempts such as hyperbaric oxygenation and hyperthermia failed to generate clinically viable treatment strategies [266,267], targeting hypoxia-related proteins may open up new strategies in overcoming hypoxic radiation resistance [17,57,68,184,247,268].

In a therapeutic perspective, multiple targeting approaches of OPN have been under investigation [269,270]. In different cancer entities, knockdown of OPN mRNA expression and protein levels by RNA interference (siRNA) resulted in a marked increase in tumor cell apoptosis, decreased invasion and cancer cell motility (by down-regulation of uPA and MMPs), colony formation and metastatic spread [211,271]. Solid results also exist for the combination with radiotherapy where silencing of OPN increased radiosensitivity [272,273].

Immunologic targeting of OPN showed promising results and interestingly, simultaneous inhibition of both OPN and VEGF by a bi-specific antibody seems to be more efficient than single-marker knockdown [274]. Using small RNA-endonucleases or anti-sense-oligonucleotides on the post-transcriptional level significantly diminished OPN-promoted tumor growth and spread in different human malignancies [229,270,275-277].

Despite promising results in-vitro and in-vivo, issues such as drug design, administration, side effects, bio-availability and specificity (in particular due to the partly bipolar function of tumor and host OPN) still limit the transfer from bench to bedside.

CAIX also has been suggested as a promising targetable biomarker for its association with treatment-resistant hypoxic tumors [150,151,278]. In-vitro and in-vivo results demonstrated successful depletion of this protein by shRNA, monoclonal antibodies or small molecule inhibitors, resulting in tumor growth attenuation and inhibition of metastasis in different tumor entities, yielding (hypoxic) targeting agents for CAIX which are currently under preclinical and clinical investigation [150,151,279,280]. Interestingly, knockdown of CAIX seems to enhance the effect of the anti-VEGF antibody bevacizumab which underlines the clinical potential of combined targeted therapy of hypoxia-related proteins [281]. Solid in-vitro results and first clinical evidence demonstrates radiosensitizing effects of a VEGF-targeted therapy in combination with radiotherapy [282-284] and underline the therapeutic exploitation of the “hypoxic crosslink” between HIF-1α, CAIX and VEGF [285,286].

2. Purpose

The purpose of this prospective clinical study was to evaluate the prognostic and predictive quality of plasma levels of the hypoxia-related proteins OPN, VEGF and CAIX in patients with lung cancer (SCLC and NSCLC) treated with radiotherapy with respect to the subgroups curative-intent NSCLC (M0-stage), palliative-intent NSCLC (M1-stage) and SCLC. The following hypotheses and questions were addressed in this work:

1. What is the plasma concentration of the aforementioned proteins and is there a correlation between plasma markers determined at different time points in lung cancer patients undergoing radiotherapy?
2. Are pre-therapeutic plasma biomarker levels associated with clinical, pathological and socio-demographic patient characteristics and do elevated plasma levels before radiotherapy reflect advanced disease and an aggressive cancer phenotype?

3. Is there a difference in absolute plasma levels between M0- and M1-stage lung cancer patients and do elevated plasma marker levels mirror metastatic tumor burden?
4. Are elevated OPN plasma levels associated with parameters of oxygenation such as hemoglobin and lung function?
5. Are absolute plasma levels of the studied biomarkers, measured before radiotherapy, associated with prognosis and outcome after radiotherapy of lung cancer and does a co-detection of baseline plasma biomarkers augment the prognostic effect?
6. How do OPN plasma levels change throughout and after radiotherapy (if measured at several time points) and are plasma level changes of prognostic and predictive relevance in the radiotherapy in lung cancer?
7. Can absolute plasma biomarker levels or their changes serve as independent prognostic predictors of outcome after radical radiotherapy of NSCLC?

3. Material and Methods

The entire patient collective, patient subgroups, inclusion and exclusion criteria, indication for radiotherapy, treatment details and the follow-up procedure are illustrated below.
In addition, the principle of enzyme-linked immunosorbent assay (ELISA) and the technical aspects of the specific ELISAs used in this study are described. The statistical methods and endpoints applied in this study are defined in 3.5.

3.1 Patient collective, inclusion criteria, indication for radiotherapy and follow-up

Between November 1st 2008 and December 31st 2010, 107 patients newly diagnosed with inoperable lung cancer (NSCLC and SCLC) who were admitted to the Department of Radiation Oncology of the Martin Luther University Halle-Wittenberg, Halle, Germany, were prospectively recruited.
Inclusion criteria were (1) histologically (by biopsy) confirmed NSCLC or SCLC, (2) no prior surgery or radiotherapy, (3) indication for curative- or palliative-intent radio(chemo)therapy, (4) age ≥ 18 years and (5) signed informed consent.
Of the 107 patients who met the inclusion criteria, 10 patients were excluded later due to patients' death before the start of radiotherapy, abortion of radiotherapy by patient will or patient transfer to another hospital for radiotherapy. Thus, a total of 97 patients could be analyzed in this study. In each patient case, indication for radiotherapy was determined by

a multidisciplinary tumor board including a medical oncologist, thoracic surgeon, radiation oncologist, radiologist, pathologist and nuclear medicine physician.
Patients were followed-up regularly at the Department of Radiation Oncology, Martin Luther University Halle-Wittenberg (initially 4 weeks after the end of radiotherapy and later at longer intervals). Survival status was obtained and monitored in cooperation with local citizen registration offices. Survival data was last updated on August 31st, 2014.
Therapy response and tumor control evaluation usually was performed 4 weeks after completion of radiotherapy at affiliated hospitals by comparatively assessing CT-image studies from before to after radiotherapy by an experienced radiologist.

3.2 Patient collective and subgroups

The entire collective includes 97 patients (n= 81 NSCLC, n=16 SCLC). Subgroups were formed according to histology and presence or absence of distant metastases, yielding a cohort of 61 patients with NSCLC in M0-stage who were treated with curative-intent (radical) radiotherapy or chemoradiation and a cohort of 20 NSCLC patients with M1-situation, treated with palliative-intent radiotherapy. The SCLC-group contained 11 patients with M0-stage and 5 patients in M1-stage. The entire patient collective and the three subgroups, i.e. curative-intent NSCLC (M0-stage), palliative-intent NSCLC (M1-stage) and SCLC have been analyzed separately.
Sociodemographic data and clinical characteristics were taken from the patients' charts and clinical tumor staging is based on the Union internationale contre le cancer (UICC) TNM classification, 7th edition.

3.2.1 Entire patient collective (n=97)

Table 1 shows sociodemographic and clinicopathological patient characteristics of the entire patient collective (n=97). In total, 41 patients (42%) were treated with radiotherapy alone while 56 patients (58%) received combined radiochemotherapy. UICC stage distribution was: 7 patients (7%) stage I, 3 (3%) stage II, 18 (19%) stage IIIa, 44 (45%) stage IIIb and 25 (26%) stage IV and among M1-stage patients, 16 (64%) had multiple and 9 (36%) had solitary metastasis.

3.2.2 Curative-intent NSCLC (M0-stage) cohort (n=61)

Clinical and sociodemographic patient characteristics of the curative-intent NSCLC (M0-stage) cohort are presented in **Table 1**. In this patient group, 19 (31%) received irradiation

treatment only and 42 (69%) were treated with simultaneous radiochemotherapy. UICC stage was: 7 patients (12%) UICC I, 2 patients (3%) UICC II, 18 patients (30%) UICC IIIa and 34 patients (56%) UICC IIIb.

3.2.3 Palliative-intent NSCLC (M1-Stage) cohort (n=20)

An overview of social, demographic and clinical patient characteristics of the palliative-intent NSCLC cohort is given in **Table 1**. Among palliative-intent NSCLC patients, 16 (80%) received radiotherapy alone and 4 (20%) were treated with combined radiochemotherapy. All patients in this cohort were staged M1 (UICC IV) with 12 (60%) having metastases at multiple organ locations while 8 patients (40%) had solitary organ metastasis.

3.2.4 SCLC cohort (n=16)

Clinicopathological and sociodemographic patient characteristics of the SCLC cohort are shown in **Table 1**. Combined radiochemotherapy was administered in 10 patients (63%), 6 patients (37%) were treated with radiotherapy alone. In this subgroup 80% of patients had multiple metastases and 20% had a solitary organ metastasis.

3.3 Curative- and palliative-intent radiotherapy and radiochemotherapy

All patients received a planning CT (Siemens Lightspeed RT) without contrast and the "Oncentra Masterplan External Beam" software (Nucletron, Elekta) was used for contouring and treatment planning. PTV included the macroscopic tumor as visible in CT image studies and regional lymph nodes with lymphatic drainage ways (plus safety margin). If available, CT studies were correlated with PET imaging for target volume delineation. The following organs at risk were contoured: ipsi- and contralateral lung, spinal cord, heart, esophagus and in some cases chest-wall.

The patients were immobilized using a positioning frame ("breast board") which was also in place when the planning CT image series were acquired. Radiotherapy was given as a 3D-conformed photon technique at the Siemens "Primus" or "MX" lineac-accelerator.

The treatment of M0-stage patients (UICC I-IIIB) consisted of a curative-intent definitive (radical) normofractionated (5 Fx/week) radiotherapy with a cumulative median total dose (TD) of 66 (59,5-66) Gy, given usually in 33 fractions with a median daily single dose (SD) of 2 (1,5-2,5) Gy. Radiotherapy was divided into a primary series (TD 50 Gy, SD 2 Gy) and a consecutive boost radiation (TD 16 Gy, SD 2 Gy) which included the tumor lesion and lymph nodes which were suspicious by CT or positive by PET imaging.

Table 1.	Sociodemographic and clinical patient characteristics of the entire patient collective, the NSCLC cohort and the SCLC cohort				
		all patients (n=97)	**curative-intent NSCLC** (n=61)	**palliative-intent NSCLC** (n=20)	**SCLC** (n=16)
sex					
	m	83 (86%)	51 (84%)	20 (100%)	12 (75%)
	f	14 (14)	10 (16%)	0 (0%)	4 (25%)
age	*(median years, range)*	65 (35-86)	65 (47-86)	64.5 (35-80)	65.5 (44-75)
smoking habits					
	never	11 (11%)	8 (13%)	1 (5%)	2 (13%)
	former	8 (8%)	5 (8%)	1 (5%)	2 (13%)
	current	77 (79%)	47 (77%)	18 (90%)	12 (75%)
	unknown	1 (1%)	1 (2%)	0 (0%)	0 (0%)
weight loss[1]					
	yes	28 (29%)	13 (21%)	12 (60%)	3 (19%)
	no	62 (64%)	44 (72%)	8 (40%)	10 (62%)
	unknown	7 (7%)	4 (7%)	0 (0%)	3 (19%)
anemia					
	yes	75 (77%)	46 (75%)	16 (80%)	13 (81%)
	no	22 (23%)	15 (25%)	4 (20%)	3 (19%)
hemoglobin	*(median g/dl, range)*	12.1 (8.3-17.4)	12.1 (8.3-15)	12.3 (8.7-17.4)	11.9 (8.5-11.1)
FeV1[2]	*(median % of normal value, range)*	67.8 (20-124%)	67.8 (27.3-124)	66.1 (20-107.6)	71.6 (35-109.2)
histology					
	SCC[3]	37 (38%)	29 (48%)	8 (40%)	n/a[6]
	adeno-carcinoma	35 (36%)	26 (43%)	9 (45%)	
	large-cell-carcinoma	3 (3%)	2 (3%)	0 (0%)	
	nos[4]	16 (17%)	1 (2%)	2 (10%)	
	unknown	6 (6%)	3 (5%)	5 (25%)	
grading					
	well	2 (2%)	2 (3%)	0 (0%)	0 (0%)
	moderately	22 (23%)	17 (28%)	5 (25%)	0 (0%)
	poor	32 (32%)	22 (36%)	8 (40%)	2 (13%)
	undifferentiated	16 (17%)	9 (15%)	2 (10%)	5 (31%)
	unknown	25 (26%)	11 (18%)	5 (25%)	9 (56%)
T-stage					
	T1	8 (8%)	8 (13%)	0 (0%)	0 (0%)
	T2	28 (29%)	18 (30%)	6 (30%)	4 (25%)
	T3	17 (18%)	10 (16%)	4 (20%)	3 (19%)
	T4	43 (44%)	25 (41%)	9 (45%)	9 (56%)
	Tx	1 (1%)	0 (0%)	1 (5%)	0 (0%)
N-stage					
	N0	16 (17%)	12 (20%)	1 (5%)	3 (19%)
	N1	3 (3%)	2 (3%)	0 (0%)	1 (6%)
	N2	33 (34%)	22 (36%)	10 (50%)	1 (6%)
	N3	44 (45%)	25 (41%)	8 (40%)	11 (69%)
	Nx	1 (1%)	0 (0%)	1 (5%)	0 (0%)
M-Stage					
	M0	72 (74%)	61 (100%)	0 (0%)	11 (69%)
	M1	25 (26%)	0 (0%)	20 (100%)	5 (31%)
GTV[5]	*(median ml, range)*	130.6 (3.3-1379.4)	140.9 (3.3-1379.4)	118 (12.8-945.1)	132.6 (7.8-578)

[1] ? 10% body weight / 6 months [2] forced expiratory volume in 1 second [3] squamous-cell carcinoma [4] not otherwise specified [5] gross tumor volume [6] not applicable

If "Eastern Cooperative Oncology Group" (ECOG) performance status allowed simultaneous chemotherapy, two courses of a double-agent cisplatin- (20mg/m^2/body surface/day) and vinorelbine-based (25 mg/m^2/body surface/day) regimen were carried out in the first and fifth treatment week (day 1-5 and day 35-40).

In total, 16 patients (24%) received radiotherapy alone, 52 patients (76%) were treated with combined radiochemotherapy and of the 68 curative-intent patients, 22 (32%) were given neoadjuvant chemotherapy before the start of radiotherapy.

Palliative-intent treatment for M1-stage patients (UICC IV) normally consisted of a hypofractionated radiotherapy given in usually 15 fractions (5 Fx/week, monday–friday) with a median single dose of 3 (2,5-3) Gy up to a median total dose of 45 (30-50) Gy.
19 patients (66%) were treated by radiotherapy alone and 10 patients (34%) received simultaneous, mostly single-agent based chemotherapy together with radiotherapy; in summa, 9 out of 29 palliative-intent patients (31%) had chemotherapy prior to radiotherapy. Chemotherapy agents mainly included etoposide, carboplatin, gemcitabine and paclitaxel.

3.4 Plasma sample acquisition, storage and processing

Blood samples of all patients were obtained by peripheral venous puncture before the start of radiotherapy (t0 time point), preferably together with routine blood sampling, and baseline (i.e. pre-treatment) plasma concentrations of OPN (t0), CAIX and VEGF was determined by ELISA. In addition, OPN plasma levels were measured at the end (t1 time point) and four weeks after radiotherapy (t2 time point), usually at the first post-radiotherapeutic follow-up at our facility.
Blood was anticoagulated (9 ml Saarstedt monovette, Nümbercht, Germany) and centrifuged at 4° Celsius for 10 minutes with 4000 rpm. Plasma was removed, aliquoted and stored at -80° Celsius until assayed.
Each plasma sample was measured in duplicate and blinded by a validated and commercially available ELISA according to the manufacturer's instructions.

3.4.1 Specifications of Human OPN ELISA Assay [287]

OPN concentration was determined using the Human Osteopontin Assay (IBL Ltd., Japan) which is a solid phase sandwich ELISA utilizing two kinds of highly specific antibodies: Anti-human OPN (O-17) rabbit IgG affinity purified coating antibody which reacts at part of the N-terminal of human OPN and anti-human OPN (10A16) mouse IgG MoAb Fab'-HRP (labeled antibody) which reacts at part of the right side from thrombin cleavage site of human OPN. The quantification of absorbance is done at 450 nm and the measurement range is from 5 to 320 ng/ml with a sensitivity of 3.33 ng/ml and a specificity (cross reactivity) of 100% (human OPN), 0.2% (mouse OPN) and ≤ 0.1% (rat OPN), respectively.

3.4.2 Specifications of Quantikine Human VEGF ELISA [288]

The Quantikine Human VEGF ELISA kit (R&D Systems, USA) was used for VEGF. It is also a sandwich assay technique using a monoclonal mouse antibody specific for $VEGF_{165}$.

It has been plated and immobilized onto the solid layer of the microtiter plate. The second antibody is polyclonal and conjugated to horseradish peroxidase as the reporter enzyme. The principle is analogue to that described in 3.4.1. In this assay, absorbance is measured at 450 nm and the minimal detectable dose of VEGF is between <5 pg/ml and <9 pg/ml (sensitivity). According to the manufacturer, cross-reactivity and interference with VEGF-related factors may be observed at levels ≥500 to 4000 pg/ml (<.5% cross-reactivity) and the assay recognizes both natural and recombinant human VEGF (specificity).

3.4.3 Specifications of Human CA IX Quantikine ELISA [289]

For CAIX, the Human CA IX Quantikine ELISA Kit (R&D Systems, USA) was conducted. The solid-phase sandwich ELISA uses a monoclonal antibody specific for CAIX and has been immobilized on a microplate. The polyclonal detection antibody is conjugated to the reporter enzyme horseradish peroxidase. The sensitivity of this assay is 4.39 pg/ml, its range is from 15.6 to 1000 pg/ml and it is specific for natural and recombinant human CAIX. Cross-reactivity with available related enzymes is reported with <.5%

3.5 Statistical analysis and endpoints

All statistical analyses were performed using the SPSS PASW software (version 18) for windows (SPSS Inc., USA). P-values were two-sided and p< .05 was regarded statistically significant. Pearson's test was used to test for correlation between biomarker plasma levels and Wilcoxon's test compared median OPN levels before, at the end and after treatment in the entire patient collective and patient subgroups.

Non-parametric tests (Mann-Whithney's u-test, Kruskal-Wallis' h-test) tested for differences in pretherapeutic (baseline) biomarker plasma levels between two groups and determined association of pre-treatment plasma levels with patient, disease and treatment characteristics in the entire patient collective and the curative-intent NSCLC M0 patient cohort. Due to the small patient number, the SCLC and palliative-intent NSCLC M1 cohort have been excluded from the aforementioned analysis.

Biomarker plasma levels were dichotomized using the median as the cutoff value with "high" marker levels referring to ≥ median and "low" marker levels to < median.

Relative changes in OPN plasma levels from one measuring time point to the other were divided into three categories according to the percent change based on the baseline OPN levels before radiotherapy (t0): fall (≥ -10%), stable (between -10% and +10%) and rise (≥ +10%). Follow-up time (i.e. from the start of radiotherapy until last seen) in living patients is

reported in months (range) and survival status of patients was last updated on August 31st 2014.

Primary endpoints included overall survival (OS, i.e. from the start of radiotherapy until death from any cause or until last seen during follow-up), progression-free survival (PFS, i.e. from the start of radiotherapy until any disease progress in recurring patients or until death or last seen during follow-up without disease progression in non-recurring patients) and time-to-progression (TTP, i.e. from the start of radiotherapy until any disease progression with death counting as a censoring variable).

Since few patients with advanced stage lung cancer (usually stage IIIa and IIIb) achieved complete remission (i.e. freedom from any disease) after radiotherapy, disease-free survival (DFS, referring to patients with complete response only) was not evaluated in this study. PFS, normally referring to patients with partial remission only, in this study refers to all patients, regardless of their tumor response.

Secondary endpoints were initial tumor control after radiotherapy (i.e. at the first post-radiotherapeutic re-staging evaluation) which was classified as complete remission (no tumor detectable), partial remission (tumor size decrease ≥ 50%), stable disease (tumor size change ≤ 25%) and progressive disease (tumor size increase of > 25%) according to comparison of CT images from before and after radiotherapy by an experienced radiologist (therapy response was rated “good” if the patients had complete or partial remission after radiotherapy while it was categorized “poor” if stable or progressive disease was noted after radiotherapy); metastasis-free survival (MFS, i.e. from the start of radiotherapy until occurrence of distant metastasis or until death or last seen during follow-up without metastasis in non-metastasizing patients with local relapse counting as a censoring variable) and freedom from local relapse (FFLR, i.e. from the start of radiotherapy until local relapse or until death or last seen during follow-up without local relapse).

Primary and secondary endpoint analysis was restricted to the entire patient collective and the curative-intent NSCLC (M0) cohort. SCLC and palliative-intent NSCLC (M1) patients were excluded from endpoint analysis for the limited patient number in these subgroups.

Survival time is reported in median months (range) and survival curves were generated using the Kaplan-Meier product-limit method and differences between survival curves were assessed with log-rank-test. To identify prognostic factors for OS, univariate and multivariate analyses were performed using the Cox proportional hazard regression model. The relative risk and hazard ratio was evaluated with the x^2-test and is reported with a 95%-confidence interval (95%-CI).

3.6 Ethics vote and informed consent

Prior to the start of the prospective recruitment of the patients, a formal proposal consisting of a detailed study description including the written information and consent form for the patient was submitted to the ethics committee of the medical faculty which approved the study and gave a positive vote for the project.

Each patient who met the inclusion criteria as stated in 3.1 was offered to participate in the study in an informative talk given by an experienced Radiation Oncology physician emphasizing the voluntary nature of the project. The scientific background, purpose and procedure of the study was explained using a detailed written information form and written informed consent was obtained for each patient using the consent form.

4. Results

Absolute baseline (pre-treatment) plasma levels of OPN, VEGF and CAIX, their combination as well as relative OPN plasma level changes over time are described below. They were assessed for interrelation, association with clinicopathological patient characteristics and their impact on prognosis.

4.1 Plasma marker concentration, changes and interrelation in all patients and subgroups

For the pre-treatment measurement time point (t0), OPN plasma samples of all patients could be acquired (n=97). At the end of treatment (t1), 91 patients (94%) had OPN plasma samples and four weeks after radiotherapy, OPN plasma samples were available in 69 patients (71%). Baseline VEGF and CAIX plasma samples could be obtained in 96 patients (99%). Median absolute pre-treatment plasma concentration of OPN, VEGF and CAIX for the entire patient collective and for subgroups is presented in **table 2.**

Table 2. Median pre-treatment plasma concentration (*min-max*) of OPN (ng/ml), VEGF (pg/ml), uPa (ng/ml), uPaR (ng/ml) and PAI (ng/ml) in the entire patient cohort and subgroups

	all patients (n=97)	*plasma samples*	**curative-intent NSCLC** (M0, n=61)	*plasma samples*	**palliative-intent NSCLC** (M1, n=20)	*plasma samples*	**SCLC** (n=16)	*plasma samples*
OPN (t0)[1]	819.8 (*223.1-4716.7*)	*97*	817 (*299-2441*)	*61*	1049.6 (*453.5-4716.7*)	*20*	740.8 (*400.4-2177.2*)	*16*
VEGF[2]	89.7 (*0-1078.1*)	*96*	92 (*0-1078.1*)	*60*	113.2 (*2.9-264.2*)	*20*	94.6 (*24.2-357.8*)	*16*
CAIX[2]	94.8 (14.8 -1000)	*96*	105 (22-420)	*61*	76.3 (14.8-179.3)	*19*	123.6 (55.5-1000)	*16*

[1] in ng/ml [2] in pg/ml

Table 3 shows intra- and post-therapeutic changes in OPN plasma levels for subgroups. Both in the entire and the curative-intent NSCLC (M0) cohort, OPN plasma levels non-significantly decreased during (t0 to t1) and after treatment (t1 to t2), **table 3**.

In palliative-intent (M1) and SCLC patients, median OPN plasma levels increased during and decreased after treatment, **table 3**. However, these changes remained insignificant.
Among curative-intent NSCLC (M0) patients, 46% had decreasing, 17% had stable and 34% had increasing OPN plasma levels during treatment (t0 to t2).

Table 3. Median OPN plasma (ng/ml) levels before (t0), at the end (t1) and four weeks after radiotherapy (t2) in the entire patient cohort and subgroups

	all patients (n=97)	*plasma samples*	**curative-intent NSCLC** (M0, n=61)	*plasma samples*	**palliative-intent NSCLC** (M1, n=20)	*plasma samples*	**SCLC** (n=16)	*plasma samples*
OPN t0	819.8 (223.1-4716.7)	*97*	760.9 (223.1-2441)	*61*	1049.6 (453.5-4716.7)	*20*	740.8 (400.4-2177.2)	*16*
OPN t1	793.1 (323-4521.6)	*91*	715.5 (323-2304.3)	*58*	1086.7 (545.7-4521.6)	*18*	779.4 (395.3-2420)	*15*
OPN t2	680.1 (71.6-4577.9)	*69*	632.5 (71.6-2855.8)	*49*	779.5 (525.4-4577.9)	*9*	770.5 (378.5-2415.2)	*11*

In palliative-intent NSCLC (M0) patients, 21% had decreasing, 7% had stable and 43% had increasing OPN levels during treatment.
Mean OPN plasma levels before (t0), at the end (t1) and 4 weeks after treatment (t2) were not significantly different. At all three time points, palliative-intent NSCLC (M1) patients had significantly higher OPN plasma levels compared to curative-intent NSCLC (M0) patients (OPN t0: 761 vs. 1050 ng/ml, p<.0001; OPN t1: 716 vs. 1087 ng/ml, p<.0001; OPN t2: 633 vs. 780 ng/ml, p<.0001).
In the entire patient collective, OPN plasma levels detected at either time point correlated with each other (t0 & t1: r=.5, p<.0001; t0 & t2: r=.3, p=.004; t1 & t2: r=.5, p<.0001) and median VEGF plasma levels inversely correlated with hemoglobin levels (r= -.2, p=.03).
No significant correlation between OPN, VEGF and CAIX was found in the entire patient collective.
In curative-intent NSCLC M0 patients, pre-treatment OPN (t0) plasma levels were positively correlated with CAIX plasma levels (r=.3, p=.03), VEGF plasma levels (r=.3, p=.03), end-of-treatment OPN (t1, r=.6, p<.0001) and OPN plasma concentration 4 weeks after radiotherapy (r=.5, p=.001). OPN t1 plasma levels also correlated with OPN plasma concentration 4 weeks after treatment (t2, r=.3, p=.03). An inverse correlation was determined between VEGF and hemoglobin concentration (r= -.3, p=.03) and between OPN t0 values and hemoglobin concentration (r=-.5, p=.001). A trend was noted for a correlation between VEGF and CAIX plasma levels (r=.2, p=.09). CAIX correlated positively with VEGF (r=.03, p=.02).
In palliative-intent NSCLC M1 patients, baseline OPN plasma levels before radiotherapy (t0) correlated with OPN levels at the end of treatment (t1, r=.5, p=.03) and OPN 4 weeks after treatment (t2) trended to correlate with pre-treatment OPN (r=.6, p=.06). In this patient group, CAIX positively correlated with pre-therapeutic OPN (t0, r=.4, p=.04) and a

trend could be determined for an inverse correlation between OPN t0 and hemoglobin (r=.4, p=.05). VEGF also correlated with CAIX (r=.5, p=.04) and an inverse correlation was noted between hemoglobin blood levels and CAIX (r=.5, p=.04).

In the SCLC patient cohort, median plasma OPN plasma levels before the start of radiotherapy (t0) correlated with VEGF (r=.5, p=.03) and CAIX (r=5, p=.03).

A positive correlation was also determined for post-treatment OPN (t2) and end-of-treatment OPN (t2, r=.8, p=.002) which also correlated with VEGF (r=.7, p=.007).

4.2 Patient and treatment characteristics and their association with pre-treatment plasma protein levels in the entire patient collective and curative-intent NSCLC M0 patients

The association of pre-treatment OPN (t0), VEGF and CAIX plasma levels with clinicopathological and sociodemographic patient characteristics is presented in **table 4** for the entire (n=97, left side of table) and the curative-intent NSCLC (M0) cohort (n=61, right side of table). Significant associations are highlighted in bold in the table.

In the entire patient collective, the association of median pre-treatment OPN plasma levels with T-stage was more pronounced if T-stage was grouped together as T1-2 vs. T3-4, with median OPN plasma levels before treatment being 651 ng/ml (T1-2) vs. 865 ng/ml (T3-4), p=.003. Pre-treatment OPN plasma levels of N+ (849 ng/ml, n=80) or N2-3 (848 ng/ml, n=77) patients were significantly higher than those of N- (642 ng/ml, n=16) or N0-1 (652 ng/ml, n=19) patients (p=.01 and .04). OPN plasma levels before treatment were almost twice as high in patients with distant metastases compared to patients in M0-stage (1140 vs. 737 ng/ml, p=.001). Consequently, UICC stage correlated with OPN t0 plasma levels where higher plasma levels were found in patients with higher disease stage (UICC I-II: 622 ng/ml, n=10 vs. UICC III: 804 ng/ml, n=62 vs. UICC IV: 1140 ng/ml, n=25, p=.001).

When SCC histology was directly compared to adeno-carcinoma, patients with SCC (n=37) had higher OPN plasma levels before treatment than patients with adeno-carcinoma (n=35; 871 vs. 706 ng/ml, p=.01) and a trend for elevated pre-treatment OPN plasma levels in NSCLC patients (n=81) compared to SCLC patients (n=16) was found (820 vs. 741 ng/ml, p.07). Anemic patients had higher VEGF levels before treatment (p=0.04) and a trend for higher T-stage in patients with elevated VEGF plasma levels before treatment was noted (T1-2: 62 ng/ml, n=35 vs. T3-4: 102 ng/ml, n=58, p=.07).

When patients with N0-2 stage were tested against those in N3 stage, baseline CAIX plasma levels were significantly higher in the latter subgroup (N0-2: 66 ng/ml, n=51 vs. N3:

123 ng/ml, n=44; p=.004). In the direct comparison between NSCLC and SCLC, the latter histology was associated with higher pre-treatment CAIX plasma levels compared to the NSCLC histologic subtype (124 ng/ml, n=80 vs. 92 ng/ml, n=80; p=.03) and a trend for elevated baseline CAIX levels was noted in patients with higher tumor grade (G1-2: 91 ng/ml, n=25 vs. G3-4: 94 ng/ml, n=47; p=.09). UICC stage was not associated with pre-therapeutic CAIX plasma concentration (p=.7).

Similar findings can be reported for the curative-intent NSCLC (M0) patient cohort. A trend for higher OPN plasma levels in patients with higher tumor grade was noted (G1-2 vs. G3-4: 691 ng/ml, n=19 vs. 838 ng/ml, n=30, p=.08). When T1-2 was evaluated against T3-4 stage, OPN plasma levels were significantly elevated in the latter group (658 ng/ml, n=26 vs. 847 ng/ml, n=34, p=.007) and trend for higher VEGF levels in T3-4 stage patients (102 pg/ml, n=34) was found compared to T1-2 stage (58 pg/ml, n=26, p=.07). A trend was also found for increased OPN (p=.08) in patients with N+ stage (versus N0 stage). Higher median OPN plasma levels were found in patients with advanced UICC stage (UICC I-II: 595 ng/ml, n=9 vs. UICC III: 820 ng/ml, n=51, p=.003) and a trend for elevated VEGF plasma levels in patients with higher UICC stage was noted (p=.08). An association between higher T-stage and increased baseline CAIX plasma levels was determined (T1-2: 86 ng/ml, n=25 vs. T3-4: 106 ng/ml, n=35, p=.04) and a statistical trend for higher pre-treatment CAIX plasma levels in patients with poorly or undifferentiated tumors (G1-2: 62 ng/ml, n=19 vs. G3-4: 106 ng/ml, n=31; p=.06) was found. Higher N-stage trended to be associated with CAIX (N0-2: 65 ng/ml, n=36 vs. N3: 151 ng/ml, n=25; p=.06).

4.3 Univariate analysis of plasma biomarker levels and their changes in the entire patient collective and in the curative-intent NSCLC M0 patient cohort

Absolute plasma biomarker levels and their relative changes were tested for an association with the primary and secondary endpoints in the entire patient collective and in the curative-intent NSCLC (M0) patient cohort. Median follow-up in surviving patients was 41 (12 – 66) months and at the time of the last survival data update (08-2014), 81 patients (84%) already had died. In 59%, death was cancer-related, in 7% it was not cancer-related and in 34% the cause of death remained unspecified.

4.3.1 Initial tumor control and response to radiotherapy

Follow up data on initial tumor control at the first post-radiotherapy response evaluation (4-6 weeks after the end of radiotherapy) was available in 71 patients (73%).

In the entire patient cohort, 2 patients (2%) had complete and 51 patients (53%) had partial remission; 13 patients (13%) had stable and 5 patients (5%) had progressive disease. Good therapy response was noted in 55% of patients (n=53) while 19% (n=18) showed poor response to radiotherapy. In 27% of cases, no information on tumor control was available.

Among curative-intent NSCLC M0 patients, complete remission could be achieved in 2 (3%), partial remission in 36 patients (59%); 9 patients (15%) had stable and 2 (3%) progressive disease. Therapy response was good in 36 patients (62%) while it was poor in 11 patients (18%). 20% of patients had no data on tumor control after radiotherapy.

In the entire patient collective, both tumor control and therapy response was not associated with OPN t0, t1, t2, CAIX and VEGF plasma levels (p=.8, .9, .3, .7 and p=.6, .5, .6, .7). However, median end-of-treatment OPN plasma levels (t1) were significantly elevated in patients with poor tumor control (p=.01): Patients with progressive disease after radiotherapy (n=5) had the highest OPN plasma levels at the end of treatment (1585.1 ng/ml), followed by patients with stable disease (n=12, 961.8 ng/ml), partial remission (n=50, 714.5 ng/ml) and those with complete remission (n=2, 815.3 ng/ml), **figure 1a**. Accordingly, OPN plasma levels at the end of treatment (t1) were significantly higher in non-responding patients compared to responding patients (1184.2 vs. 715.5 ng/ml, p=.002), **figure 1b**.

Relative OPN plasma level changes after radiotherapy (t1 to t2) were not related to tumor control and therapy response (p=.2, and .4) but intratherapeutic plasma level changes (t0 to t1) were associated with therapy response: Responding patients had a median decrease in OPN t0 to t1 plasma levels by -4.7% as opposed to a median increase by +14.7% which was noted in non-responding patients (p=.04), **figure 1c**.

In addition, relative OPN t0t1 plasma levels were related to tumor control, however, these findings remained a statistical trend (p=.1): Patients with progressive disease (n=5) after radiotherapy had a median OPN plasma level change from before (t0) to the end of radiotherapy (t1) by +43.7%, patients with stable disease (n=13) had a median increase by +14.4%. OPN t0t1 plasma levels of patients with partial remission (n=50) decreased by -4.2% and patients with complete remission (n=2) displayed a median decrease in their intratherapeutic plasma levels by -15.9%, **figure 1d**.

In cross-table analysis, among responding patients, 30 had low (i.e. below the) median OPN t1 levels and 22 had OPN t1 plasma levels above the median while among non-responding patients, 13 had OPN t1 plasma levels above and 4 below the median (p=.01).

Table 4. Association of pre-treatement OPN (t0), VEGF and CAIX plasma levels with clinicopathological patient characteristics in the entire patient cohort (n=97)

Characteristic	OPN	VEGF	CAIX
	ng/ml	*pg/ml*	*ng/ml*
age	***.03***	*.2*	*.2*
> median	886 (n=50)	72 (n=48)	106 (n=49)
≤ median	744 (n=47)	99 (n=46)	88 (n=47)
Sex	***.03***	*.2*	*.3*
male	850 (n=83)	92 (n=82)	98 (n=82)
female	678 (n=14)	70 (n=14)	95 (n=14)
FeV1[1]	***.002***	*.8*	*.8*
> median	690 (n=44)	92 (n=43)	93 (43)
≤ median	910 (n=44)	87 (n=42)	98 (n=44)
weight loss[2]	***.001***	*.3*	*.6*
yes	999 (n=28)	79 (n=27)	90 (n=27)
no	726 (n=62)	102 (n=61)	93 (n=62)
hemoglobin	*.3*	***.04***	*.7*
> median	791 (n=51)	72 (n=51)	95 (n=50)
≤ median	849 (n=46)	102 (n=43)	97 (n=46)
histology	*.1*	*.3*	*.6*
SCC[3]	871 (n=37)	107 (n=37)	86 (n=37)
adeno-carcinoma	706 (n=35)	88 (n=33)	92 (n=34)
large-cell-carcinoma	652 (n=3)	356 (n=3)	183 (n=3)
SCLC	866 (n=16)	122 (n=16)	109 (n=16)
unknown	626 (n=6)	33 (n=5)	83 (n=6)
Grading	*.7*	*.4*	*.3*
well (G1)	844 (n=2)	35 (n=2)	76 (n=2)
moderate (G2)	712 (n=22)	94 (n=22)	91 (n=22)
poor (G3)	855 (n=32)	100 (n=32)	90 (n=31)
undifferentiated (G4)	858 (n=16)	90 (n=16)	106 (n=16)
nos[4]	671 (n=25)	60 (n=25)	89 (n=24)
T-stage	***.02***	*.3*	*.3*
T1	561 (n=8)	64 (n=8)	54 (n=8)
T2	677 (n=28)	62 (n=27)	86 (n=26)
T3	919 (n=17)	107 (n=17)	190 (n=17)
T4	1034 (n=43)	97 (n=41)	103 (n=42)
Tx	n/a (n=1)	n/a (n=1)	n/a (n=1)
N-stage	***.08***	.7	***.04***
N0	642 (n=16)	78 (n=16)	76 (n=16)
N1	850 (n=3)	88(n=3)	109 (n=3)
N2	867 (n=33)	102 (n=33)	62 (n=32)
N3	825 (n=44)	72 (n=44)	123 (n=44)
Nx	n/a (n=1)	n/a (n=1)	n/a (n=1)
M-stage	***.001***	*.2*	*.3*
M0	737 (n=72)	83 (n=71)	100 (n=72)
M1	1140 (n=25)	133 (n=23)	92 (n=24)
GTV[5]	***.01***	***.002***	*.5*
> median	859 (n=48)	126 (n=47)	105 (n=47)
≤ median	689 (n=47)	58 (n=47)	93 (n=47)

Association of pre-treatement OPN (t0), VEGF and CAIX plasma levels with clinicopathological patient characteristics in the curative-intent NSCLC M0 patient cohort (n=61)

Characteristic	OPN	VEGF	CAIX
	ng/ml	*pg/ml*	*ng/ml*
age	***.09***	*.3*	*.8*
> median	831 (n=30)	67 (n=30)	109 (n=31)
≤ median	726 (n=31)	95 (n=31)	86 (n=30)
Sex	*.9*	*.4*	*.5*
male	746 (n=51)	88 (n=51)	104 (n=51)
female	768 (n=10)	86 (n=10)	85 (n=10)
FeV1[1]	***.01***	*.5*	*.1*
> median	691 (n=29)	60 (n=29)	96 (n=29)
≤ median	848 (n=28)	118 (n=28)	93 (n=29)
weight loss[2]	***.001***	*.7*	*.6*
yes	1001 (n=13)	93 (n=13)	106 (n=13)
no	692 (n=43)	88 (n=43)	86 (n=44)
hemoglobin	*.6*	***.04***	*.6*
> median	712 (n=32)	57 (n=32)	110 (n=32)
≤ median	770 (m=28)	104 (n=28)	86 (n=29)
histology	*.2*	***.08***	*.8*
SCC[3]	820 (n=29)	107 (n=29)	86 (n=20)
adeno-carcinoma	706 (n=25)	88 (n=25)	110 (n=26)
large-cell-carcinoma	507 (n=2)	44 (n=2)	123 (n=2)
nos	n/a (n=1)	n/a (n=1)	n/a (n=1)
unknown	460 (n=3)	24 (n=3)	109 (n=3)
Grading	*.3*	*.4*	*.2*
well (G1)	844 (n=2)	35 (n=2)	86 (n=2)
moderate (G2)	691 (n=17)	85 (n=17)	62 (n=17)
poor (G3)	830 (n=21)	92 (n=21)	96 (n=22)
undifferentiated (G4)	841 (n=9)	83 (n=9)	114 (n=9)
unknown	706 (n=11)	72 (n=11)	171 (n=11)
T-stage	***.02***	*.4*	*.1*
T1	561 (n=8)	64 (n=8)	54 (n=8)
T2	690 (n=18)	58 (n=18)	115 (n=18)
T3	847 (n=10)	97 (n=10)	177 (n=10)
T4	840 (n=24)	102 (n=24)	96 (n=25)
N-stage	*.4*	*.3*	*.3*
N0	651 (n=12)	85 (n=12)	74 (n=12)
N1	805 (n=2)	88 (n=2)	86 (n=2)
N2	852 (n=22)	102 (n=22)	64 (n=22)
N3	768 (n=24)	67 (n=24)	151 (n=25)
Nx	n/a (n=1)	n/a (n=1)	n/a (n=1)
GTV[5]	***.03***	***<.0001***	*.6*
> median	819 (n=30)	132 (n=30)	86 (n=31)
≤ median	677 (n=30)	52 (n=30)	108 (n=30)

[1] forced expiratory volume in 1 second [2] **≥ 10% body weight / 6 months** [3] squamous-cell carcinoma [4] not otherwise specified [5] gross tumor volume

Relative intratherapeutic OPN (t0 to t1) plasma level changes were associated with therapy response in cross-table analysis: Among responding patients, 29 had decreasing and 23 had increasing OPN t0t1 plasma levels, while in non-responding patients, 13 had increasing and 4 decreasing OPN t01 plasma levels (p=.02).

When patients were classified in rising vs. falling vs. stable intratherapeutic OPN (t0t1) plasma levels, among responding patients, 23 had falling, 13 had stable and 16 had increasing OPN t0t1 plasma levels while in non-responding patients, 4 had falling, 3 had stable and 11 had increasing OPN t0t1 plasma levels (p=.07).

In curative-intent NSCLC (M0) patients, absolute OPN, CAIX and VEGF plasma levels were not associated with tumor control or therapy response but a trend was noted for better therapy response in patients with decreasing intratherapeutic OPN (t0 to t1) plasma levels: OPN plasma levels in responding patients decreased by -3.6% during radiotherapy while they increased by 14.9% in non-responding patients (p=.04).

In cross-table analysis, a significant association between therapy response and relative intratherapeutic OPN plasma level changes (t0 to t1) was observed: Among responding patients, 16 had decreasing, 10 stable and 11 increasing OPN plasma levels during radiotherapy while in non-responding patients, 2 had falling, 1 stable and 8 increasing OPN plasma levels (p=.03). Tumor control was not related to OPN plasma level changes.

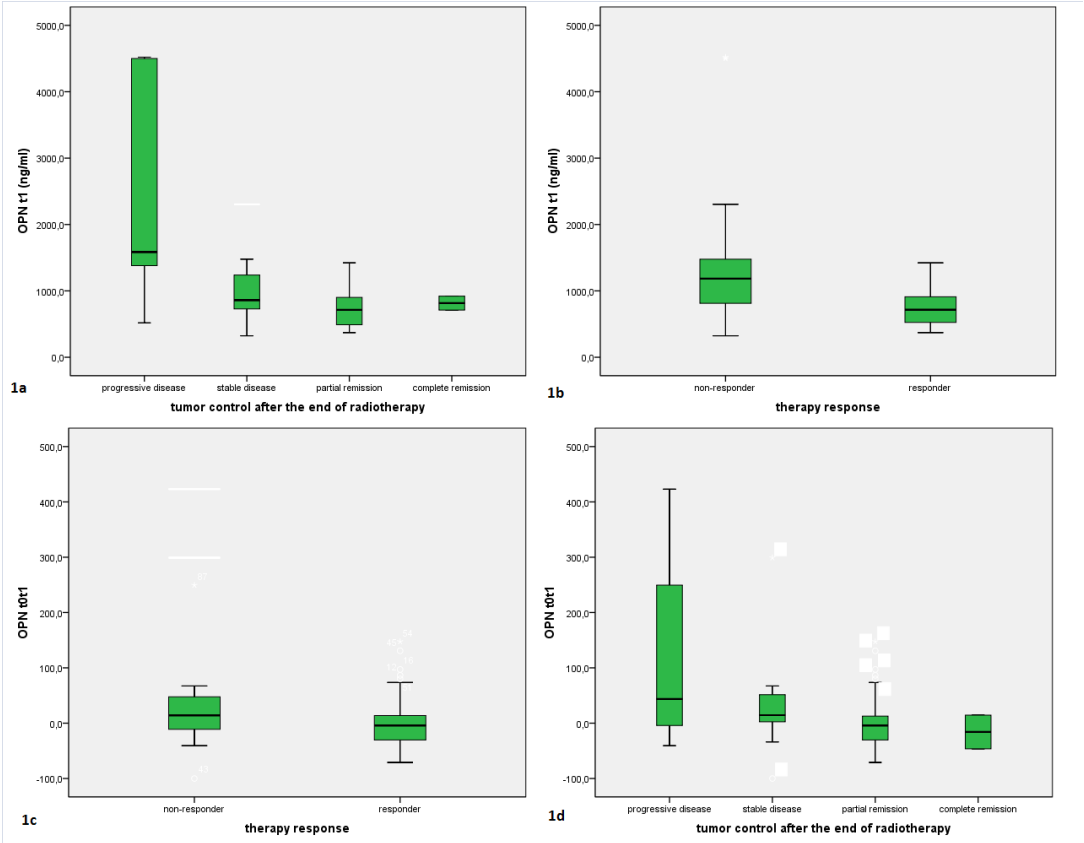

Figure 1 **Histogram of the association of median post-treatment OPN plasma levels and their relative changes with tumor control and therapy response in the entire patient cohort (n=97). Black line indicates the median, error bars in black and range in green.**

1a Post-treatment OPN (t1) plasma levels in patients with progressive, stable disease, partial or complete remission four weeks after the end of radiotherapy.

1b Post-treatment OPN (t1)plasma levels in responding and non-responding patients.

1c Intratherapeutic OPN plasma level changes (t1 to t2) in responding and non-responding patients.

1d Intratherapeutic OPN plasma levels changes (t0 to t1) in patients with progressive, stable disease, partial or complete remission four weeks after the end of radiotherapy.

4.3.2 Overall survival (OS)

Median OS was 11 (0 – 66) months in all patients and in curative-intent NSCLC M0 patients, it was 16 (1 – 66) months. In the latter subgroup, 50 deaths (82%) were registered at the last survival data update and 56% of deaths were cancer-related, 8% were not cancer-related and in 64% of cases, death cause could not be specified.

Cancer-specific survival (i.e. disease-specific survival) was 7 (0 – 37) months in the entire patient cohort (n=57) and it was 13 (2 – 37) months in the curative-intent cohort (n=14).

In the entire patient collective, absolute OPN plasma levels before (t0), at the end (t1) and 4 weeks after radiotherapy (t2) were associated with OS. Patients with high pre-treatment OPN plasma levels (i.e. t0 ≥ median, n=57) had a median OS of 7.6 months compared to patients with low t0 plasma levels (i.e. < median, n=40) who lived 16.1 months (p=.04). Patients with elevated baseline OPN plasma levels also had a significantly increased risk of death compared to patients with low OPN t0 levels (rr=1.6, 95%-CI [1.01-2.5], p=.04), **figure 2a**. OS was 6.9 months in patients with elevated OPN t1 plasma levels (n=46) as opposed to 15.7 months in patients with low OPN plasma levels at the end of treatment (n=45, p=.004). Patients also had a significantly increased risk of death if their plasma levels were elevated at the end of treatment (t1, rr=1.9, 95%-CI [1.2-3], p=.005), **figure 2b**. Superior OS was also found in patients with low OPN plasma levels 4 weeks after radiotherapy (t2) when compared to patients with high plasma levels (25.5 months, n=35 vs. 11.8 months, n=34, p=.02) who also had a significantly increased risk of death (rr=1.8, 95%-CI [1.1-3.1], p=.03), **figure 2c**. Absolute CAIX and VEGF plasma levels before treatment were not associated with OS in all 97 patients (p=.7 and p= .5). No survival differences were determined in patients with increasing, stable or decreasing OPN plasma levels during treatment (t0 to t1, p=.8) but a trend for superior survival was noted in patients with falling (n=37) or stable (n=7) post-therapeutic OPN plasma levels compared to patients with increasing (n=26) plasma levels from t1 to t2 time point (p=.09). The latter patients also had an increased risk of death (rr=1.2, 95%-CI [.7-2.1], p=.07), **figure 2d**.

In the curative-intent NSCCL M0 cohort, OPN baseline (t0) and t1 plasma levels were not associated with OS but I found a trend for prolonged survival in patients with low post-treatment (t2) OPN plasma levels (26.5 months, n=27 vs. 12.7 months, n=22, p=.08) and an increased risk of death in patients with high OPN t2 plasma levels (rr=1.7, 95%-CI [.9-3.2], p=.09), **figure 3a**.

Median pre-treatment plasma levels of VEGF and CAIX were not associated with OS.

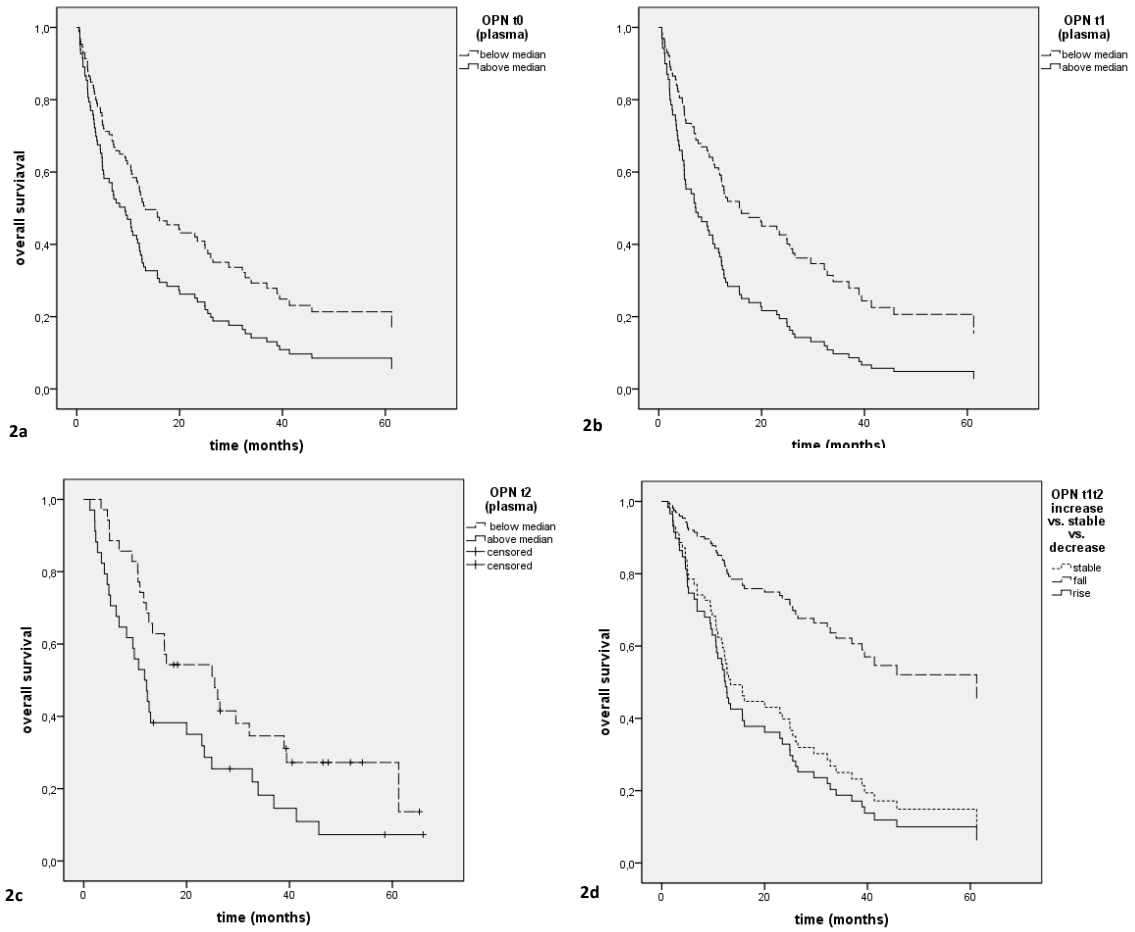

Figure 2 **Association of plasma marker levels with overall survival (OS) in the entire patient collective (n=97).**
2a baseline OPN (t0) plasma levels and overall survival in Cox proportional hazards model
2b end-of-treatment OPN (t1) plasma levels and overall survival in Cox proportional hazards model
2c post-treatment OPN (t2) plasma levels and overall survival in Kaplan Meier analysis
2d increasing vs. stable vs. decreasing post-treament OPN (t1 to t2) plasma levels and overall survival in Cox proportional hazards model

Relative OPN plasma level changes during radiotherapy (t0 to t1) were not related to survival but patients with increasing OPN plasma levels after treatment (t1 to t2) had a poorer OS than patients with decreasing post-treatment OPN plasma levels (10.9 months, n=18 vs. 26.5 months, n=29, p=.1), **figure 3b**. This trend was more pronounced if patients were divided into increasing (n=16) vs. stable (n=5) vs. decreasing (n=28) OPN t1 to t2 plasma levels as defined in 3.5 (13 vs. 14.4 vs. 15.7 months, p=.07), **figure 3c**.

4.3.3 Progression-free survival (PFS)

Median PFS was 6 (0 – 65) months in all patients. In this group, 47 patients (49%) developed a disease progress during follow-up (n=23: 24% local relapse, n=11: 11% distant metastasis, n=13: 13% both), 19 patients (20%) remained without progressive

disease during follow-up (i.e. until death or last seen) and in 31 patients (32%), no data on disease progress was available.

In curative-intent NSCLC M0 patients, median PFS was 9 (1 – 65) months and disease progress during follow-up was noted in 26 patients (43%). 18 patients (30%) had no disease progress until their death or last seen during follow-up and disease progress data was missing in 17 patients (28%). In this patient group, 23% (n=14) had local, 7% (n=4) distant and 13% (n=8) had both local and distant disease progression.

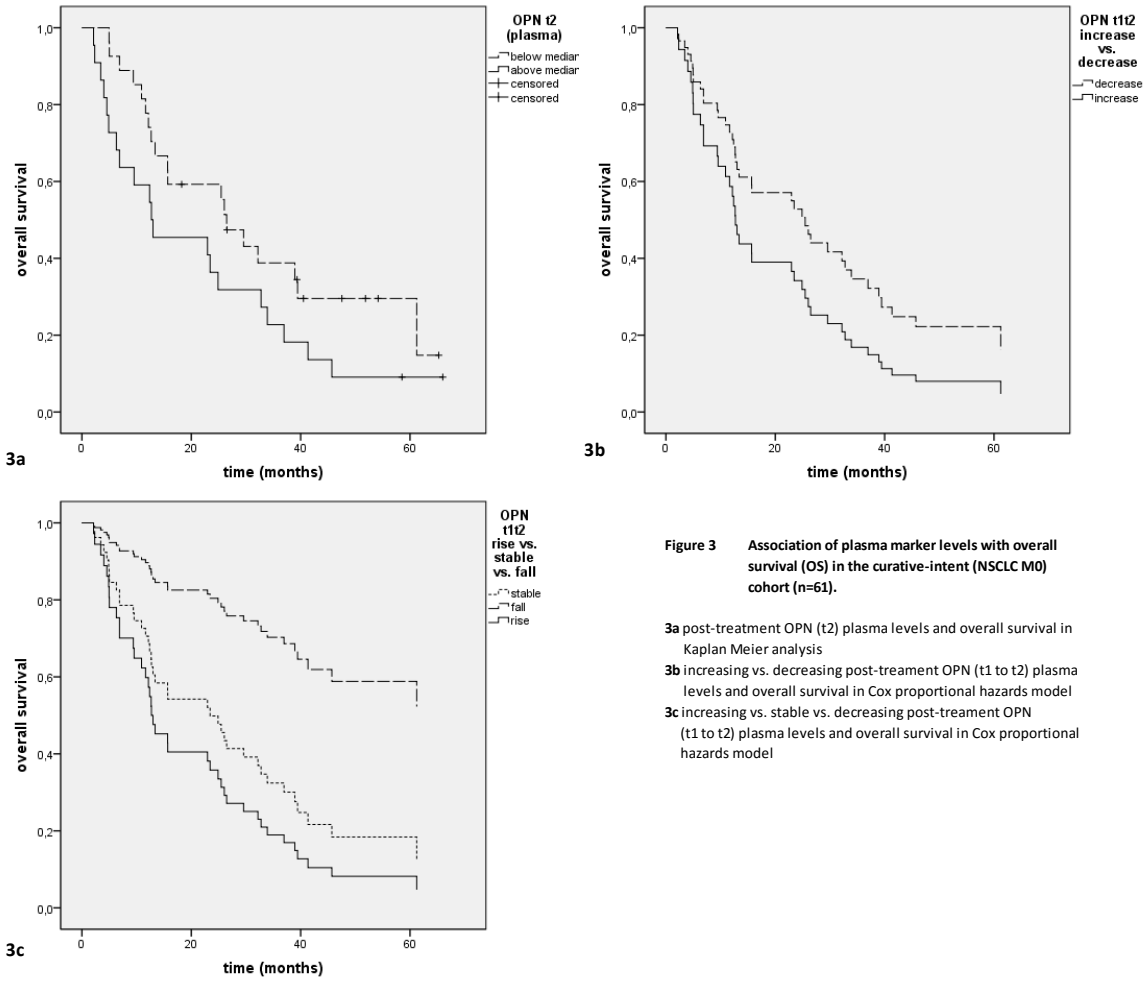

Figure 3 **Association of plasma marker levels with overall survival (OS) in the curative-intent (NSCLC M0) cohort (n=61).**

3a post-treatment OPN (t2) plasma levels and overall survival in Kaplan Meier analysis

3b increasing vs. decreasing post-treament OPN (t1 to t2) plasma levels and overall survival in Cox proportional hazards model

3c increasing vs. stable vs. decreasing post-treament OPN (t1 to t2) plasma levels and overall survival in Cox proportional hazards model

In the entire patient cohort, elevated OPN plasma levels at the end of (t1) and four weeks after radiotherapy (t2) were significantly associated with PFS.

Patients with elevated end-of-treatment OPN plasma levels (t1, n=46) had a median PFS of 5.6 months compared to patients with OPN t1 levels below the median (n=45, 9.1

months, p=.02). The former patients also had a significantly elevated risk for disease progression (rr=1.7, 95%-CI [1.1-2.7], p=.02), **figure 4a**.

Patients with low OPN plasma levels four weeks after the end of radiotherapy (t2, n=35) had a median PFS of 12.2 months as opposed to patients with elevated OPN t2 levels (n=34, 7.5 months, p=.04) who also had a significantly increased risk for disease progress (rr=1.7, 95%-CI [1.1-3], p=.04), **figure 4b**. A trend for reduced PFS in patients with elevated pre-treatment OPN plasma levels (t0, n=49) compared to patients with low baseline plasma OPN levels (n=48) was noted (p=.06), **figure 4c**. Absolute pre-treatment VEGF and CAIX plasma levels were not associated with PFS (p=.77 and p=.97).

Relative OPN plasma level changes during treatment (t0 to t1) did not show any relation to PFS but post-radiotherapy OPN level changes (t1 to t2) were significantly related to PFS. Patients with increasing post-treatment OPN plasma levels (n=26) had a median PFS of 5.2 months compared to 9.1 months in patients with stable (n=7) and 15.6 months in patients with decreasing OPN t1t2 plasma levels (n= 37, p=.03). Correspondingly, a trend for an elevated relative risk for disease progression in patients with increasing OPN t1t2 plasma levels (rr=1.5, 95%-CI [.9-2.5], p=.05) was observed, **figure 4d**.

In the curative-intent NSCLC M0 patient cohort, absolute pre-treatment plasma levels of OPN, VEGF and CAIX were not associated with PFS (p=.5, p=.8 and p=.5).

OPN plasma levels at the end of (t1) and four weeks after radiotherapy (t2) were also not related to PFS (p=.4 and p=.1). Relative OPN plasma level changes after (t1 to t2) but not during radiotherapy (t0 to t1, p=.9) were associated with PFS. A trend for prolonged PFS was noted in patients with decreasing (n=29) compared to increasing OPN plasma levels (n=18) after treatment (t1t2, 14.3 months vs. 5.3 months, p=.08). The latter patients also had an increased risk for disease progression (rr=1.7, 95%-CI [.9-3.3], p=.09).

When patients were classified according to their relative OPN plasma level changes after radiotherapy in increasing vs. stable vs. decreasing OPN t1t2 levels, the effect on PFS was more pronounced. Patients with increasing OPN t1t2 plasma levels (n=16) had a median PFS of 6.2 months, median PFS in patients with stable plasma levels (n=5) was 10.3 months and 22 months in patients with decreasing plasma levels (n=28) after radiotherapy (p=.009). Patients with decreasing OPN plasma levels after radiotherapy also had the lowest risk for disease progression, followed by patients with stable and those with increasing OPN t1t2 plasma levels whose relative risk was elevated by a factor 1.9 (95%-CI [1.1-3.8], p=.02), **figure 5**.

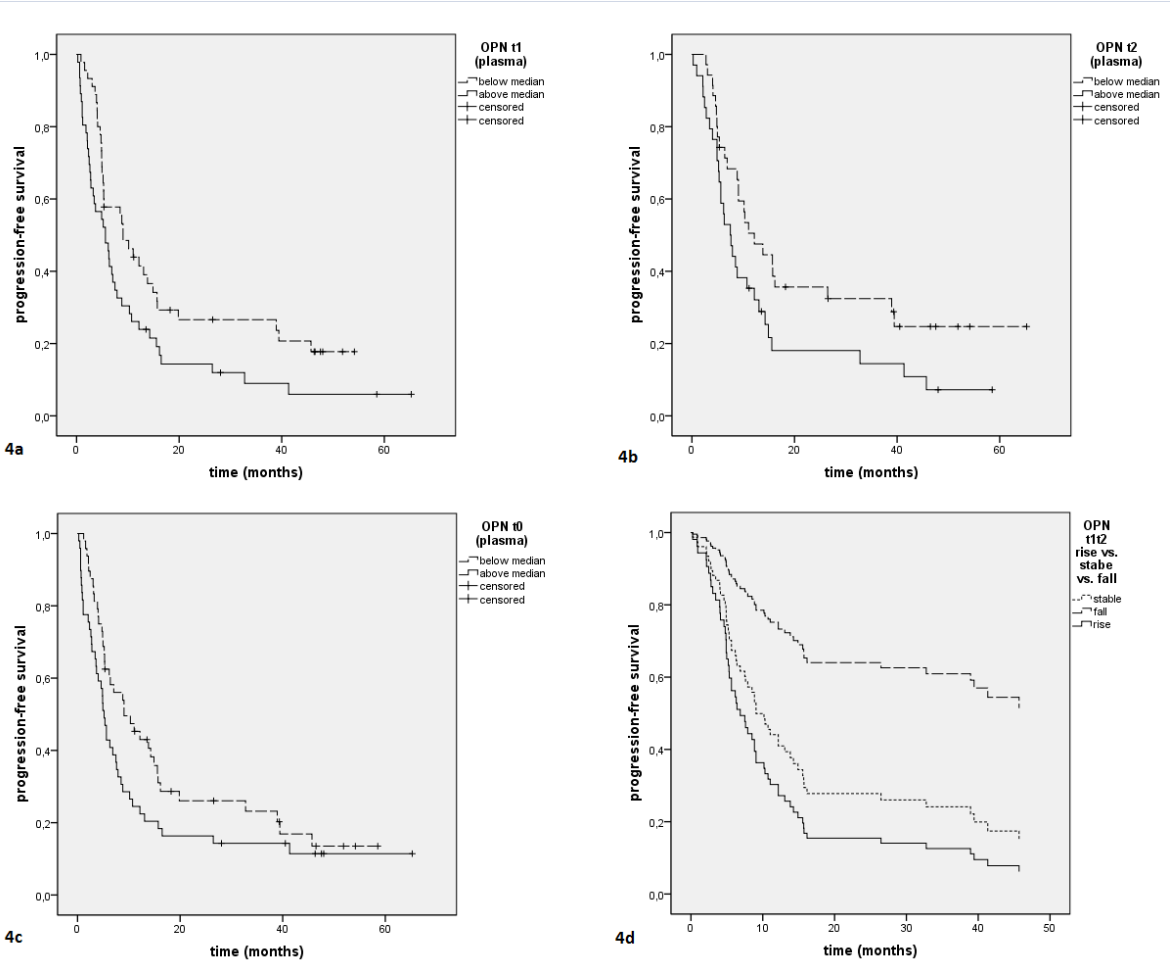

Figure 4 **Association of plasma marker levels with progression-free survival (PFS) in the entire patient collective (n=97).**
4a end-of-treatment OPN (t1) plasma levels and progression-free survival in Kaplan Meier analysis
4b post-treament OPN (t2) plasma levels and progression-free survival in Kaplan Meier analysis
4c pre-treament OPN (t0) plasma levels and progression-free survival in Kaplan Meier analysis
4d increasing vs. stable vs. decreasing post-treament OPN (t1 to t2) plasma levels and progression-free survival in Cox proportional hazards model

Additionally, patients whose relative increase in OPN t1t2 plasma levels was higher than the median increase of all patients had a significantly reduced PFS compared to patients whose plasma level increase was lower than the median increase (6.3 months, n=21 vs. 15.7 months, n=26, p=.04). The relative risk for disease progression was also significantly elevated on the former patient group (rr=1.9, 95%-CI [1.02-3.6], p=.04).

4.3.4 Time to progression (TTP)

Median TTP in the entire patient collective was 7 (0 – 17) months and in the curative-intent patient cohort it was 9 (2 – 17) months.

TTP was not associated with absolute baseline plasma levels of OPN (t0, p=.2), VEGF (p=.2) and CAIX (p=.4) or with end-of-treatment (t1) and post-treatment (t2) OPN plasma levels (p= .6 and .7).

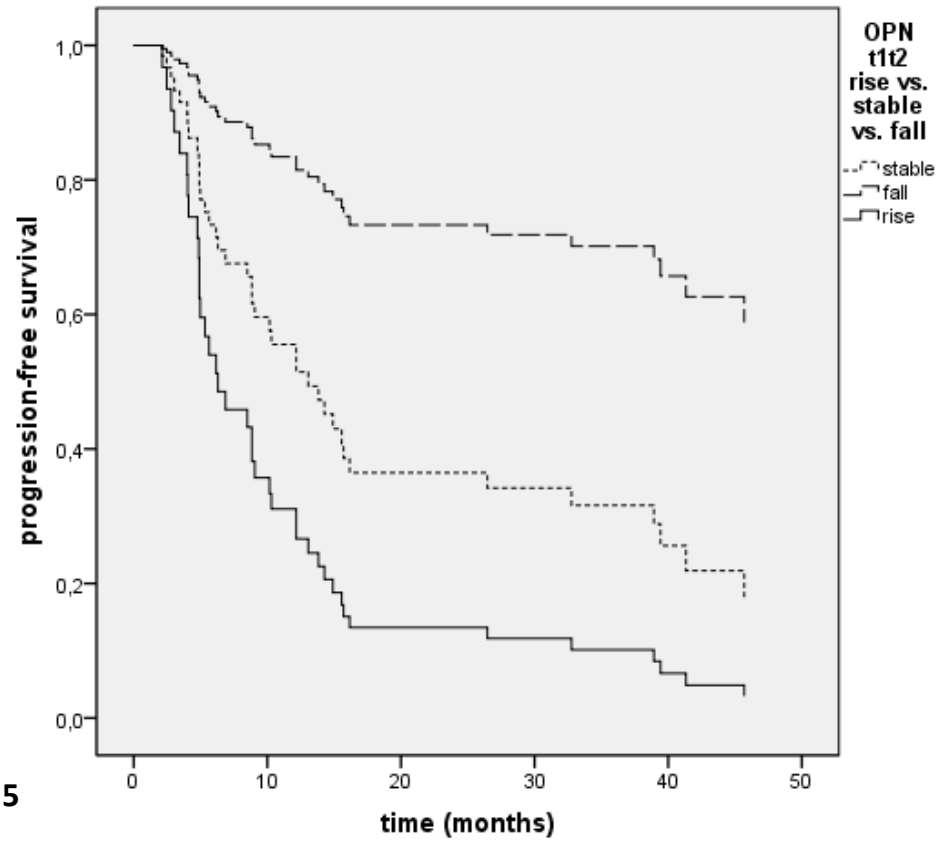

Figure 5 **Association of plasma marker levels with progression-free survival (PFS) in the curative-intent (NSCLC M0) cohort (n=61).**
Increasing vs. stable vs. decreasing post-treament OPN (t1 to t2) plasma levels and progression-free survival in Cox proportional hazards model

Relative OPN plasma level changes during (t0 to t1) and after radiotherapy (t1 to t2) were not related to TTP (p=.2 and .5) even though patients with increasing OPN plasma levels during and after treatment had an overall reduced TTP and an increased risk for progression compared patients with decreasing or stable OPN plasma levels in the same timeframe.

In the curative-intent patient cohort, median post-treatment OPN (t2), pre-treatment OPN (t0), CAIX and VEGF plasma levels were not linked to TTP (p=.7, .4 and .5) but patients with elevated end-of-treatment (t1) OPN plasma levels had a significantly reduced TTP compared to patients with low OPN t2 plasma levels (8.8 months, n=12 vs. 5.3 months, n=12, p=.04). Also, patients with increasing OPN plasma levels during therapy (t0 to t1, n=9) had an inferior TTP compared to patients with stable (n=4) or decreasing (n=12)

plasma levels (5.3 vs. 8.8 vs. 14.9 months, p=.01). No correlation of relative OPN plasma level changes after radiotherapy (t1 to t2) and TTP was determined (p=.4).

4.3.5 Metastasis-free survival (MFS)

Median MFS in the entire patient collective was 12 (0 – 66) months and in 24 patients (24%), distant metastasis occurred during follow-up. In NSCLC M0 patients, MFS was 16 (3 – 66) months (12 patients developed distant metastasis during follow-up, 20%).

Table 5 shows the association of absolute OPN plasma levels and their changes with MFS. In all patients, VEGF and CAIX plasma levels as well as OPN t0t1 plasma level changes were not related to MFS and in curative-intent NSCLC (M0) patients, MFS was not associated with baseline OPN, CAIX and VEGF plasma levels.

4.3.6 Freedom from local relapse (FFLR)

In the entire patient cohort, median FFLR was 9 (1 – 66) months and 36 patients (37%) were diagnosed with local relapse during follow-up. In the curative-intent NSCLC M0 cohort median FFLR was 12 (2 – 66) months and 22 patients (36%) had local recurrence during follow-up. The association of absolute baseline biomarker plasma levels and relative OPN plasma level changes with FFLR is presented in **table 6**.

In the entire patient cohort, absolute plasma biomarker levels of OPN, CAIX and VEGF were not associated with FFLR (OPN t0, t1, t2: p=.2, .3, .3; CAIX: p=.7; VEGF: p=.5).

Relative OPN plasma level changes during radiotherapy (t0 to t1) were not related to FFLR (p=.9).

In the curative-intent patient cohort (NSCLC M0), no correlation between FFLR and absolute plasma concentration of OPN, CAIX and VEGF was found (OPN t0: p=.6, t1: p=.7, t2: p=.5, CAIX: p=.4, VEGF: p=.3). Relative OPN plasma level changes during radiotherapy (t0t1) were not associated with FFLR (p=.5).

4.4 Univariate analysis of the association of clinical patient characteristics with prognosis in the entire and curative-intent NSCLC M0 patient cohort

Clinical patient and tumor characteristics have been tested for their association with prognosis in both the entire and the curative-intent NSCLC M0 patient cohort with restriction to OS and PFS as the primary endpoints in univariate analysis.

In the entire collective, weight loss (p=.02), T-stage (p=.003), M-stage (p<.0001), UICC-stage (p<.0001) and GTV (p<.0001) but not N-stage (p=.16), histology (p=.8) or grade

(p=.21) were associated with OS in univariate analysis. A trend for reduced OS in patients with N+ stage compared to N0 stage was noted (p=.05). Weight loss (p=.02), T-stage (p=.02), N-stage (p=.04) and GTV (p=.001) were significantly associated with PFS in the entire patient collective and a trend for reduced PFS in patients with hemoglobin levels below the median (compared to above the median) was noted (p=.05).

Table 5. Association of median OPN plasma levels and their changes with metastasis-free survival (MFS) in the entire (left side) and the curatvive-intent patient cohort (right side)

	MFS	p [1]	rr [2]	95%-CI	p [3]		MFS	p [1]	rr [2]	95%-CI	p [3]
OPN t0		***.04***				**OPN t0t1**		*.06*			
<median	16.2 (n=20)					increase	12.2 (n=8)				
≥median	5.6 (n=22)		*2.1*	*.98-4.3*	*.05*	decrease	39.4 (n=17)		*.36*	*.12-1.1*	*.07*
OPN t1		***.02***				**OPN t1t2**		***.04***			
<median	15.7 (n=20)					increase	5.3 (n=7)		*3.5*	*1.2-10.1*	***.02***
≥median	6.4 (n=20)		*2.3*	*1.1-4.7*	***.02***	decrease	39.4 (n=15)				
OPN t2		*.06*				**OPN t1t2**		*.05*			
<median	16.2 (n=19)					increase	12.2 (n=3)		*2.9*	*.7-11.2*	*.09*
≥median	7.9 (n=16)		*2.1*	*.9-4.8*	*.07*	stable	16 (n=4)				
						decrease	23.7 (n=17)				
OPN t1t2		*.05*									
increase	15.7 (n=12)		*2.2*	*.97-5.1*	*.05*						
decrease	7.5 (n=21)										
OPN t1t2		***.04***									
increase	11 (n=7)		*2.4*	*.9-6.4*	*.06*						
stable	12.2 (n=6)										
decrease	20.1 (n=22)										

[1] p-value referring to differences in MFS according to high vs. low OPN plasma levels [2] relative risk [3] p-value referring to rr

Table 6. Association of median OPN plasma levels and their changes with freedom from local relapse (FFLR) in the entire (left side) and the curative-intent patient cohort (right side)

	FFLR	p [1]	rr [2]	95%-CI [3]	p [4]		FFLR	p [1]	rr [2]	95%-CI [3]	p [4]
OPN t1t2		***.02***				**OPN t1t2**		***.01***			
increase	8.8 (n=16)		*2.4*	*1.1-5*	***.03***	increase	9.8 (n=13)		*3*	*1.2-7.3*	***.01***
decrease	13.8 (n=26)					decrease	16.2(n=21)				
OPN t1t2		***.004***				**OPN t1t2**		***.002***			
increase	6.2 (n=15)		*2.6*	*1.2-5.6*	***.01***	increase	6.2 (n=11)		*3.7*	*1.5-9.5*	***.007***
stable	10.7 (n=4)					stable	16.2 (n=4)				
decrease	16.7 (n=27)					decrease	25.7 (n=21)				

[1] p-value referring to differences in FFLR according to OPN plasma levels [2] relative risk [3] 95-confidence interval

[4] p-value referring to relative risk

In the curative-intent NSCLC M0 patient group, T-stage (p=.003), grade (p=.02), UICC stage (p=.02) and GTV (p=<.0001) but not weight loss (p=.25), N-stage (p=.66) or nodal involvement (i.e. N0 vs. N+, p=.93) significantly predicted OS in the univariate analysis.

For the association of clinicopathological parameters with PFS in this patient group, significant results could be determined for GTV (p=.002), UICC stage (p=.04) and T-stage (p=.03) while a trend was noted for inferior PFS with poorer tumor differentiation (p=.06).

4.5 Multivariate and combined analysis of plasma biomarker levels and their changes in the curative-intent NSCLC M0 patient cohort

Relative OPN plasma level changes and absolute OPN, CAIX and VEGF plasma levels have been evaluated for their association with prognosis (overall-, OS, and progression-free survival, PFS) in multivariate analysis including known prognostic and clinical factors.
Additionally, biomarker combination of baseline (i.e. pre-treatment) OPN, CAIX and VEGF plasma levels was assessed for their prognostic quality in multivariate analysis. Since the informative value of prognostic models is most useful in patients treated with curative-intent, the aforementioned analyses have been restricted to the NSCLC M0 patient cohort. Both palliative-intent and SCLC patients (in order to ensure adequate homogeneity of the studied patient cohort) have been excluded from uni- and multivariate analysis.

4.5.1 Absolute plasma OPN levels in a multivariate prognostic model

A prognostic baseline model for OS was created which consisted of the factors anemia (yes vs. no), gender (male vs. female), forced expiratory volume (FeV1, above vs. below median), tumor grading (G1 vs. G2 vs. G3 vs. G4), age (above vs. below median), OPN t0 plasma levels (above vs. below median), weight loss (yes vs. no), T-stage (T1 vs. T2 vs. T3 vs. T4) and N-stage (N0 vs. N1 vs. N2 vs. N3).
Then, a stepwise backward logistic regression was used to determine the parameters which most significantly predicted OS. The final model (p<.0001, **table 7**) included anemia (p=.02), gender (p=.09), weight loss (p=.006), grade (p=.004), age (p=.03), T-stage (p<.0001) and OPN t0 (p=.02). N-stage (p=.5) and FeV1 (p=.8) were not significantly associated with OS and thus have been removed from the prognostic model.
When T-stage was replaced by gross-tumor-volume (GTV, above vs. below median) and N-stage was replaced by nodal involvement (N0 vs. N+) in the same baseline model, the final model (p<.0001) consisted of grade (p=.04) and GTV (p<.0001) only while all other parameters including OPN t0 (p=.5) were removed from the initial model.
When baseline OPN (t0) plasma levels were replaced by end-of-treatment OPN (t1) plasma levels in the same initial model, they did not prove to be independently associated

with OS in multivariate analysis (p=.3). Only weight loss (p=.04), grade (p=.005) and T-stage (p<.0001) remained independent predictors for OS in the final model (p<.0001).

I finally tested post-treatment OPN (t2) plasma levels in the same baseline prognostic model and found it was significantly associated with OS. The final model (p=.001) consisted of OPN t2 (rr=2.6 for patients with plasma levels above the median, 95%-CI [1.2-5.6], p=.01) and T-stage (rr=1.5 for T4-stage patients, 95%-CI [.5-4.7], p=.003).

For PFS, the same multivariate prognostic models have been calculated and evaluated.

In the baseline model consisting of anemia, FeV1, age, gender, tumor grading, T-stage, N-stage, weight loss and pre-treatment OPN plasma levels (t0), only age (p=.08), grading (p=.02), T-stage (p=.01) and weight loss (p=.06) remained in the final model (p=.009) after a stepwise logistic regression. OPN t0 plasma levels remained insignificant (p=.3).

When T-stage was replaced by GTV in the same model, the final model (p=.007) consisted of GTV only which significantly predicted PFS (p=.009; rr=2.2, 95%-CI [1.2-4.1]).

When OPN t0 plasma levels were replaced by end-of treatment OPN plasma levels (t1) in the same baseline model, only weight loss (p=.04), grading (p=.005), T-stage (p<.0001) and N-stage (p=.007) significantly predicted PFS in the final model (p<.0001) while OPN t1 plasma levels were not significant (p=.4). Substituting T-stage by GTV resulted in a change of the final model (p<.0001) which then contained grading (p=.03) and GTV (p<.0001) only. When absolute OPN plasma levels four weeks after treatment (t2) were evaluated in the same baseline prognostic model for PFS, the final model (p=.001) consisted of T-stage (p=.003; rr=1.2, 95%-CI [1.1-4.2] for T2-3 vs. T1; rr=2.6, 95%-CI [1.2-5.7] for T4 vs. T1) and OPN t2 plasma levels (p=.02; rr=2.6, 95%-CI [1.2-5.7]).

When T-stage was replaced by GTV in the same model, only the latter parameter remained significant (p=.003; rr=2.9, 95%-CI [1.5-5.8]) in the final model (p=.002) while OPN dropped out of the model due to low significance (p=.9).

Table 7. Multivariate Cox regression model for overall survival in curative-intent NSCLC M0 patients

Variable	Compared groups	Subject group[2]	Hazard ratio[3]	95%-CI	*p*
anemia	*yes vs. no*	yes	2.8	1.2-6.6	*.02*
sex	*male vs. female*	female	.4	.1-1.1	*.09*
weight loss	*yes vs. no*	yes	3.6	1.5-9	*.006*
grade	*1 vs. 2 vs. 3 vs. 4*	1	.04	.006-.3	*.004*
age	*above vs. below median*	above median	2.3	1.1-4.7	*.03*
T-stage	*T1 vs. T2 vs. T3 vs. T4*	T4	6.3	1.6-25.8	*<.0001*
OPN t0[1]	*above vs. below median*	above median	2.5	1.6-12.3	*.02*

[1] osteopontin before treatment [2]discriminated or favored subgroup [3]>1 reflecting an increased risk of death, <1 reflecting a reduced risk of death

4.5.2 Relative OPN plasma level changes in a multivariate prognostic model

For the evaluation of the prognostic quality of relative OPN plasma level changes over time, the latter have been integrated in the same prognostic baseline model, consisting of gender, anemia, FeV1, grading, age, T-stage, N-stage and in addition, OPN t0t1 plasma level changes (increase vs. decrease).

After a stepwise regression, only grading (p=.002) and T-stage (p<.0001) but not OPN level changes (t0 to t1 time point, p=.8), FeV1 (p=.9), age (p=.5), N-stage (p=.3), anemia (p=.6) or gender (p=.8) were significantly associated with OS in the final model (p<.0001).

When T-stage was replaced by gross tumor volume (GTV), the final model (p<.0001) contained grading (p=.03) and GTV (p<.0001) while the other clinical factors including OPN t0t1 were removed.

Different prognostic models have been further calculated such as a model initially including OPN t0t1, gender, weight loss, grading, T-stage and N-stage. However, intratherapeutic OPN t0t1 plasma level changes did not prove as an independent predictor for OS besides weight loss (p=.08), grading (p=.002) and T-stage (p=.001) in any model.

When intratherapeutic OPN plasma level changes (to to t1 time point) were grouped in decreasing vs. stable vs. increasing plasma levels during radiotherapy and included into the same prognostic model with gender, anemia, FeV1, grading, age, T- and N-stage, only grade and T-stage (p<.0001) remained significant predictors for OS in the final model (p<.0001) while relative OPN t0t1 plasma level changes remained insignificant (p=.3).

When weight loss was added and T-stage replaced by GTV in the same model with OPN t0t1 (increase vs. stable vs. decrease), anemia, FeV1, age, gender and grade, the final model which significantly predicted OS (p<.0001) consisted of GTV (p<.0001), N-stage (p=.004), gender (p=.02) and OPN t0t1 plasma level changes (p=.05).

The same prognostic model was used to evaluate the predictive quality of post-treatment OPN plasma level changes (t1 to t2, increase vs. decrease). The initial model included the latter in addition to anemia, FeV1, age, gender, weight loss, T-stage, grading and N-stage. The final model (p=.002) consisted of T-stage which remained the only independent predictor for OS (p=.004) while all other parameters, including OPN (p=.1), were not significantly associated with prognosis. When T-stage was replaced by GTV in the same model, only the latter significantly predicted OS in the final model (p<.0001).

When post-treatment OPN plasma level changes, grouped into increasing vs. stable vs. decreasing OPN levels from t1 to t2 time point, were analyzed in the same prognostic model together with anemia, FeV1, age, gender, weight loss, grading, T-stage and N-

stage, the final model (p<.0001) consisted of T-stage (p<.0001), gender (p=.06), anemia (p=.03) and post-treatment OPN t1t2 plasma levels (p<.0001) which significantly predicted OS (rr=6.02, 95%-CI [2.3-15.6], **table 8**. When T-stage was replaced by GTV in the same model, the final model which significantly predicted OS (p=.002) consisted of GTV (p=.001) and OPN t1t2 plasma levels (rr=2.1, 95%-CI [1.1-4.3], p=.06).

PFS was evaluated, using the same baseline prognostic model consisting of anemia, FeV1, age, gender, weight loss, tumor grade, T-stage, N-stage and relative OPN t0t1 plasma level changes (i.e. increase vs. decrease). The final model (p=.008) contained grade (p=.02) and T-stage (p=.02) which significantly predicted PFS while relative OPN t0t1 plasma level changes remained without significance (p=.09).

When T-stage was replaced by GTV in the same model, only grade (p=.06) and GTV (p=.001) remained in the final model (p=.002).

Table 8. Multivariate Cox regression model for overall survival in curative-intent NSCLC M0 patients (n=61)

Variable	Compared groups	Subject group[1]	Hazard ratio[2]	95%-CI	*p*
OPN t1t2[3]	*increase vs.*	stable	.9	.2-4.4	*<.0001*
	stable vs.	increase	6.02	2.3-15.6	
	decrease				
T-stage	*T1 vs. T2 vs.*	T3	1.5	.4-5.2	*<.0001*
	T3 vs. T4	T4	1.8	.6-5.6	
gender	*male vs. female*	female	.4	.13-1.1	*.065*
anemia	*yes vs. no*	yes	2.6	1.1-6.2	*.034*

[1] discriminated or favored subgroup [2] reflected in increased (>1) or reduced (<1) risk of death

[3] osteopontin plasma level changes (increase, +10%; decrease, -10%; stable, between +10% rise and -10% fall) from the end (t1) to four weeks after treatment (t2)

Intratherapeutic OPN plasma level changes were further grouped in increasing vs. stable vs. decreasing t0t1 plasma levels and were included in the same baseline model.

The final model only consisted of T-stage (p=.02) and grade (p=.004) which significantly predicted PFS (p=.008) while OPN t0t1 plasma levels did not reach statistical significance (p=.24). When T-stage was replaced by GTV in the same model, only the latter parameter (p=.001) and grade (p=.06) remained in the final model (p=.002) which significantly predicted PFS (OPN t0t1, p=.3).

I then included post-treatment OPN plasma level changes (t1t2 increase vs. decrease) in the same model. The final model (p=.02) consisted of T-stage (p=.03) and OPN t1t2 plasma levels (p=.07) which predicted PFS. Patients with increasing OPN plasma levels after radiotherapy had an increased risk to die by a factor 1.9 (95-% CI [.9-4.1] and the

relative risk to die was 1.4 (95%-CI [1.1-4.8] in T3/T4-stage patients compared to T1/T2-stage patients. When GTV was included in the same model instead of T-stage, only GTV remained in the final model (p=.003) as an independent significant predictor for PFS (rr=2.7; 95%-CI [1.4-5.5], p=.004).

OPN t1t2 plasma levels then were further grouped in increasing vs. stable vs. decreasing plasma levels after treatment and were included in the same baseline model.

I found that in the final model which significantly predicted PFS (p=.001), both T-stage (p=.006) and OPN t1t2 (p=.001) were independent predictors for PFS, **table 9**.

Replacing T-stage by GTV resulted in a change in the final prognostic model (p=.001) which then consisted of OPN t1t2 plasma levels (rr=3; 95%-CI [1.5-6.3], p=.004) and GTV (rr=3; 95%-CI [1.5-6.3], p=.003).

Table 9. Multivariate Cox regression model for progression-free survival (PFS) in curative-intent NSCLC M0 patients (n=61)

Variable	Compared groups	Subject group[1]	Hazard ratio[2]	95%-CI	*p*
OPN t1t2[3]	*increase vs. stable vs. decrease*	stable increase	.5 4.1	.1-2.4 1.8-8.9	*.001*
T-stage	*T1 vs. T2 vs. T3 vs. T4*	T3 T4	1.2 1.4	.3-4.4 .4-4.6	*.006*

[1] discriminated or favored subgroup [2] reflected in increased (>1) or reduced (<1) risk of death [3] osteopontin plasma level changes (increase, +10%; decrease, -10%; stable, between +10% rise and -10% fall) from the end (t1) to four weeks after treatment (t2)

4.5.3 Absolute baseline CAIX and VEGF plasma levels in a multivariate prognostic model

Baseline, i.e. pre-radiotherapy, plasma levels of VEGF and CAIX have also been assessed for their prognostic impact on OS and PFS in a multivariate analysis using the same prognostic model as described above.

When pre-treatment VEGF plasma levels were evaluated together with other potential prognostic factors for OS including age, gender, anemia, weight loss, T-stage, N-stage, grade and FeV1, the final model which significantly predicted OS (p<.0001) contained T-stage (p<.0001), grade (p=.002), weight loss (p=.007), age (p=.08) and VEGF (p=.07). When T-stage was replaced by GTV in the same model, the final model (p<.0001) then

consisted of gender (p=.03), weight loss (p=.07), GTV (p<.0001) and VEGF (p=.004) which remained independent predictors for OS, **table 10**.

VEGF was also evaluated for its impact on PFS in a multivariate analysis using the same prognostic model. After a stepwise backwards logistic regression, only T-stage (p=.01), grade (p=.01), weight loss (p=.04) and age (p=.1) remained in the final model which significantly predicted PFS (p=.006). When T-stage was replaced by GTV in the same model, the final model (p=.003) now included VEGF (p=.009; rr=.4, 95%-CI [.2-.8] for VEGF levels below the median), gender (p=.05; rr=.4, 95%-CI [.2-1] for female gender) and GTV (p=.001; rr=3.4, 95%-CI [1.7-7.1] for GTV above the median).

I then evaluated baseline CAIX plasma levels using the same prognostic model and found that it was not significant (p=.71) while age (p=.06), weight loss (p=.03), grade (p=.001) and T-stage (p=.001) significantly predicted OS in the final model (p<.0001).

Table 10. Multivariate Cox regression model for overall survival in curative-intent NSCLC M0 patients (n=61)

Variable	Compared groups	Subject group[1]	Hazard ratio[2]	95%-CI	*p*
VEGF	*above vs. below median*	below median	.4	.2-.7	*.004*
GTV[3]	*above vs. below median*	above median	4.9	2.3-10.4	*<.0001*
gender	*male vs. female*	female	.4	.2-.9	*.03*
weight loss	*yes vs. no*	yes	2	.9-4.2	*.07*

[1] discriminated or favored subgroup [2] reflected in increased (>1) or reduced (<1) risk of death
[3] gross tumor volume

Exchanging T-stage by GTV changed the final model (p=<.0001) which then only consisted of grade (p=.04) and GTV (p=.001).

For PFS, similar results were found: the baseline prognostic model which initially included CAIX plasma levels, age, gender, weight loss, anemia, T-stage, N-stage, grade and FeV1 consisted of age (p=.08), weight loss (p=.06), grade (p=.02) and T-stage (p=.01) after the logistic regression (p=.009) while CAIX remained insignificant (p=.6).

Replacing T-stage by GTV resulted in a final model for PFS (p=.007) which only contained GTV as an independent predictor for PFS (p=.009; rr=2.2, 95%-CI [1.2-4.1] for GTV higher than the median).

4.5.4 Combination of OPN, CAIX and VEGF and their impact on OS and PFS

Combined biomarker baseline (pre-therapeutic) plasma levels were evaluated for OS and PFS, i.e. the double-biomarker pairs OPN-VEGF, OPN-CAIX and VEGF-CAIX and the triple combination OPN-VEGF-CAIX. Each biomarker pair was analyzed according to the plasma concentration based on the median, yielding 4 subgroups for double-biomarker pairs and 6 subgroups for the triple marker combination (i.e. plasma levels of all markers above the median vs. plasma levels all biomarkers below the median vs. intermediate groups with one plasma marker below or above the median and vice versa).

For the pairs OPN-VEGF (p=.62) and VEGF-CAIX (p=.98), no significant differences in OS were found in subgroups. OS however, significantly differed between subgroups for OPN-CAIX: Median OS was 29.6 (16.7-42.5) month in the group with plasma levels of both biomarkers below the median, it was 26 (20.1-32) months in the group OPN high/CAIX low, 15.7 (7.9-23.5) months in the group OPN low/CAIX high and 6.7 (0-12.7) months in the group with plasma levels of both markers above the median (p=.03). Accordingly, the relative risk to die was significantly different, **figure 6a**. Compared to patients with plasma levels of both markers below the median, patients whose plasma levels were higher than the median had a significantly increased risk of death (rr=1.3, 95%-CI [1.1-2.6], p=.04).

The triple biomarker combination OPN-CAIX-VEGF showed a significantly different OS of subgroups (p=.02), **figure 6b**: Median OS was 41.3 (9.9-72.1) months in patients with low plasma levels of all three markers compared to 5.3 (4.5-6.1) months in patients with high plasma levels of all three biomarkers.

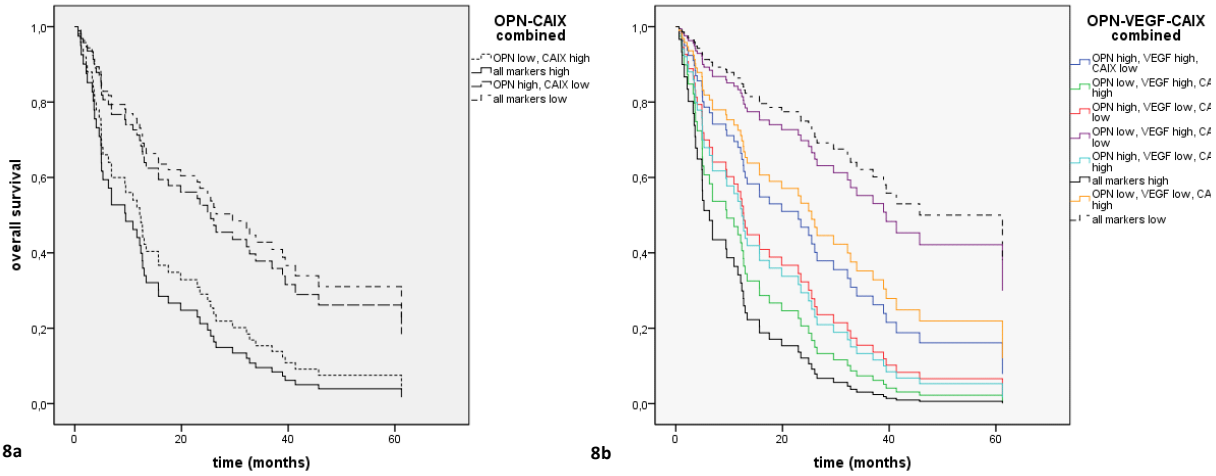

Figure 6 **Association of combined plasma marker levels with OS in n=61 curative-intent NSCLC M0 patients in Cox regression analysis.**
6a Absolute pre-therapeutic plasma levels of the double biomarker combination OPN and CAIX (*high* refers to plasma levels above the median, *low* refers to plasma levels below the
6b Absolute pre-therapeutic plasma levels of the triple biomarker combination OPN, VEGF and CAIX (*high* refers to plasma levels above the median, *low* refers to plasma levels belo

The latter patient group also had a significantly increased risk to die compared to the index group of patients with low levels of all markers (rr=2.7, 95%-CI [1.1-7.6], p=.04), **table 11**. Median PFS was not significantly different between subgroups for the double biomarker combination OPN-VEGF (p=.91) and VEGF-CAIX (p=.8). However, a trend (p=.07) for superior PFS in patients with low plasma levels of OPN-CAIX was noted: Median PFS was 14.3 (9.7-18.8) month in patients with low plasma levels of both OPN and CAIX, it was 10.2 (3-17.4) month in patients with high OPN/low CAIX plasma concentration, 5.6 (3.8-7.4) months in patients with high CAIX/low OPN plasma levels and 5.2 (2.7-7.8) months in patients with high plasma levels of both OPN and CAIX. Accordingly, the relative risk of death was increased in patients with high levels of both CAIX and OPN compared to patients with low plasma levels of both markers (rr=1.1, 95%-CI [.5-2.1], p=.09).

Table 11. Overall survival (Kaplan-Meier analysis) and hazard ratio (Cox proportional hazards model) in curative-intent NSCLC M0 patients (n=61) according to plasma levels of the triple biomarker combination

Plasma level	**Median OS[1] (range)**	**Hazard ratio[2]**	**95%-CI[3]**	***p*[4]**
all markers low[5]	41.3 (9.9-72.1)	n/a[7]	n/a	*n/a*
OPN high[6], VEGF low, CAIX low	17.5 (5.8-27.6)	1.5	.47-4.7	*.5*
OPN high, VEGF high, CAIX low	22.9 (.9-45.1)	.83	.32-2.2	*.71*
OPN high, VEGF low, CAIX high	16 (6.7-24.6)	1.6	.52-5	*.41*
OPN low, VEGF high, CAIX low	36.9 (0-110.4)	.4	.08-1.8	*.22*
OPN low, VEGF low, CAIX high	25.5 (4.5-46.4)	.47	.14-1.6	*.23*
OPN low, VEGF high, CAIX high	14.3 (4.1-24.4)	2.1	.74-5.8	*.17*
all markers high	5.3 (4.5-6.1)	2.7	1.1-7.6	***.04***

[1] overall survival in months [2] reflecting the risk to die in comparison to the subgroup with low levels of all three markers as the comparison group (values >1 increased risk, <1 reduced risk) [3] confidence interval [4] p-value corresponds to hazard ratio which is referring to the comparison group with low levels of all three markers [5] below the median [6] pre-treatment (OPN t0), above the median [7] not applicable

The triple biomarker combination OPN-VEGF-CAIX non-significantly impacted PFS which was highest in patients with low plasma concentration of all three biomarkers (41.3 [0-108] months) and lowest in patients with high plasma levels of OPN-VEGF-CAIX (4.9 [2.2-7.5] months, p=.11). The latter patients also had an elevated risk to die (rr=1.6, 95%-CI [.6-4.2], p=.15) compared to patients with low plasma levels of all three biomarkers.

4.5.5 Combined analysis of baseline OPN, CAIX and VEGF plasma levels in a multivariate prognostic model

Pre-treatment (baseline) plasma levels of OPN, VEGF and CAIX have been evaluated in combination for their impact on OS and PFS in multivariate analysis.

At first, I included absolute baseline plasma levels of pre-therapeutic OPN (t0), CAIX and VEGF in the same prognostic model for OS as described above (i.e. containing age, gender, weight loss, FeV1, tumor grade, N-stage, T-stage and anemia).

After stepwise logistic regression, T-stage (p<.0001), grade (p=.002), weight loss (p=.002), age (p=.08), gender (p=.08), anemia (p=.09), OPN (p=.02) and VEGF (p=.04) were independent predictors for OS in the final model (p<.0001), **table 12**.

With T-stage replaced by GTV in the same baseline model, the final model which significantly predicted OS (p<.0001), included VEGF (p=.004), gender (p=.03), weight loss (p=.07) and GTV (p<.0001).

For the evaluation of PFS, the initial model included the same clinicopathological parameters and in addition, OPN t0, CAIX and VEGF plasma levels as described above.

After a stepwise logistic regression, only age (p=.09), weight loss (p=.05), grade (p=.03) and T-stage (p=.008) remained in the final model which significantly predicted PFS (p=.006) while VEGF (p=.2), CAIX (p=.7) and OPN (p=.2) were not significant.

When T-stage was replaced by GTV in the same baseline model, the final model (p=.003) consisted of VEGF (rr=.4, 95%-CI [.2-.8] for plasma levels below the median, p=.009), gender (rr=.4, 95%-CI [.2-1.1] for female patients, p=.05) and GTV (rr=3.5, 95%-CI [1.7-7.1] for GTV above the median, p=.001). OPN and CAIX remained insignificant (p=.3 and .6).

Since only the triple marker combination OPN-VEGF-CAIX and the double marker combination OPN-CAIX but not OPN-VEGF and VEGF-CAIX significantly impacted OS (trend for PFS) in the univariate analysis (4.5.4), only the combination OPN-CAIX and the triple combination have been evaluated in multivariate analysis for OS and PFS using the same prognostic model described above (containing anemia, FeV1, age, gender, T-stage, weight loss, tumor grade and N-stage).

Table 12. Multivariate Cox regression model for overall survival in curative-intent NSCLC M0 patients (n=61)

Variable	Compared groups	Subject group[1]	Hazard ratio[2]	95%-CI[3]	*p*
anemia	*yes vs. no*	yes	2	.9-4.3	*.09*
age	*above vs. below median*	above median	1.9	.9-3.9	*.08*
Grade	*G1-2 vs. G3-4*	G1-2	.05	.007-.3	*.002*
T-stage	*T1-2 vs.* T3-4	T3-4	3.7	1.1-14.4	*<.0001*
VEGF	*above vs. below median*	below median	.4	.2-.9	*.04*
OPN t0	*above vs. below median*	below median	.07	.5-.2	*.02*
gender	*male vs. female*	male	2.5	.9-7.4	*.08*
weight loss	*yes vs. no*	yes	4.8	01.08.2013	*.002*

[1] discriminated or favored subgroup [2] reflected in increased (>1) or reduced (<1) risk of death
[3] confidence interval

After a stepwise backward logistic regression, the final model which significantly predicted OS (p<.0001) consisted of anemia (p=.1), T-stage (p=.001) and OPN-CAIX (p=.002) which were all independent predictors for OS. When T-stage was replaced by GTV in the same model, the final model (p<.0001) contained anemia (p=.11), OPN-CAIX (p=.03) and GTV (p=.001). Replacing N-stage (i.e. N0, N1, N2, N3) by nodal status (N0 vs. N+) resulted in a change in the final model (p<.0001), now consisting of anemia (p=.08), OPN-CAIX (p=.03), GTV (p<.0001) and nodal status (p=.08), **table 13**, **figure 7**.

Table 13. Multivariate Cox regression model for overall survival in curative-intent NSCLC M0 patients (n=61)

Variable	Compared groups	Subject group[1]	Hazard ratio[2]	95%-CI[3]	*p*
anemia	*yes vs. no*	yes	1.9	.9-4.9	*.08*
GTV[4]	*above vs. below median*	above median	3.8	1.8-7.9	*<.0001*
nodal status	*N0 vs. N+*	N0	.4	.2-1.1	*.08*
OPN t0-CAIX	*OPN-CAIX low vs. OPN-CAIX high vs. OPN high/CAIX low vs. OPN low/CAIX high*	OPN-CAIX high	2.1	1.2-4.9	*.03*

[1] discriminated or favored subgroup [2] reflected in increased (>1) or reduced (<1) risk of death
[3] confidence interval [4] gross tumor volume

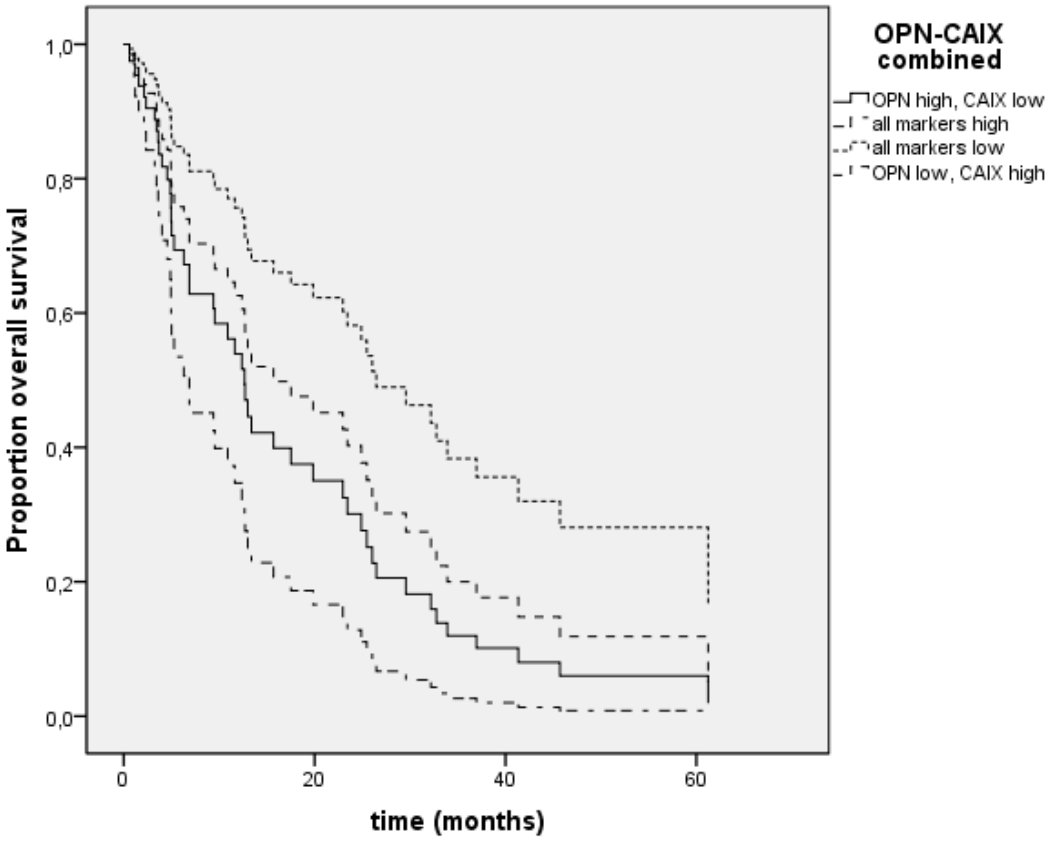

Figure 7 **Association of combined plasma marker levels OPN and CAIX with OS in n=61 curative-intent NSCLC M0 patients in multivariate Cox regression analysis.**
Absolute pre-therapeutic plasma levels of the double biomarker combination OPN and CAIX (high refers to plasma levels above the median, low refers to plasma levels below the median).

I then evaluated the prognostic impact of the triple biomarker combination OPN-VEGF-CAIX in the same multivariate model for OS.

After a stepwise backward logistic regression, the final model (p=.001) contained T-stage (p=.002), OPN-VEGF-CAIX (p=.005) and N-stage (p=.08).

Compared to patients with low plasma levels of all three markers, those with high levels of OPN-VEGF-CAIX had a significantly elevated risk to die (rr=9.1, 95%-CI [1.1-19.3]) as had patients with higher T-stage (rr=3.3, 95%-CI [1.1-15.2] for T2; rr=3.9, 95%-CI [1.3-15.7] for T3-4). When T-stage was replaced by GTV in the same baseline model, the final model which significantly predicted PFS (p<.0001) then contained N-stage (p=.001, rr=.1, 95%-CI [.03-.39] for patients with N0 compared to N+), GTV (p<.0001, r=6.3, 95%-CI [2.6-15.3] for

patients with GTV higher than the median) and OPN-VEGF-CAIX (p=.006, rr=8.8, 95%-CI [1.7-44.9] for patients with high plasma levels of all three markers).

For PFS, also the double and triple combination OPN-CAIX and OPN-VEGF-CAIX were evaluated in a multivariate analysis.

If OPN-CAIX was integrated in the baseline model including age, gender, FeV1, weight loss, T-stage, N-stage, grade and anemia, the final model (p=.02) consisted of the independent prognostic factors T-stage (p=.04) and OPN-CAIX (p=.03).

When T-stage was replaced by the GTV, the final model (p=.007) included GTV only (rr=2.2, 95%-CI [1.2-4.1], p=.009) while OPN-CAIX remained insignificant (p=.11).

When the triple plasma marker combination OPN-VEGF-CAIX was assessed using the same baseline model, the final model (p=.06) contained T-stage (p=.05) and OPN-VEGF-CAIX (p=.06). When T-stage was replaced by GTV in the same baseline model, the final model which significantly predicted PFS (p=.01) consisted of gender (p=.04), OPN-VEGF-CAIX (p=.006) and GTV (p=.001). In this model, female patients had a lower risk to die (rr=.4, 95&-CI [.2-.9]) while patients with a GTV above the median had increased risk of death (rr=3.7, 95%-CI [1.7-8.2]. Patients whose OPN-VEGF-CAIX were lower than the median had a reduced risk of death by a factor .15 (95%-CI [.04-.58]).

5. Discussion

In the following section, the results of this work are discussed and interpreted against the background of the current literature and the limitations of this work are clarified.

5.1 Influence of radiotherapy on the OPN plasma level course over time

This is the first work to evaluate the serial detection of OPN plasma levels before, during and after radical radiotherapy for lung cancer [290,291].

I found that OPN plasma levels remained mostly constant during radiotherapy in both curative-intent (M0) NSCLC patients (from 761 ng/ml, t0 to 716 ng/ml, t2) and palliative-intent (M1) NSCLC patients (from 1050 ng/ml, t0 to 1087 ng/ml, t1).

In the entire patient collective, OPN plasma levels slightly decreased during treatment whereas a considerable decline in OPN plasma levels was noted after radiotherapy (median -113 ng/ml). However, in both the entire patient collective and in subgroups the aforementioned overall OPN plasma level changes remained insignificant, which is consistent with the findings of Snitcovsky et al. who reported pre- and post-treatment OPN plasma levels in patients with head-and-neck cancer undergoing radiochemotherapy not to

be significantly different [238]. Nevertheless, my results could indicate that in non-metastasized patients, OPN plasma levels decrease during radiotherapy because the primary tumor, as the main source for (increased) OPN, shrinks in response to radiation while in metastasized patients, the failure to decrease of OPN levels may be related to their metastatic tumor load not being affected by radiation treatment [292,293]. This hypothesis is substantiated by my finding that OPN plasma levels not only further decreased after the end of radiotherapy but also that the most prominent OPN plasma level decrease could be observed after radiotherapy in NSCLC M0 patients (from 716 ng/ml, t1 to 633 ng/ml, t2). This is in accordance with the results of Blasberg et al. who reported a significant reduction in OPN plasma levels after resection of early stage NSCLC [256]. In contrast to the work of Blasberg however, in my study, patients were diagnosed with advanced-stage NSCLC and treatment was radiotherapy. Assuming that the malignant tumor is the primary source of increased OPN plasma concentration, it is conceivable that an early and significant decrease in OPN plasma levels may be observed after surgical removal of the tumor whereas with radiotherapy, tumoricidal effects are not as instant since tumor shrinkage occurs over the whole treatment course and tumor regression continues after the end of radiotherapy. This is supported by my finding that most prominent OPN level changes were noted after radiotherapy and stands in line with the results of Blasberg et al. who also observed the most obvious plasma level changes when OPN was evaluated after treatment [256].

5.2 Baseline biomarker plasma levels and their interrelation

Baseline OPN, VEGF and CAIX plasma levels in this study were 830 ng/ml, 90 pg/ml and 95 pg/ml (entire patient collective). Comparison with marker plasma levels published elsewhere is difficult due to the plasma level dependency on the ELISA system used [264]. The biomarkers investigated in this study and OPN in particular, have been shown to be expressed in numerous human tissues where they are involved in various physiological and pathological processes including infections and sepsis [294,295], lung disease [295,296], vascular disease, inflammation, autoimmune [297] and cardiovascular diseases where OPN is crucially involved artherosclerotic plaque formation [200-202,298].

Evidently, OPN has many sources and can also be elevated in benign disease as well which is why this protein is considered a multi-modal mediator [296,298] and despite its crucial role in cancer [189,299] cannot be regarded as a cancer-specific marker, limiting the informative value of crude biomarker plasma levels alone.

Nevertheless, the majority of studies demonstrated that OPN, VEGF and CAIX expression and circulating levels are considerably increased in (lung) cancer patients distinguishing them from healthy controls [133,135,208,300-304].

Despite the fact that clear cut-off values discriminating healthy individuals from cancer patients are not known so far [301], OPN expression and plasma levels have been suggested as a potential diagnostic tool in some human cancer entities [302-305].

In my work, only cancer patients were evaluated and a control group of healthy individuals for comparison of median OPN plasma levels was not used which can be regarded as a weak point. Since numerous studies demonstrated an overexpression of OPN in various human cancers, including lung cancer [206,232,300,306] and showed that OPN plasma levels are significantly elevated in cancer patients compared to healthy controls [233,307], scientific evidence is sufficient to dispense with a healthy control group in this study.

Most of the patients in this study had additional comorbidities. Therefore an effect of the latter on overall biomarker plasma levels may not be excluded. It also cannot be answered by this work whether these effects are of clinical significance or not but since most published studies show that for instance OPN plasma levels are considerably elevated in cancer patients, the impact of benign comorbidities on overall OPN plasma levels in my patient collective might be rather of fluctuating nature and supposedly negligible small.

In my study, I found a positive correlation between OPN plasma levels measured at different time points and baseline OPN, VEGF and CAIX were positively interrelated which strengthens the rationale for a co-detection of these biomarkers [308].

Phuoc et al. reported an inverse correlation between VEGF and CAIX in renal cell cancer patients [308]. Notably, I determined an inverse correlation between hemoglobin levels and both OPN ($p=.08$) and VEGF ($p=.04$) which could be indicative of a poor oxygenation status of patients [129,251,309,310]. In the context of a rather poorly oxygenated patient with a tumor featuring extensive hypoxia and neo-angiogenesis, reflected by increased overall plasma concentrations of OPN, VEGF and CAIX (HIF-1α mediated), my finding that OPN and VEGF were linearly correlated in curative-intent NSCLC M0 patients could be indicative of the cooperative role of these proteins in tumor growth [51,311-314]

5.3 Pre-therapeutic plasma biomarker levels as indictors of advanced disease and biologically aggressive tumor behavior

In this work, baseline (i.e. pre-treatment) plasma levels of OPN, VEGF and CAIX were evaluated for their association with clinicopathological patient and tumor characteristics.

OPN plasma levels were higher in male (p=.03) and in older patients (p=.03, entire patient collective and p=.09, NSCLC M0 cohort) which is in contrast to prior studies reporting no significant correlation between OPN plasma levels and age in cancer patients [239,240,256]. One study however, reported a significant age-dependent increase in OPN expression and serum levels which negatively impacted muscle regeneration in the context of inflammation [315].

In my patient collective, median OPN plasma levels were significantly higher in patients with squamous-cell carcinoma compared to adenocarcinoma (p=.01) and a trend for higher OPN plasma levels in NSCLC was noted in comparison to SCLC histology (p=.07).

A differential OPN expression in lung cancer was published before [232]: Zhang et al. reported a predominant expression of OPN in squamous-cell carcinoma (69%) and a lower expression in adenocarcinoma (21%); the lowest OPN expression rate was found in SCLC (11%) which is supportive of my results.

High OPN plasma levels were associated with low hemoglobin levels (p=.08), poor lung function (p=.002 and .01), weight loss (p=.001), high tumor grade (p=.08), large tumor volume (GTV, p=.01 and .03), higher UICC-stage (p=.001 and .003) and T-stage (p=.02).

VEGF plasma levels were also related to GTV (p=.002 and <.0001), T-stage (p=.07) and UICC-stage (p=.08) and elevated baseline CAIX plasma levels were found in patients with higher N-stage (p=.04, .06 and .004), grade (p=.09 and .06), T-stage (p=.04) and SCLC histology (p=.03) which is in accordance with the current literature reporting a significant association of increased VEGF with advanced tumor disease [301,316].

Interestingly, Fuhrmann-Benzakein et al. reported baseline VEGF plasma levels to be significantly related to tumor metastasis [316] which is contrasting my results where baseline VEGF plasma levels were not significantly different in M0- and M1-stage patients.

The aforementioned findings could in summa be indicative of a rapidly progressing, highly invasive tumor [311] with an aggressive and biologically unfavorable phenotype [317-320] which exhibits extensive hypoxia and angiogenesis and is accompanied by a significant paraneoplastic systemic inflammatory reaction which in turn drives (muscle) wasting and cachexia [321]. These observations are in accordance with current literature, confirming the association of elevated OPN plasma levels with characteristics of advanced disease in (lung) cancer patients [206,236,303,307,318,319,322,323].

In my work, it is demonstrated that median OPN plasma levels before (t0), at the end (t1) and four weeks after completion of radiotherapy (t2) are significantly higher in metastasized (M1-stage) NSCLC patients compared to those with M0-stage (p<.0001).

These findings are concordant with the current literature, reporting significantly increased (plasma/tumor) OPN in metastatic patients which is of prognostic relevance in many human cancers including lung cancer [325-328], underlining the association of OPN with the metastatic and invasive cancer phenotype [40,222,224,292,303,307,328,329].

5.4 The predictive power of the biomarkers OPN, VEGF and CAIX in the radiotherapy of NSCLC

Apart from the prognostic quality of OPN, VEGF and CAIX plasma levels, their predictive power has been evaluated in this work.

Previously, Poon et al. investigated a substantial number of studies on the prognostic and predictive effects of circulating VEGF in cancer patients. He found that apart from its association with advanced disease stage, which is in accordance with my own results (4.2 and 5.3), VEGF might be useful in predicting tumor response after cancer therapy [330].

In the curative-intent (NSCLC M0) patient collective in my study, absolute biomarker plasma levels were not associated with tumor control and therapy response after radiotherapy but OPN plasma levels at the end of radiotherapy (t1) were significantly lower in responding patients who achieved complete or partial remission after radiotherapy in the entire patient collective (p=.002): Among non-responders, significantly more patients had elevated OPN t1 plasma levels when compared to responding patients (p=.01).

No significant relation between absolute CAIX, VEGF and OPN plasma levels and TTP or FFLR in the entire or curative-intent patient cohort was found. However, NSCLC-M0 patients with high OPN plasma levels at the end of radiotherapy (t1) had a significantly shorter TTP compared to patients with low OPN t1 plasma levels (p=.04). This finding agrees with current literature, suggesting a negative influence of elevated baseline OPN on tumor recurrence, freedom-from-relapse and event-free survival in cancer patients [209,323,331].

Interestingly, not post-treatment OPN plasma level changes (t1 to t2) but intra-therapeutic plasma level changes were significantly related to therapy response in the entire patient collective (p=.04). Decreasing OPN plasma levels were noted in responding patients as opposed to increasing plasma levels in non-responders. Analog findings could be obtained in curative-intent NSCLC M0 patients, however they remained a statistical trend (p=.05). Yet, cross-table analysis revealed a significantly higher number of patients with increasing OPN plasma levels during radiotherapy (t0 to t1) in the non-responders group (p=.03).

In the entire patient cohort, no association between relative OPN plasma level changes during or after radiotherapy and TTP was observed but increasing OPN plasma levels from the end of therapy (t1) to four weeks after radiotherapy (t2) significantly predicted shorter time to local progression (FFLP, p=.02 and .004) and were associated with an increased risk of relapse after radiotherapy (rr=2.4, p=.03; rr=2.6, p=.01).

In curative-intent NSCLC M0 patients, TTP was lower in patients with increasing OPN plasma levels during but not after radiotherapy (p=.01). FFLR was almost double in patients with decreasing OPN plasma levels after radiotherapy compared to those with increasing OPN t1 to t2 plasma levels (p=.01 and p=.002). The latter patients also had a significantly elevated risk of local relapse (rr=3, p=.03 and rr=3.7, p=.007).

To the author's knowledge, this is the first study to suggest a potential predictive quality of OPN plasma levels and their changes after radiotherapy of NSCLC. My findings amend previously published data on the association of increased absolute, mostly pre-therapeutic OPN levels with reduced disease-/relapse-free survival and cancer progression [323].

In the surgical therapy of NSCLC, Takenaka et al. reported preoperative OPN plasma levels to significantly predict prognosis [332] and Blasberg et al. suggested increasing OPN plasma levels after resection of early-stage NSCLC as indicators of tumor recurrence [256]. In head-and-neck cancer, both pre- and post-treatment OPN plasma levels significantly predicted tumor response after chemoradiotherapy [237].

Hui et al. not only reported a significant association of baseline OPN plasma levels with tumor control after radiotherapy of nasopharyngeal carcinoma but also observed a more than doubled complete response rate in patients with low baseline OPN plasma levels compared to patients with high pre-treatment plasma OPN (88% vs. 40%, p=.009) [245].

However in my work, both the small patient numbers of compared subgroups and the insufficient follow-up data on tumor control which was available in 73% of curative-intent patients, needs to be considered when interpreting the results presented here.

Therapy response evaluation in my work was restricted to a single time point 4-6 weeks after radiotherapy following the rationale that radiation-induced inflammation and edema in tumor-surrounding tissue disappears and tumor regression continues after the end of radiotherapy. Evaluation of tumor control at more than one time point after radiotherapy however, is critical since patients will have received widely differing treatments after the end of radiotherapy. Nevertheless, the predictive potential of absolute OPN plasma levels and their changes remains to be investigated by larger studies incorporating more measurement time points in order to validate the hypotheses generated by this study.

5.5 The prognostic value of serial plasma OPN detection in the curative-intent radiotherapy of NSCLC

This is the first study to evaluate the prognostic impact of sequential detection of OPN plasma levels before, at the end of and four weeks after radiotherapy of lung cancer.

In the chemotherapy of NSCLC, a significant association of baseline OPN plasma levels and both OS and PFS has been reported by several studies [239,240]. Interestingly, Mack et al. observed no association between baseline VEGF plasma levels and outcome in NSCLC patients treated with chemotherapy [239] despite the clinical significance of the cooperative role of OPN and VEGF in lung cancer biology [311,312].

Also in other tumor entities such as head-and-neck cancer or malignant melanoma, elevated pre-treatment OPN plasma levels were linked to inferior prognosis, underlining the prognostic potential of serial detection of this biomarker [209,238,252,333].

Here, I found that in the entire patient collective but not in the curative-intent patient cohort, absolute OPN plasma levels before (t0), at the end of (t1) and four weeks after radiotherapy (t2) were significantly related to survival (OPN t0, t1 and t2: p=.04, .004 and .02) with elevated plasma levels being associated with an increased risk of death (t0: rr=1.6, p=.04; t1: rr=1.9, p=.005 and t2: rr=1.8, p=.03). In the curative-intent NSCLC (M0) cohort, a trend for lower OS in patients with high post-treatment OPN plasma levels (t2) was noted (p=.08). In both the entire and the curative-intent NSCLC-M0 patient cohort, absolute baseline VEGF and CAIX plasma levels were not related to survival.

Similar findings could be obtained for PFS: Both end-of-treatment (t1) OPN plasma levels and those measured four weeks after radiotherapy (t2) were significantly associated with tumor progression (p=.02 and .04) in the entire patient cohort while baseline (t0) plasma levels of OPN, VEGF and CAIX did not show a relationship with PFS as it was in the curative-intent NSCLC-M0 patient cohort. Unlike my own findings, current literature suggests a negative prognostic impact of elevated tumor expression and plasma levels of VEGF and CAIX in (lung) cancer [137,138,169,303,308].

In both the entire and curative-intent NSCLC (M0) patient cohort in my study, OPN plasma level changes during radiotherapy (t0 to t1) were not related to OS or PFS. However, a trend for reduced OS in patients with increasing post-treatment OPN plasma levels (t1 to t2) was noted in the entire (p=.07) and the curative-intent cohort (p=.07). A significant association between OPN plasma levels changes after radiotherapy (t1 to t2) and PFS was determined in both the entire (p=.03) and the curative-intent NSCLC (M0) cohort (p=.009). Patients with increasing OPN plasma levels after treatment had a reduced PFS

and an increased risk for tumor progression compared to patients with stable or falling OPN t1 to t2 plasma levels (rr=1.5, p=.05, entire patient collective; rr=1.9, p=.02).

Since in my study, OS, PFS and MFS were best in patients with decreasing OPN plasma levels after radiotherapy, intermediate in patients with stable and worst in patients with increasing post-treatment OPN plasma levels, it can be assumed that while decreasing OPN plasma levels after radiotherapy indicate a major reduction in tumor volume (good response to radiotherapy), stable post-treatment OPN plasma levels might reflect residual or a less radiation-responsive tumor. Increasing OPN plasma levels after radiotherapy might be related to a largely radio-resistant, progressive tumor and / or growth of initially present but occult micrometastasis [334], translating into poor OS, PFS and MFS.

Since OPN plasma levels have been shown to be associated with parameters of advanced disease as well as tumor burden in cancer patients and since patients with higher T-stage (p=.003 and .007), larger tumor volume (GTV) and lymphonodal spread (p=.01, .04 and .08) in my study had significantly elevated OPN plasma levels in both the entire and the curative NSCLC M0 patient cohort, it needs to be discussed to what extent OPN plasma levels are affected by tumor volume and its changes during or after radiotherapy.

Assumed that OPN merely is a surrogate of tumor burden, relative OPN plasma level changes would then rather reflect tumor volume changes during or after radiotherapy.

In this case, decreasing OPN plasma levels after treatment might simply be an expression of tumor shrinkage, translating into a superior prognosis [335,336].

Therefore, a potential “tumor volume effect” on OPN plasma levels should be investigated in future studies in order to determine the correlation between OPN plasma level- and tumor volume changes during radiotherapy. Assessment of tumor volume (i.e. GTV) and its changes by integration of serial CT- or preferably PET imaging at the time of OPN readings during and after radiotherapy could prove to be a suitable approach.

Nevertheless, multivariate analyses in my study demonstrate that baseline OPN plasma levels (t0, p=.02), end-of-treatment OPN (t1, p=.01) and relative OPN plasma level changes after radiotherapy (t1t2, p<.0001) remained significant predictors for OS independent from other prognostic factors such as GTV (p<.0001), T-stage (p<.0001) or N-stage (p=.004, 4.5.1 and 4.5.2) which, in part, reflect tumor volume. Accordingly, post-treatment OPN (t2, p=.02) and relative OPN plasma level changes after radiotherapy (t1t2, p=.007 and p=.004) were predictive for PFS beyond T-stage (p=.003 and .006) or GTV (p=.003), 4.5.2 and 4.5.1.

5.6 The prognostic role of serial OPN detection in tumor metastasis

In the entire patient collective, MFS was considerably shorter in patients with elevated OPN plasma levels before (t0, p=.04), at the end of treatment (t1, p=.02) and four weeks after radiotherapy (t2, p=.07). The risk to die from metastasis during follow-up was also significantly increased in patients with high pre-treatment (rr=2.1, p=.05) and end-of-treatment OPN plasma levels (rr=2.3, p=.02). In the curative-intent NSCLC patient cohort, absolute plasma levels of OPN, VEGF and CAIX were not associated with MFS.

The current literature mostly evaluated absolute pre-treatment OPN plasma levels.

Here, I report that increasing OPN plasma levels after radiotherapy (t1 to t2) translate into a reduced MFS compared to patients with stable or decreasing post-treatment OPN plasma levels (entire patient cohort, p=.04). Similar findings could be obtained for curative-intent NSCLC (M0) patients: Patients with increasing post-treatment OPN plasma levels had a shorter MFS and an increased risk of death (rr=3.5, p=.02) than patients with decreasing (or stable) OPN plasma levels after therapy (p=.04 and p=.05).

In summa, my findings that not only pre-treatment OPN was significantly higher in metastasized patients (5.3) but also that OPN levels after radiotherapy and particularly their increase was significantly associated with reduced MFS and the development of metastasis during follow-up, strengthens and amends current literature where OPN plasma levels have been shown to be associated with tumor metastasis in many human cancers [40,292,327,328,337]. In patients with hepatocellular carcinoma for instance, OPN overexpression was associated with early disease recurrence, occurrence of metastasis and poor survival [229]. Interestingly, the role of metastasis-promoting OPN in the context of tumor hypoxia has been demonstrated for many human cancers [338,339].

My results underline the crucial role of OPN in cancer progression and dissemination where an induction of this protein has been associated with the metastatic and invasive (lung) cancer phenotype [269,292,325,340-342]. Against this background, it needs to be discussed whether OPN plasma levels merely reflect metastatic tumor burden which is supported by the fact that in my study, elevated (pre-treatment) OPN plasma levels were significantly increased in M1-stage patients and that M0- and M1-stage patients did not significantly differ in tumor size (T stage) or nodal involvement (N stage). The prognostic effects of OPN might then rather be surrogative of metastatic tumor load [293,325]. However, I demonstrated that OPN plasma levels and particularly their changes were also of prognostic significance in non-metastasized (M0) curative-intent NSCLC patients (4.3, 4.5 and 5.5). In this patient collective, increasing OPN plasma levels after radiotherapy

significantly correlated with poor prognosis, that is MFS (besides OS and PFS). Since the majority of (lung) cancer patients die as a result of distant metastasis, monitoring of OPN plasma levels after definitive radiotherapy of NSCLC may help to identify patients with a high risk for the development of metastasis and death [326,335], prompting more rigorous systemic therapy after radiotherapy.

5.7 The use of a co-detection of the potential hypoxia-related proteins OPN, CAIX and VEGF for a plasma hypoxia score in the curative-intended radiotherapy of NSCLC

With respect to the intricacy of tumor hypoxia and the fact that none of the studied hypoxia markers can be currently considered “gold standard” in the detection of tumor hypoxia or a direct surrogate of the latter [69,160], a combination of hypoxia-related proteins could integrate different aspects of tumor hypoxia and hypothetically prove to be more robust in predicting prognosis than a single marker [65]. The association of tumor hypoxia with OPN and CAIX expression [161] and the cooperative role of OPN and VEGF in lung cancer [311,343] further support the hypothesis that OPN (together with other hypoxia-related proteins [344]), might be a potential predictor of clinically significant tumor hypoxia [249].

In the literature, there is solid evidence for a relation of OPN, VEGF and CAIX with prognosis in (lung) cancer patients undergoing treatment [130,137,138,169,208,301,323, 345-347]. Yet, most of these studies were single marker based and investigated the prognostic value of baseline circulating biomarkers in surgery or chemotherapy of cancer [140,238,246]. Thus, equivalent data for the radiotherapy of NSCLC, particularly the prognostic effect of a co-detection of the aforementioned biomarkers, is still missing [348].

In my study, unlike OPN, baseline VEGF and CAIX plasma levels were not related to OS, PFS or MFS in univariate analysis (entire and curative-intent NSCLC cohort).

However, when I evaluated baseline VEGF and CAIX plasma levels in multivariate analysis, I found that VEGF (and OPN) but not CAIX significantly predicted OS and PFS ($p=.004$ and .009) independent from GTV ($p<.0001$ and .001) and other prognostic factors (4.5.3). One explanation for the lack of a significant association of VEGF and CAIX with prognosis in my study could be the limited patient number of compared subgroups. This is underlined by my finding that VEGF and CAIX trended to be related to prognosis and suggests that significant results could be obtained in studies with higher patient numbers.

The rationale for a co-detection of baseline plasma levels of OPN, VEGF and CAIX is based on their cooperative role in cancer progression such as tumor neo-vascularization

[256,349,311] which is of clinical importance [119] on the one hand and on the correlation of the biomarkers reported in this study (4.1) on the other hand. Additional support for a combination of the aforementioned biomarkers comes from the fact that each single marker has been related to prognosis in cancer patients in the literature previously.

I assumed that co-detection of the aforementioned biomarkers could provide additional prognostic information, augmenting the prognostic value of a single biomarker in the curative-intent radiotherapy of NSCLC. My results show that when plasma levels of OPN and CAIX were combined, the double marker combination had an increased prognostic effect with elevated plasma levels of both biomarkers being associated with a significantly increased risk of death (4.5.4). The prognostic effect was more pronounced when all three markers were combined, yielding a median OS of 41 months in patients with low compared to 5 months in patients with high plasma levels of all three markers (4.5.4).

In the multivariate analysis, combined plasma levels of OPN-CAIX and also the triple biomarker combination OPN-VEGF-CAIX significantly predicted OS and PFS independent from know prognostic factors such as T-stage, N-stage or GTV (4.5.5).

These findings support the hypothesis that the prognostic effect of a co-detection of the studied biomarkers could be superior and more robust than single biomarker evaluation which is strengthened by the literature [350]. Phuoc et al. for instance reported a superior prognostic impact of the co-expression of VEGF and CAIX in renal cell carcinoma [308].

The combined evaluation of OPN, VEGF and CAIX is further supported by the clinically relevant relation between VEGF and CAIX as downstream effectors of HIF 1α in the cellular response to hypoxia on the one hand [120,351] and by the cooperative role of OPN and VEGF in tumor growth and neo-angiogenesis [51,312-314] on the other hand. Interestingly, co-expression of OPN and VEGF has been suggested as a surrogate marker of tumor recovery after radiotherapy [313,314] and also for VEGF and CAIX, co-expression has been linked to response after radiotherapy of NSCLC.

These observations are supported by my findings that both OPN and VEGF were positively correlated and that OPN plasma level increases after radiotherapy were related to reduced OS and PFS [311] which could indicate remaining or recurrent disease after radiotherapy with the underlying cause being hypoxic radiation resistance of the tumor.

Thus, my data in principle justifies further combined evaluation of the hypoxia-related proteins OPN, VEGF and CAIX, for instance as part of an individual prognostic hypoxia patient profile, i.e. “plasma hypoxia score” [344,352], helping to identify patients with significant hypoxic tumor burden before the start of radiotherapy.

Incorporating other methods of detection of clinically significant tumor hypoxia such as exogeneous hypoxia markers [353] or hypoxia-specific imaging [354-356] could enhance the validity of this approach by integrating different aspects of hypoxia such as dynamic changes in tumor oxygenation and re-oxygenation, monitored for instance by sequential F-MISO-PET readings [357,358]. Ultimately, this could provide useful information for selecting patients with largely hypoxic tumors for anti-hypoxic treatment approaches [359] which may be available in future [184,270,360].

Numerous studies for instance reported first promising results in targeting OPN and other hypoxia-related proteins, thereby opening up a therapeutic perspective besides the potential prognostic and predictive role of these biomarkers [40,269,361]. Kou et al. for instance followed an immunologic approach by developing a bi-specific antibody targeting both OPN and VEGF which resulted in a significant reduction of tumor volume, microvessel density and metastatic lesions in hepatocellular carcinoma patients [274].

5.8 Methodological limitations of this work

When interpreting the results of this work, all limitations inherent to such prospective study design have to be considered. The overall patient number (n=97) and particularly the number of patients in the respective subgroups was small which underlines the exploratory character of this work and limits the conclusions made when interpreting my results.

The basis of this study was a heterogeneous patient collective consisting of non-metastasized (M0) patients treated with curative-, metastasized (M1) patients treated with palliative intent (NSCLC and SCLC). This implies that both histology and treatment concepts (radiation dose, anti-cancer agents) were different among patients and additionally, some patients (even though constituting only a minor part of the entire patient collective) previously received induction chemotherapy. To reduce this bias and to ensure adequate patient collective homogeneity for statistical analysis, patient subgroups have been formed according to histology and M-status, yielding three subgroups (NSCLC-M0, curative-intent cohort; NSCLC-M1, palliative-intent cohort and SCLC cohort) which have been evaluated separately. Increasing patient cohort homogeneity however, comes at the cost of smaller patient numbers in subgroups.

The impact of biomarker plasma levels on the clinical endpoints OS, PFS, FFLR, tumor control, MFS and TTP has been evaluated in univariate and multivariate analysis which have been restricted to the entire patient collective (to ensure endpoint evaluation in a patient collective with adequate size) and the curative-intent NSCLC M0 patient cohort.

The latter patient cohort constitutes the main subject group of survival and clinical outcome analyses in this study since NSCLC M0-stage patients are of particular interest in radiation oncology for their curative treatment chance. Thus, both prognostic and predictive biomarkers are of considerable value in these patients.

Due to the limited value of prognostic biomarkers in palliative-intent patients (poor prognosis and survival time, absence of indication for curative-intent treatment), the latter have been excluded from endpoint analyses. Also the SCLC cohort has been excluded from analysis of the association of baseline protein levels with clinical patient characteristics and survival analysis for the small patient number in this group (n=16).

While follow-up time (median 41 months in surviving patients) and integrity of survival data in this study was solid to allow adequate conclusions on the impact of biomarker plasma levels on patient survival, clinical data on therapy response, tumor progression and remission was rather incomplete. Since the patient number with available follow-up data on tumor control was small, particularly the evaluation of the endpoints TTP and FFLR is critical. In addition, re-staging and therapy response evaluation after radiotherapy was carried out in a decentralized manner. Hospitals taking over patients for follow-up care after radiotherapy were responsible for imaging studies and their assessment (without central review). This may have compromised standardization of tumor response evaluation.

The primary endpoint cancer(disease)-specific survival was not analyzed in this work because in most patients, death was clearly cancer-related so that overall and cancer-specific survival can be regarded as almost identical in the studied patient collective.

The statistical concern may be raised that the number of statistical tests relative to the number of events might imply a risk of mass significance. As part of the present prospective pilot study, I evaluated a number of clinical factors and biomarkers in an exploratory session in univariate analysis. In the multivariate analysis, the number of factors entered into the model appears adequate with regard to the number of events. However, future studies will require stricter biometric study planning.

In order to evaluate the prognostic impact of relative OPN plasma level changes over time, patients have been grouped into increasing vs. decreasing (vs. stable) intra- or post-therapeutic OPN plasma levels. In future studies, patient subgroups with falling or rising OPN plasma levels during or after radiotherapy could also be further classified by OPN velocity [362]. In this work, it is demonstrated that OPN plasma level changes over time, particularly in the post-radiotherapy time window, possess prognostic and maybe predictive quality. At this point, it is difficult to envision how to individualize radio-oncologic

therapies according to post-radiotherapy biomarker plasma level changes. However, not only OPN plasma level changes during and after radiotherapy but also absolute pre-treatment plasma levels were evaluated. While (high) baseline OPN levels before radiotherapy (indicating a hypoxic, aggressive and radioresistant cancer phenotype) could influence radiotherapy individualization by means such as hypoxic modification during radiotherapy, which might be available in future, post-treatment OPN level changes (identifying patients with high risk for death and relapse after radiotherapy) could help in the decision-making process for ongoing (intensified) cancer treatment after radiotherapy.

As far as biomarker plasma samples are concerned, the number of both baseline samples which were acquired before the start of radiotherapy and of those obtained at the end of radiotherapy was sufficient whereas a total of 69 patients (71%) had OPN plasma samples four weeks after radiotherapy. The reduced number of OPN t2 plasma samples can be explained by logistic problems and loss of follow-up in some patients which limits the prognostic conclusions based on post-treatment OPN plasma levels.

Another constraining factor is the fact that after the end of radiotherapy, patients will have received widely differing therapies according to their tumor situation during follow-up. However, since the last measurement time point for OPN was 4 weeks after radiotherapy, the majority of patients probably will not yet have started with ongoing consolidation treatment, especially because treatment response evaluation by post-radiotherapy imaging (i.e. re-staging) usually is not performed earlier than 4-6 weeks after radiotherapy.

In summa, further larger, preferably multi-center prospective studies utilizing pre-hoc biometric study planning are needed to validate the results of this study which was a hypothesis-generating study. Additionally, verification of the results and corroboration of the hypotheses generated by this study in an independent data set would be desirable.

6. Conclusions

My results suggest that baseline plasma levels of OPN and the other investigated biomarkers reflect tumor biology and disease extent. Here, increased plasma levels could indicate an aggressive, biologically unfavorable and invasive cancer phenotype, advanced disease stage and extensive hypoxia [46]. Consequently, detection of baseline biomarker levels before radiotherapy might help selecting patients with radioresistant tumors who need individualized and more rigorous treatment, i.e. radiation dose escalation in hypoxic tumor areas or hypoxia modification using hypoxic radiosensitizers [252].

In contrast, relative OPN plasma level changes during and especially after radiotherapy provide additional prognostic information beyond T-stage, N-stage and tumor volume (GTV). Increasing post-treatment plasma levels are associated with poor OS, PFS and MFS which could be indicative of tumor persistence or recurrence and a high risk for the development of distant disease spread (i.e. metastasis) after radiotherapy [227,325].

Thus, monitoring OPN plasma levels during and after radiotherapy could be potentially useful in the decision-making process for consolidating treatment after definitive radiotherapy of NSCLC in order to ultimately reduce both death and relapse rates [256].

References

1. Newlin HE, Ivengar M, Morris CG, Olivier K (2009) Unresectable squamous cell carcinoma of the lung: an outcomes study. Int H Radiat Oncol Biol Phys 74:370-376
2. Jemal A, Thun MJ, Ries LA, Howe HL, Weir HK, Center MM, Ward E, Wu XC, Eheman C, Anderson R, Ajani UA, Kohler B, Edwards BK (2008) Annual report of cancer, 1975-2005, featuring trends in lung cancer, tobacco use, and tobacco control. J Natl Cancer Inst 100:1672-1694.
3. Höckel M, Vaupel P (2001) Biological consequences of tumor hypoxia. Semin Oncol 28:36-41.
4. Vaupel P, Mayer A (2007) Hypoxia in cancer: significance and impact on clinical outcome. Cancer Metastasis Rev 26:225-239.
5. Vaupel P, Kelleher DK, Höckel M (2001) Oxygen status of malignant tumors: pathogenesis and significance for tumor therapy. Semin Oncol 28:29-35.
6. Thomlinson RH, Gray LH (1955) The histological structure of some human lung cancers and the possible implications for radiotherapy. Br J Cancer 9:539-549.
7. Duncan W (1973) Exploitation of the oxygen enhancement ratio in clinical practice. Br Med Bull 29:33-38.
8. Yaromina A, Thames H, Zhou X, Hering S, Eicheler W, Dörfler A, Leichtner T, Zips D, Baumann M. (2010) Radiobiological hypoxia, histological parameters of tumour microenvironment and local tumour control after fractionated irradiation. Radiother Oncol 96:116-122.
9. Gray LH, Conger AD, Ebert M, Hornsey S, Scott OC (1953) The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br J Radiol 26:638-648.
10. Moulder JE, Rockwell S (1984) Hypoxic fractions of solid tumors: experimental techniques, methods of analysis, and a survey of existing data. Int J Radiat Oncol Biol Phys 10:695-712.
11. Pries AR, Cornelissen AJ, Sloot AA, Hinkeldey M, Dreher MR, Höpfner M, Dewhirst MW, Secomb TW (2005) Structural adaptation and heterogeneity of normal and tumor microvascular networks. PLoS Comput Biol 5:e1000394.
12. Bussink J, Kaanders JH, van der Kogel AJ (2003) Tumor hypoxia at the micro-regional level: clinical relevance and predictive value of exogenous and endogenous hypoxic cell markers. Radiother Oncol 67:3-15.
13. Brown JM (1999) The hypoxic cell: a target for selective cancer therapy--eighteenth Bruce F Cain Memorial Award lecture. Cancer Res 59:5863-5870.
14. Kennedy AS, Raleigh JA, Perez GM, Calkins DP, Thrall DE, Novotny DB, Varia MA (1997) Proliferation and hypoxia in human squamous cell carcinoma of the cervix: first report of combined immunohistochemical assays. Int J Radiat Oncol Biol Phys 37:897-905.
15. Ljungkvist AS, Bussink J, Rijken PF, Kaanders JH, van der Kogel AJ, Denekamp J (2002) Vascular architecture, hypoxia, and proliferation in first-generation xenografts of human head-and-neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 54:215-228.
16. Webster L, Hodgkiss RJ, Wilson GD (1995) Simultaneous triple staining for hypoxia, proliferation, and DNA content in murine tumours. Cytometry 21:344-351.
17. Wilson WR, Hay MP (2011) Targeting hypoxia in cancer therapy. Nature Rev Cancer 11:393-410.
18. Vaupel P, Thews O, Kehlleher DK, Höckel M (1998) Oxygenation of human tumors: the Mainz experience. Strahlenther Onkol 174:6-12.
19. Padhani AR, Krohn KA, Lewis JS, Alber M (2007) Imaging oxygenation of human tumours. Eur Radiol 17:861-872.
20. Vaupel P, Harrison L (2004) Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. Oncologist 9:4-9.
21. Kolstad P (1963) OXYGEN TENSION IN CERVICAL CANCER. Nord Med 70:1128-1130.
22. Kolstad P (1968) Clinical trial with atmospheric oxygen breathing during radiotherapy of cancer of the cervix. Scand J Clin Lab Invest Suppl 106:167-171.
23. Höckel M, Schlenger K, Aral B, Mitze M, Schaffer U, Vaupel P (1996) Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 56:4509-4515.

24. Gatenby RA, Kessler HB, Rosenblum JS, Coia LR, Moldofsky PJ, Hartz WH, Broder GJ (1988) Oxygen distribution in squamous cell carcinoma metastases and its relationship to outcome of radiation therapy. Int J Radiat Oncol Biol Phys 14:831-838.
25. Nordsmark M, Overgaard M, Overgaard J (1996) Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 41:31-39.
26. Dasu A, Denenkamp J (1998) New insights into factors influencing the clinically relevant oxygen enhancement ratio. Radiother Oncol 46:269-277.
27. Wouters BG, Brown JM (1997) Cells at intermediate oxygen levels can be more important than the "hypoxic fraction" in determining tumor response to fractionated radiotherapy. Radiat Res 147:541-550.
28. Nordsmark M, Bentzen SM, Overgaard J (1994) Measurement of human tumour oxygenation status by a polarographic needle electrode. An analysis of inter- and intratumour heterogeneity. Acta Oncol 33:383-389.
29. Overgaard J (1989) Sensitization of hypoxic tumour cells--clinical experience. Int J Radiat Biol 56:801-811.
30. Luoto KR, Kumareswaran R, Bristow RG (2013) Tumor hypoxia as a driving force in genetic instability. Genome Integr 4:5.
31. Erler JT, Cawthorne CJ, Williams KJ, Koritzinsky M, Wouters BG, Wilson C, Miller C, Demonacos C, Stratford IJ, Dive C (2004) Hypoxia-mediated down-regulation of Bid and Bax in tumors occurs via hypoxia-inducible factor 1-dependent and -independent mechanisms and contributes to drug resistance. Mol Cell Biol 24:2875-2889.
32. Bristow RG, Hill RP (2008) Hypoxia and metabolism. Hypoxia, DNA repair and genetic instability. Nature Rev Cancer 8:180-192.
33. Graeber TG, Osmanian C, Jacks T, Housman DE, Koch CJ, Lowe SW, Giaccia AJ (1996) Hypoxia-mediated selection of cells with diminished apoptotic potential in solid tumours. Nature 379:88-91.
34. Hall EJ, Giaccia AJ: Radiologist: Remodelling of the Tumor Microenvironment by Combined Treatment with a Novel Radiosensitizer, α-Sulfoquinovosylmonoacylglycerol (α-SQMG) and X-irradiation. 6th edition Lippincott Williams and Wilkins, Philadelphia, 2006, pp. 85-105.
35. Yotnda P, Wu D, Swanson AM (2010) Hypoxic tumors and their effect on immune cells and cancer therapy. Methods Mol Biol 651:1-29.
36. Kioi M, Vogel H, Schultz G, Hoffman RM, Harsh GR, Brown JM (2010) Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J Clin Invest 120:694-705.
37. Liu Y, Song X, Wang X, Wei L, Liu X, Yuan S, Lv L (2010) Effect of chronic intermittent hypoxia on biological behavior and hypoxia-associated gene expression in lung cancer cells. J Cell Biochem 11:554-563.
38. Schmaltz C, Hardenbergh PH, Wells A, Fisher DE (1998) Regulation of proliferation-survival decisions during tumor cell hypoxia. Mol Cell Bio 18:2845-2854.
39. Nordsmark M, Hoyer M, Keller J, Nielsen OS, Jensen OM, Overgaard J (1996) The relationship between tumor oxygenation and cell proliferation in human soft tissue sarcomas. Int J Radiat Oncol Biol Phys 35:701-708.
40. Vordermark D: Hypoxia: Causes, Types and Management. Nova Publishers, New York, 2013, pp.255-318.
41. Semenza GL (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Crit Rev Biochem Mol Biol 35:71-103.
42. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, Comoglio PM (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3:347-361.

43. Chang Q, Jurisica I, Do T, Hedley DW (2011) Hypoxia predicts aggressive growth and spontaneous metastasis formation from orthotopically grown primary xenografts of human pancreatic cancer. Cancer Res 78:3110-3120.

44. Hill RP, Marie-Egyptienne DT, Hedley DW (2009) Cancer stem cells, hypoxia and metastasis. Semin Radiat Oncol 19:106-111.

45. Hoeckel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93:266-276.

46. Vaupel P (2008) Hypoxia and aggressive tumor phenotype: implications for therapy and prognosis. Oncologist 13:21-26.

47. Giaccia AJ (1996) Hypoxic Stress Proteins: Survival of the Fittest. Semin Radiat Oncol 6:46-58.

48. Sutherland RM, Ausserer WA, Murphy BJ, Laderoute KR (1996) Tumor Hypoxia and Heterogeneity: Challenges and Opportunities for the Future. Semin Radiat Oncol 6:59-70.

49. Rofstad EK, Galappathi K, Matthiesen B, Ruud EB (2007) Fluctuating and diffusion-limited hypoxia in hypoxia-induced metastasis. Clin Cancer Res 13:1971-1978.

50. Vaupel P (2004) The role of hypoxia-induced factors in tumor progression. Oncologist 9:10-17.

51. Vaupel P, Thews O, Hoeckel M (2001) Treatment resistance of solid tumors: role of hypoxia and anemia. Med Oncol 18:243-259.

52. Rohwer N, Cramer T (2011) Hypoxia-mediated drug resistance: novel insights on the functional interaction of HIFs and cell death pathways. Drug Resist Updat 14:191-201.

53. Nordsmark M, Alsner J, Keller J, Nielsen OS, Jensen OM, Horsman MR, Overgaard J (2001) Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. Br J Cancer 84:1070-1075.

54. Rofstad EK, Sundfor K, Lyng H, Trope CG (2000) Hypoxia-induced treatment failure in advanced squamous cell carcinoma of the uterine cervix is primarily due to hypoxia-induced radiation resistance rather than hypoxia-induced metastasis. Br J Cancer 83:354-359.

55. Vaupel P, Hoeckel M (1999) Predictive power of the tumor oxygenation status. Adv Exp Med Biol 471:533-539.

56. Vaupel P (2009) Prognostic potential of the pre-therapeutic tumor oxygenation status. Adv Exp Med Biol 645:241-246.

57. Nordsmark M, Bentzen SM, Rudat V, Brizel D, Lartigau E, Stadler P, Becker A, Adam M, Molls M, Dunst J, Terris DJ, Overgaard J (2005) Prognostic value of tumor oxygenation in 397 head and neck tumors after primary radiation therapy. An international multi-center study. Radiother Oncol 77:18-24.

58. Nordsmark M, Overgaard M, Overgaard J (1996) Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. Radiother Oncol 41:31-39.

59. Raleigh JA, Dewhirst MW, Thrall DE (1996) Measuring Tumor Hypoxia. Semin Radiat Oncol 6:37-45.

60. Stone HB, Brown JM, Phillips TL, Sutherland RM (1993) Oxygen in human tumors: correlations between methods of measurement and response to therapy. Summary of a workshop held November 19-20, 1992, at the National Cancer Institute, Bethesda, Maryland. Radiat Res 136:422-434.

61. Kallinowski F, Zander R, Hoeckel M, Vaupel P (1990) Tumor tissue oxygenation as evaluated by computerized-pO2-histography. Int J Radiat Oncol Biol Phys 319:953-961.

62. Hoeckel M, Schlenger K, Knoop C, Vaupel P (1991) Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. Cancer Res 51:6098-6102.

63. Brizel DM, Rosner GL, Harrelson J, Prosnitz LR, Dewhirst MW (1994) Pretreatment oxygenation profiles of human soft tissue sarcomas. Int J Radiat Oncol Biol Phys 30:635-642.

64. Brizel DM, Rosner GL, Prosnitz LR, Dewhirst MW (1995) Patterns and variability of tumor oxygenation in human soft tissue sarcomas, cervical carcinomas, and lymph node metastases. Int J Radiat Oncol Biol Phys 32:1121-1125.

65. Le QT, Courter D (2008) Clinical biomarkers for hypoxia targeting. Cancer Metastasis Rev 27:351-362.

66. Collingrdige DR, Young WK, Vojnovic B, Wardman P, Lynch EM, Hill SA, Chaplin DJ (1997) Measurement of tumor oxygenation: a comparison between polarographic needle electrodes and a time-resolved luminescence-based optical sensor. Radiat Res 147:329-334.

67. Bussink J, Kaanders JH, Strik AM, Vojnovic B, van Der Kogel AJ (2000) Optical sensor-based oxygen tension measurements correspond with hypoxia marker binding in three human tumor xenograft lines. Radiat Res 154:547-555.

68. Le QT (2007) Identifying and targeting hypoxia in head and neck cancer: a brief overview of current approaches. Int J Radiat Oncol Biol Phys 69:S56-S58.

69. Mayer A, Hoeckel M, Vaupel P (2008) Endogenous hypoxia markers: case not proven! Adv Exp Med Biol 614:801-811.

70. Goethals L, Debucguoy A, Perneel C, Geboes K, Ectors N, De Schutter H, Penninckx F, McBride WH, Beqq AC, Haustermans KM (2006) Hypoxia in human colorectal adenocarcinoma: comparison between extrinsic and potential intrinsic hypoxia markers. Int J Radiat Oncol Biol Phys 65:246-254.

71. Vaupel P, Schlenger K, Knoop C, Höckel M (1991) Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O2 tension measurements. Cancer Res 51:3316-3322.

72. Nordsmark M, Grau C, Horsman MR, Jörgensen HS, Overgaard J (1995) Relationship between tumour oxygenation, bioenergetic status and radiobiological hypoxia in an experimental model. Acta Oncol 34:329-334.

73. Loncaster JA, Harris AL, Davidson SE, Loque JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Stratford IJ, West CM (2001) Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 61:6394-6399.

74. Yaromina A, Eckhardt A, Zips D, Eicheler W, Schuetze C, Thames H, Baumann M (2009) Core needle biopsies for determination of the microenvironment in individual tumours for longitudinal radiobiological studies. Radiother Oncol 92:460-465.

75. Hou H, Lariviere JP, Demidenko E, Gladstone D, Swartz H, Khan N (2009) Repeated tumor pO(2) measurements by multi-site EPR oximetry as a prognostic marker for enhanced therapeutic efficacy of fractionated radiotherapy. Radiother Oncol 91:126-131.

76. Menon C, Fraker DL (2004) Tumor oxygenation status as a prognostic marker. Cancer Lett 221:225-235.

77. Christian N, Deheneffe S, Bol A, De Bast M, Labar D, Lee JA, Gregoire V (2010) Is (18)F-FDG a surrogate tracer to measure tumor hypoxia? Comparison with the hypoxic tracer (14)C-EF3 in animal tumor models. Radiother Oncol 97:183-188.

78. Gulliksrud K, Mathiesen B, Galappathi K, Rofstad EK (2010) Quantitative assessment of hypoxia in melanoma xenografts by dynamic contrast-enhanced magnetic resonance imaging: intradermal versus intramuscular tumors. Radiother Oncol 97:233-238.

79. Howe FA, Robinson SP, McIntyre DJ, Stubbs M, Griffiths JR (2001) Issues in flow and oxygenation dependent contrast (FLOOD) imaging of tumours. NMR Biomed 14:497-506.

80. Taylor NJ, Baddeley H, Goodchild KA, Powell ME, Thoumine M, Culver LA, Stirling JJ, Saunders MI, Hoskin PJ, Phillipd H, Padhani AR, Griffiths JR (2001) BOLD MRI of human tumor oxygenation during carbogen breathing. J Magn Reson Imaging 14:156-163.

81. Li L, Yu J, Xing L, Ma K, Zhu H, Guo H, Sun X, Li J, Yang G, Li W, Yue J, Li B (2006) Serial hypoxia imaging with 99mTc-HL91 SPECT to predict radiotherapy response in nonsmall cell

lung cancer. Am J Clin Oncol 29:628-633.

82. Piert M, Machulla HJ, Piocchio M, Reischl G, Ziegler S, Kumar P, Wester HJ, Beck R, McEwan AJ, Wiebe LI, Schwaiger M (2005) Hypoxia-specific tumor imaging with 18F-fluoroazomycin arabinoside. J Nucl Med 46:108-113.

83. Busk M, Horsman MR, Jakobsen S, Keiding S, van der Kogel AJ, Bussink J, Overgaard J (2008) Imaging hypoxia in xenografted and murine tumors with 18F-fluoroazomycin arabinoside: a comparative study involving microPET, autoradiography, PO2-polarography, and fluorescence microscopy. Int J Radiat Oncol Biol Phys 70:1202-1212.

84. Dehdashti F, Mintun MA, Lewis JS, Bradley J, Govindan R, Laforest R, Welch MJ, Siegel BA (2003) In vivo assessment of tumor hypoxia in lung cancer with 60Cu-ATSM. Eur J Nucl Med Mol Imaging 30:844-850.

85. Chapman JD, Engelhardt EL, Stobbe CC, Schneider RF, Hanks GE (1998) Measuring hypoxia and predicting tumor radioresistance with nuclear medicine assays. Radiother Oncol 46:229-237.

86. Mason RP, Constantinescu A, Hunjan S, Le D, Hahn EW, Antich PP, Blum C, Peschke P (1999) Regional tumor oxygenation and measurement of dynamic changes. Radiat Res 52:239-249.

87. Krishna MC, English S, Yamada K, Yoo J, Murugesan R, Devasahayam N, Cook JA, Golman K, Ardenkjaer-Larsen JH, Subramanian S, Mitchell JB (2002) Overhauser enhanced magnetic resonance imaging for tumor oximetry: coregistration of tumor anatomy and tissue oxygen concentration. Proc Natl Acad Sci USA 99:2216-2221.

88. Thureau S, Chaumet-Riffaud P, Modzelewski R, Fernandez P, Tessonnier L, Vervueren L, Cachin F, Berriolo-Riedinger A, Olivier P, Kolesnikov-Gauthier H, Blagosklonov O, Bridji B, Devillers A, Collombier L, Courbon F, Gremillet E, Houzard C, Caignon JM, Roux J, Aide N, Brenot-Rossi I, Doyeux K, Dubray B, Vera P (2013) Interobserver agreement of qualitative analysis and tumor delineation of 18F-fluoromisonidazole and 3'-deoxy-3'-18F-fluorothymidine PET images in lung cancer. J Nucl Med 54:1543-1550.

89. Okamoto S, Shiga T, Yasuda K, Ito YM, Magota K, Kasai K, Kuge Y, Shirato H, Tamaki N (2013) High reproducibility of tumor hypoxia evaluated by 18F-fluoromisonidazole PET for head and neck cancer. J Nucl Med 54:201-207.

90. Mönnich D, Troost EG, Kaanders JH, Oyen WJ, Alber M, Zips D, Thorwarth D (2013) Correlation between tumor oxygenation and 18F-fluoromisonidazole PET data simulated based on microvessel images. Acta Oncol 52:1308-1313.

91. Henriques de Figueiredo B, Zacharatou C, Galland-Girodet S, Benech J, De Clermont-Gallerande H, Lamare F, Hatt M, Digue L, De Mones del Pujol E, Fernandez P (2015) Hypoxia imaging with [18F]-FMISO-PET for guided dose escalation with intensity-modulated radiotherapy in head-and-neck cancers. Strahlenther Onkol 191:217-224.

92. Eschmann SM, Paulsen F, Reimold M, Dittmann H, Welz S, Reischl G, Machulla HJ, Bares R (2005) Prognostic impact of hypoxia imaging with 18F-misonidazole PET in non-small cell lung cancer and head and neck cancer before radiotherapy. J Nucl Med 46:253-260.

93. Rischin D, Hicks RJ, Fisher R, Binns D, Corry J, Porceddu S, Peters LJ, Trans-Tasman Radiation Oncology Group Study 98.02 (2006) Prognostic significance of [18F]-misonidazole positron emission tomography-detected tumor hypoxia in patients with advanced head and neck cancer randomly assigned to chemoradiation with or without tirapazamine: a substudy of Trans-Tasman Radiation Oncology Group Study 98.02. J Clin Oncol 24:2098-2104.

94. Hicks RJ, Rischin D, Fisher R, Binns D, Scott AM, Peters LJ (2005) Utility of FMISO PET in advanced head and neck cancer treated with chemoradiation incorporating a hypoxia-targeting chemotherapy agent. Eur J Nucl Med Mol Imaging 32:1384-1391.

95. Ng P, Peterson LM, Schwartz DL, Scharnhrost J, Krohn KA (2003) Can F-18 fluoromisonidazole PET imaging predict treatment response in head and neck cancer? J Nucl Med 44:416.

96. Spence AM, Muzi M, Swanson KR, O'Sullivan F, Rockhill JK, Rajendran JG, Adamsen TC, Link JM, Swanson PE, Yagle KJ, Rostomily RC, Silbergerld DL, Krohn KA (2008) Regional hypoxia in glioblastoma multiforme quantified with [18F]fluoromisonidazole positron emission tomography before radiotherapy: correlation with time to progression and survival. Clin Cancer Res 14:2623-2630.

97. Rajendran JG, Wilson DC, Conrad EU, Peterson LM, Bruckner JD, Rasey JS, Chin LK, Hofstrand PD, Grierson JR, Eary JF, Lrohn KA (2003) [(18)F]FMISO and [(18)F]FDG PET imaging in soft tissue sarcomas: correlation of hypoxia, metabolism and VEGF expression. Eur J Nucl Med Mol Imaging 30:696-704.

98. Koh WJ, Bergman KS, Rasey JS, Peterseon LM, Evans ML, Graham MM, Grierson JR, Lindsley KL, Lewellen TK, Krohn KA (1995) Evaluation of oxygenation status during fractionated radiotherapy in human nonsmall cell lung cancers using [F-18]fluoromisonidazole positron emission tomography. Int J Radiat Oncol Biol Phy 33:391-398.

99. Troost EG, Laverman P, Philippens ME, Lok J, van der Kogel AJ, Oyen WJ, Boerman OC, Kaanders JH, Bussink J (2008) Correlation of [18F]FMISO autoradiography and pimonidazole [corrected] immunohistochemistry in human head and neck carcinoma xenografts. Eur J Nucl Med Mol Imaging 35:1803-1811.

100. Zimny M, Gagel B, DiMartino E, Hamacher K, Coenen HH, Westhofen M, Eble M, Buell U, Reinartz P (2006) FDG--a marker of tumour hypoxia? A comparison with [18F]fluoromisonidazole and pO2-polarography in metastatic head and neck cancer. Eur J Nucl Med Mol Imaging 12:1426-1431.

101. Thorwarth D, Eschmann SM, Scheiderbauer J, Paulsen F, Alber M (2005) Kinetic analysis of dynamic 18F-fluoromisonidazole PET correlates with radiation treatment outcome in head-and-neck cancer. BMC Cancer 5:152.

102. Troost EG, Laverman P, Kaanders JH, Philippens M, Lok J, Oyen WJ, van der Kogel AJ, Boermann OC, Bussink J (2006) Imaging hypoxia after oxygenation-modification: comparing [18F]FMISO autoradiography with pimonidazole immunohistochemistry in human xenograft tumors. Radiother Oncol 80:157-164.

103. Bussnik J. Quantification of tumour hypoxia. Functional histology and autoradiography. Nuklear Medizin 6:S37-S40.

104. Hoeben BA, Kaanders JH, Franssen JM, Troost EG, Rijken PF, Oosterwijk E, van Dongen GA, Oyen WJ, Boermann OC, Bussink J (2010) PET of hypoxia with 89Zr-labeled cG250-F(ab')2 in head and neck tumors. J Nucl Med 51:1076-1083.

105. Burgman P, Odonoghue JA, Humm JL, Ling CC (2001) Hypoxia-Induced increase in FDG uptake in MCF7 cells. J Nucl Med 42:170-175.

106. Anaira CC, Wahl RL (1996) Effects of hypoxia on the uptake of tritiated thymidine, L-leucine, L-methionine and FDG in cultured cancer cells. J Nucl Med 37:502-506.

107. Anaira CC; Brown RS; Wahl RL (1995) Fluorodeoxyglucose uptake in human cancer cell lines is increased by hypoxia. J Nucl Med 36:1625-1632.

108. Ljungkvist AS, Bussink J, Kaanders JH, van der Kogel (2007) Dynamics of tumor hypoxia measured with bioreductive hypoxic cell markers. Radiat Res 167:127-145.

109. Kaanders JH, Wijffels KI, Marres HA, Ljungkvist AS, Pop LA, van den Hoogen FJ, de Wilde PC, Bussink J, Raleigh JA, van der Kogel AJ (2002) Pimonidazole binding and tumor vascularity predict for treatment outcome in head and neck cancer. Cancer Res 62:7066-7074.

110. Vordermark D, Brown JM (2003) Endogenous markers of tumor hypoxia predictors of clinical radiation resistance? Strahlenther Onkol 179:801-811.

111. Nordsmark M, Loncaster J, Aquino-Parsons C, Chou SC, Ladekarl M, Havsteen H, Lindegaard JC, Davidson SE, Varia M, West C, Hunter R, Overgaard J, Raleigh JA (2003) Measurements of hypoxia using pimonidazole and polarographic oxygen-sensitive electrodes in human cervix carcinomas. Radiother Oncol 67:35-44.

112. Ljungkvist AS, Bussink J, Rijken PF, Raleigh JA, Denekamp J, Van Der Kogel AJ (2000) Changes in tumor hypoxia measured with a double hypoxic marker technique. Int J Radiat Oncol Biol Phys 48:1529-1538.

113. Busk M, Mortensen LS, Nordsmark M, Overgaard J, Jakobsen S, Hansen KV, Theil J, Kallehauge JF, D'Andrea FP, Steiniche T, Horsman MR (2013) PET hypoxia imaging with FAZA: reproducibility at baseline and during fractionated radiotherapy in tumour-bearing mice. Eur J Nucl Med Mol Imaging 40:186-197.

114. Jewell UR, Kvietikova I, Scheid A, Bauer C, Wegner RH, Gassmann M (2001) Induction of HIF-1alpha in response to hypoxia is instantaneous. FASEB J 15:1312-1314.

115. Jiang BH, Semenza BL, Bauer C, Marti HH (1996) Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O2 tension. Am J Physiol 271:C1172-1180.

116. Bilton RL, Booker GW (2003) The subtle side to hypoxia inducible factor (HIFalpha) regulation. Eur J Biochem 270:791-798.

117. Semenza GL (2007) Evaluation of HIF-1 inhibitors as anticancer agents. Drug Discov Today 12:853-859.

118. Giaccia A, Siim BG, Johnson RS (2003) HIF-1 as a target for drug development. Nature Rev Drug Discov 2:803-811.

119. Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M, Heeboll S, Horwich A, Huddart R, Khoo V, Eeles R, Cooper C, Sydes M, Dearnaley D, Parker C (2008) Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 9:342-351.

120. Ren W, Mi D, Yang K, Cao N, Tian J, Li Z, Ma B (2013) The expression of hypoxia-inducible factor-1α and its clinical significance in lung cancer: a systematic review and meta-analysis. Swiss Med Wkly 143:w13855.

121. Helbig L, Yaromina A, Sriramareddy SN, Böke S, Koi L, Thames HD, Baumann M, Zips D (2012) Prognostic value of HIF-1α expression during fractionated irradiation. Strahlenther Onkol 188:1031-1037.

122. Dellas K, Bache M, Pigorsch SU, Taubert H, Kappler M, Holzapfel D, Zorn E, Holzhausen HJ, Haensgen G (2008) Prognostic impact of HIF-1alpha expression in patients with definitive radiotherapy for cervical cancer. Strahlenther Onkol 184:169-174.

123. Koukourakis MI, Bentzen SM, Giatromanolaki A, Wilson GD, Daley FM, Saunders MI, Dische S, Sivridis E, Harris AL (2006) Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. J Clin Oncol 24:727-735.

124. Hung JJ, Yang MH, Hsu HS, Hsu WH, Liu JS, Wu KJ (2009) Prognostic significance of hypoxia-inducible factor-1alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. Thorax 64:1082-1089.

125. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075-7083.

126. Eckert AW, Kappler M, Schubert J, Taubert H (2012) Correlation of expression of hypoxia-related proteins with prognosis in oral squamous cell carcinoma patients. Oral Maxillofac Surg 16:189-96.

127. Vordermark D, Kaffer A, Riedl S, Katzer A, Flentje M (2005) Characterization of carbonic anhydrase IX (CA IX) as an endogenous marker of chronic hypoxia in live human tumor cells. Int. J Radiat Oncol Biol Phys 61:1197-1207.

128. Mayer A, Hoeckel M, Wree A, Vaupel P (2005). Microregional expression of glucose

transporter-1 and oxygenation status: lack of correlation in locally advanced cervical cancers. Clin Cancer Res 11:2768-2773.

129. Dunst J, Stadler P, Becker A, Kuhnt T, Lautenschläger C, Molls M, Haensgen G (2001) Tumor hypoxia and systemic levels of vascular endothelial growth factor (VEGF) in head and neck cancers. Strahlenther Onkol 177:469-73

130. Kim SJ, Rabbani ZN, Dewhirst MW, Vujaskovic Z, Vollmer RT, Schreiber EG, Oosterwijk E, Kelley MJ (2005) Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer 49:325-35.

131. Minchenko A, Bauer T, Salceda S, Caro J (1994) Hypoxic stimulation of vascular endothelial growth factor expression in vitro and in vivo. Lab Invest 71:374-379.

132. Deacon K, Onion D, Kumari R, Watson SA, Knox AJ (2012) Elevated SP-1 transcription factor expression and activity drives basal and hypoxia-induced vascular endothelial growth factor (VEGF) expression in non-small cell lung cancer. J Biol Chem 287:39967-39981.

133. Kishiro I, Kato S, Fuse D, Yoshida T, Machida S, Kaneko N (2002) Clinical significance of vascular endothelial growth factor in patients with primary lung cancer. Respirology 7:93-98.

134. Vergis R, Corbishley CM, Norman AR, Bartlett J, Jhavar S, Borre M, Heeboll S, Horwich A, Huddart R, Khoo V, Eeles R, Cooper C, Sydes M, Dearnaley D, Parker C (2008) Intrinsic markers of tumour hypoxia and angiogenesis in localised prostate cancer and outcome of radical treatment: a retrospective analysis of two randomised radiotherapy trials and one surgical cohort study. Lancet Oncol 9:342-351.

135. Tamura M, Ohta Y (2003) Serum vascular endothelial growth factor-C level in patients with primary nonsmall cell lung carcinoma: a possible diagnostic tool for lymph node metastasis. Cancer 98:1217-22.

136. Zhan P, Wang J, Lv XJ, Wang Q, Qiu LX, Lin XQ, Yu LK, Song Y (2009) Prognostic value of vascular endothelial growth factor expression in patients with lung cancer: a systematic review with meta-analysis. J Thorac Oncol 4:1094-1103.

137. Delmotte P, Martin B, Paesmans M, Berghmans T, Mascaux C, Meert AP, Steels E, Verdebout JM, Lafitte JJ, Sculier JP (2002) [VEGF and survival of patients with lung cancer: a systematic literature review and meta-analysis]. Rev Mal Respir 19:577-584.

138. Bremnes RM, Camps C, Sirera R (2006) Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines VEGF and bFGF in tumours and blood. Lung Cancer 51:143-158.

139. Tang XP, Li J, Yu LC, Chen YC, Shi SB, Zhu LR, Chen P (2013). Clinical significance of survivin and VEGF mRNA detection in the cell fraction of the peripheral blood in non-small cell lung cancer patients before and after surgery. Lung Cancer 81:273-279.

140. Beasley NJ, Wykoff CC, Watson PH, Leek R, Turley H, Gatter K, Pastorek J, Cox GJ, Ratcliffe P, Harris AL (2001) Carbonic anhydrase IX, an endogenous hypoxia marker, expression in head and neck squamous cell carcinoma and its relationship to hypoxia, necrosis, and microvessel density. Cancer Res 61:5262-5267.

141. Koukourakis MI, Giatromanolaki A, Sivridis E, Simopoulos K, Pastorek J, Wykoff CC, Gatter KC, Harris AL (2001) Hypoxia-regulated carbonic anhydrase-9 (CA9) relates to poor vascularization and resistance of squamous cell head and neck cancer to chemoradiotherapy. Clin Cancer Res 7:3399-33403.

142. Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ (2001) Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158:905-919.

143. Malentacchi F, Simi L, Nannelli C, Andreani M, Janni A, Pastorekova S, Orlando C (2009) Alternative splicing variants of carbonic anhydrase IX in human non-small cell lung cancer. Lung Cancer 64:271-276.

144. McDonald PC, Dedhar S (2014) Carbonic anhydrase IX (CAIX) as a mediator of hypoxia-induced stress response in cancer cells. Subcell Biochem 75:255-269

145. Benej M, Pastorekova S, Pastorek J (2014) Carbonic anhydrase IX: regulation and role in cancer. Subcell Biochem 75:199-219.

146. Giatromanolaki A, Koukourakis MI, Siviridis E, Pastorek J, Wykoff CC, Gatter KC, Harris AL (2001) Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res 61:7992-7998.

147. Wykoff CC, Beasley N, Watson PH, Campo L, Chia SK, English R, Pastorek J, Sly WS, Ratcliffe P, Harris AL (2001) Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. Am J Pathol 158:1011-1019.

148. Chia SK, Wykoff CC, Watson PH, Han C, Leek RD, Pastorek J, Gatter KC, Ratcliffe P, Harris AL (2001) Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 19:3660-3668.

149. Zatovicova M, Jelenska L, Hulikova A, Csaderova L, Ditte Z, Ditte P, Goliasova T, Pastorek J, Pastorekova S (2010) Carbonic anhydrase IX as an anticancer therapy target: preclinical evaluation of internalizing monoclonal antibody directed to catalytic domain. Curr Pharm Des 16:3255-3263.

150. De Simone G, Supuran CT (2009) Carbonic anhydrase IX: Biochemical and crystallographic characterization of a novel antitumor target. Biochim Biophys Acta 1804:404-409.

151. Tafreshi NK, Lloyd MC, Bui MM, Gillies RJ, Morse DL (2014) Carbonic anhydrase IX as an imaging and therapeutic target for tumors and metastases. Subcell Biochem 75:221-254.

152. Schrijvers ML, van der Laan BF, de Bock GH, Pattje WJ, Mastik MF, Menkema L, Langendijk JA, Kluin PM, Schuuring E, van der Wal JE (2008) Overexpression of intrinsic hypoxia markers HIF1alpha and CA-IX predict for local recurrence in stage T1-T2 glottic laryngeal carcinoma treated with radiotherapy. Int J Radiat Oncol Biol Phys 72:161-169.

153. Koukourakis MI, Giatromanolaki A, Danielidis V, Sivridis E (2008) Hypoxia inducible factor (HIf1alpha and HIF2alpha) and carbonic anhydrase 9 (CA9) expression and response of head-neck cancer to hypofractionated and accelerated radiotherapy. Int J Radiat Biol 84:47-52.

154. Wachters JE, Schrijvers ML, Slagter-Menkema L, Mastik M, de Bock GH, Langendijk JA, Kluin PM, Schuuring E, van der Laan BF, van der Wal JE (2013) Prognostic significance of HIF-1a, CA-IX, and OPN in T1-T2 laryngeal carcinoma treated with radiotherapy. Laryngoscope 123:2154-2160.

155. Jankovic B, Aquino-Parsons C, Raleigh JA, Stanbridge EJ, Durand RE, Banath JP, MacPhail SH, Olive PL (2006) Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. Cytometry B Clin Cytom 70:44-55.

156. Sorensen BS, Hao J, Overgaard J, Vorum H, Homore B, Alsner J, Horsman MR (2005) Influence of oxygen concentration and pH on expression of hypoxia induced genes. Radiother Oncol 76:187-193.

157. Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S (2004) HIF activation by pH-dependent nucleolar sequestration of VHL. Nat Cell Biol 6:642-647.

158. Rafajova M, Zatavicova M, Kettmann R, Pastorek J, Pastorekova S (2004) Induction by hypoxia combined with low glucose or low bicarbonate and high posttranslational stability upon reoxygenation contribute to carbonic anhydrase IX expression in cancer cells. Int J Oncol 24:995-1004.

159. Boado RJ, Pardridge WM (2002) Glucose deprivation and hypoxia increase the expression of the GLUT1 glucose transporter via a specific mRNA cis-acting regulatory element. J Neurochem 80:552-554.

160. Mayer A, Höckel M, Vaupel P (2006) Endogenous hypoxia markers in locally advanced

cancers of the uterine cervix: reality or wishful thinking? Strahlenther Onkol 182:501-510.

161. Le QT, Chen E, Salim A, Cao J, Kong CS, Whyte R, Donington J, Cannon W, Wakelee H, Tibshirani R, Mitchell JD, Richardson D, O'Byrne KJ, Koong AC, Giaccia AJ (2006) An evaluation of tumor oxygenation and gene expression in patients with early stage non-small cell lung cancers. Cancer Res 12:1507-1514.

162. Airley R, Loncaster J, Davidson S, Bromley M, Roberts S, Patterson A, Hunter R, Stratford I, West C (2001) Glucose transporter glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. Clin Cancer Res 7:928-934.

163. Hedley D, Pintilie M, Woo J, Morrison A, Birle D, Fyles A, Milosevic M, Hill R (2003) Carbonic anhydrase IX expression, hypoxia, and prognosis in patients with uterine cervical carcinomas. Clin Cancer Res 9:5666-5674.

164. Haugland HK, Vukovic V, Pintilie M, Dyles AW, Milosevic M, Hill RP, Hedley DW (2002) Expression of hypoxia-inducible factor-1alpha in cervical carcinomas: correlation with tumor oxygenation. Int J Radiat Oncol Biol Phys 53:854-861.

165. Hutchison GJ, Valentine HR, Loncaster JA, Davdison SE, Hunter RD, Roberts SA, Harris AL, Stratford IJ, Price PM, West CM (2004) Hypoxia-inducible factor 1alpha expression as an intrinsic marker of hypoxia: correlation with tumor oxygen, pimonidazole measurements, and outcome in locally advanced carcinoma of the cervix. Clin Cancer Res 10:8405-8412.

166. Mayer A, Wree A, Hoeckel M, Leo C, Pilch H, Vaupel P (2004) Lack of correlation between expression of HIF-1alpha protein and oxygenation status in identical tissue areas of squamous cell carcinomas of the uterine cervix. Cancer Res 64:5876-5881.

167. Mayer A, Hoeckel M, Vaupel P (2005) Carbonic anhydrase IX expression and tumor oxygenation status do not correlate at the microregional level in locally advanced cancers of the uterine cervix. Clin Cancer Res 11:7220-7225.

168. Hoogsteen IJ, Marres HA, Bussink J, van der Kogel AJ, Kaanders JH (2007) Tumor microenvironment in head and neck squamous cell carcinomas: predictive value and clinical relevance of hypoxic markers. A review. Head Neck 29:591-604.

169. Ilie M, Mazure NM, Hofman V, Ammadi RE, Ortholan C, Bonnetaud C, Havet K, Venissac N, Mograbi B, Mouroux J, Pouysségur J, Hofman P (2010) High levels of carbonic anhydrase IX in tumour tissue and plasma are biomarkers of poor prognostic in patients with non-small cell lung cancer. Br J Cancer 102:1627-1635.

170. Senger D, Wirth DF, Hynes RO (1979) Transformed mammalian cells secrete specific proteins and phosphoproteins. Cell 16:885-893.

171. Oldberg A, Franzen A, Heinegard D (1986) Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell-binding sequence. Prot Natl Acad Sci 83:8819-8823.

172. Prince CW, Butler WT (1987) 1, 25-Dihydroxyvitamin D3 regulates the biosynthesis of osteopontin, a bone-derived cell attachment protein, in clonal osteoblast-like osteosarcoma cells. Coll Relat Res 7:305-313.

173. Butler WT (1989) The nature and significance of osteopontin. Connect Tissue Res 23:123-136.

174. Reinholt FP, Hullenby K, Oldberg A, Heinegard D (1999) Osteopontin--a possible anchor of osteoclasts to bone. Proc Natl Acad Sci USA 87:4473-4475.

175. Kasugai S, Nagata T, Sodek J (1992) Temporal studies on the tissue compartmentalization of bone sialoprotein (BSP), osteopontin (OPN), and SPARC protein during bone formation in vitro. J Cell Physiol 152:467-477.

176. Flores ME, Norgard M, Heinegard D, Reinholt FP, Andersson G (1992) RGD-directed attachment of isolated rat osteoclasts to osteopontin, bone sialoprotein, and fibronectin. Exp Cell Res 201:526-530.

177. Suzuki K, Zhu B, Rittling SR, Denhardt DT, Goldberg HA, McCulloch CA, Sodek J (2002)

Colocalization of intracellular osteopontin with CD44 is associated with migration, cell fusion, and resorption in osteoclasts. J Bone Miner 8:1489-1497.

178. Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, Kawahata H, Inaguma N, Kitamura Y, Nomura S (1999) Role of osteopontin in bone remodeling caused by mechanical stress. J Bone Miner Res 6:839-849.

179. Toma CD, Ashkar S, Gray ML, Schaffer JL, Gerstenfeld LC (1997) Signal transduction of mechanical stimuli is dependent on microfilament integrity: identification of osteopontin as a mechanically induced gene in osteoblasts. J Bone Miner Res 12:1626-1636.

180. Brown LF, Berse B, Van de Water L, Papadopoulos-Serqiou A, Perruzzi CA, Manseau EJ, Dvorak HF, Senger DR (1992) Expression and distribution of osteopontin in human tissues: widespread association with luminal epithelial surfaces. Mol Biol Cell 3:1169-1180.

181. Pollack SB, Linnemeyer PA, Gill S (1994) Induction of osteopontin mRNA expression during activation of murine NK cells. J Leukoc Biol 55:398-400.

182. Young MF, Kerr JM, Termine JD, Wewer UM, McBride OQ, Fisher LW (1990) cDNA cloning, mRNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomic 7:491-502.

183. Shanmugam V, Chackalaparampil I, Kundu GC, Mukherjee AB, Mukherjee BB (1997) Altered sialylation of osteopontin prevents its receptor-mediated binding on the surface of oncogenically transformed tsB77 cells. Biochemistry 36:5729-5738.

184. Güttler A, Giebler M, Cuno P, Wichmann H, Keßler J, Ostheimer C, Söling A, Strauss C, Illert J, Kappler M, Vordermark D, Bache M (2013) Osteopontin and splice variant expression level in human malignant glioma: radiobiologic effects and prognosis after radiotherapy. Radiother Oncol 108:535-540.

185. Gimba ER, Tilli TM. Human osteopontin splicing isoforms: known roles, potential clinical applications and activated signaling pathways. Cancer Lett 331:11-17.

186. Goparaju CM, Pass HI, Blasberg JD, Hirsch N, Donington JS (2010) Functional heterogeneity of osteopontin isoforms in non-small cell lung cancer. J Thorac Oncol 5:1516-1523.

187. Weber GF, Ashkar S, Glimcher MJ, Cantor H (1996) Receptor-ligand interaction between CD44 and osteopontin (Eta-1). Science 271:509-512.

188. Katagiri YU, Sleeman J, Fujii H, Herrlich P, Hotta H, Tanaka K, Chikuma S, Yagita H, Okumura K, Murakami M, Saiki I, Chambers AF, Uede T (1999) et al. CD44 variants but not CD44s cooperate with beta1-containing integrins to permit cells to bind to osteopontin independently of arginine-glycine-aspartic acid, thereby stimulating cell motility and chemotaxis. Cancer Res 59:219-226.

189. El-Tanani MK, Campbell FC, Kurisetty V, Jin D, McCann M, Rudland PS (2006) The regulation and role of osteopontin in malignant transformation and cancer. Cytokine Growth Factor Rev 17:463-474.

190. Wang KX, Denhardt DT (2008) Osteopontin: role in immune regulation and stress responses. Cytokine Growth Factor Rev 19:333-345.

191. Weber GF, Zawaideh S, Hikita S, Kumar VA, Cantor H, Ashkar S (2002) Phosphorylation-dependent interaction of osteopontin with its receptors regulates macrophage migration and activation. J Leuk Bio 72:752-761.

192. Wang X, Louden C, Yue TL, Ellison JA, Barone FC, Solleveld HA, Feuerstein GZ (1998) Delayed expression of osteopontin after focal stroke in the rat. J Neurosci 18:2075-2083.

193. Tuck AB, Hota C, Wilson SM, Chambers AF (2003) Osteopontin-induced migration of human mammary epithelial cells involves activation of EGF receptor and multiple signal transduction pathways. Oncogene 22:1198-1205.

194. Giachelli CM, Lombardi D, Johnson RJ, Murry CE, Almeida M (1998) Evidence for a role of osteopontin in macrophage infiltration in response to pathological stimuli in vivo. Am J Pathol 152:353-358.

195. Liaw L, Skinner MP, Raines EW, Ross R, Cheresh DA, Schwartz SM, Giachelli CM (1995) The adhesive and migratory effects of osteopontin are mediated via distinct cell surface integrins. Role of alpha v beta 3 in smooth muscle cell migration to osteopontin in vitro. J Clin Invest 95:713-725.

196. Medico E, Gentile A, Lo Celso C, Williams TA, Gambarotta G, Trusolini L, Comoglio PM (2001) Osteopontin is an autocrine mediator of hepatocyte growth factor-induced invasive growth. Cancer Res 61:5861-5868.

197. Scatena M, Almeida M, Chaisson ML, Fausto N, Nicosia RF, Giachelli CM (1998) NF-kappaB mediates alphavbeta3 integrin-induced endothelial cell survival. J Cell Biol 141:1083-1093.

198. Leali D, Dell'Era P, Stabile H, Sennino B, Chambers AF, Naldini A, Sozzani S, Nico B, Ribatti D, Presta M (2003) Osteopontin (Eta-1) and fibroblast growth factor-2 cross-talk in angiogenesis. J Immunol 171:1085-109.

199. Liaw L, Almeida M, Hart CE, Schwartz SM, Giachelli CM (1994) Osteopontin promotes vascular cell adhesion and spreading and is chemotactic for smooth muscle cells in vitro. Circ Res 74:214-224.

200. Mazzone A, Parri MS, Giannessi D, Ravani M, Vaghetti M, Altieri P, Casalino L, Maltini M, Balbi M, Barsotti A, Berti S (2011) Osteopontin plasma levels and accelerated atherosclerosis in patients with CAD undergoing PCI: a prospective clinical study. Coron Artery Dis 22:179-187.

201. Panda D, Kundu GC, Lee BI, Peri A, Fohl D, Chackalaparampil I, Mukherjee BB, Li XD, Mukherjee DC, Seides S, Rosenberg J, Stark K, Mukherjee AB (1997) Potential roles of osteopontin and alphaVbeta3 integrin in the development of coronary artery restenosis after angioplasty. Proc Natl Acad Sci USA 94:9308-9313.

202. Georgiadou P, Iliodromitis EK, Kolokathis F, Mavroidis M, Andreadou I, Demopouplou M, Varounis Ch, Capetanaki Y, Boudoulas H, Kremastinos DT (2008) Plasma levels of osteopontin before and 24 h after percutaneous coronary intervention. Expert Opin Ther Targets 12:1477-1480.

203. Tuck AB, Chambers AF, Allan AL (2007) Osteopontin overexpression in breast cancer: knowledge gained and possible implications for clinical management. J Cell Biochem 102:859-868.

204. Agrawal A, Chen T, Irby R, Quackenbush J, Chambers AF, Szabo M, Cantor A, Coppola D, Yeatman TJ (2002) Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. J Nat Cancer Inst 94:513-521.

205. Salitoh Y, Kuratsu J, Takeshima H, Yamamoto S, Ushio Y (1995) Expression of osteopontin in human glioma. Its correlation with the malignancy. Lab Invest 72:55-63.

206. Coppola D, Szabo M, Boulware D, Muraca P, Alsarraj M, Chambers AF, Yeatman TJ (2004) Correlation of osteopontin protein expression and pathological stage across a wide variety of tumor histologies. Clin. Cancer Res. 2004, 10, 184-190.

207. Cho H, Kang ES, Kim YT, Kim JH (2009) Diagnostic and prognostic impact of osteopontin expression in endometrial cancer. Cancer Invest 27:313-323.

208. Fedarko NS, Jain A, Karadag A, Van Eman MR, Fisher LW (2001) Elevated serum bone sialoprotein and osteopontin in colon, breast, prostate, and lung cancer. Clin Cancer Res 7:4060-4066.

209. Petrik D, Lavori PW, Cao H, Zhu Y, Wong P, Christofferson E, Kaplan MJ, Pinto HA, Sitphin P, Koong AC, Giaccia AJ, Le QT (2006) Plasma osteopontin is an independent prognostic marker for head and neck cancers. J Clin Oncol 24:5291-5297.

210. Cook AC, Tuck AB, McCarthy S, Turner JG, Irby RB, Bloom GC, Yeatman TJ, Chambers AF (2005) Osteopontin induces multiple changes in gene expression that reflect the six "hallmarks of cancer" in a model of breast cancer progression. Mol Carcinog 43:225-236.

211. Wai PY, Kuo PC (2008) Osteopontin: regulation in tumor metastasis. Cancer Metastasis Rev

27:103-118.

212. Takafuji V, Forgues M, Unsworth E, Goldsmith P, Wang XW (2007) An osteopontin fragment is essential for tumor cell invasion in hepatocellular carcinoma. Oncogene 26:6361-6371.

213. He B, Mirza M, Weber GF (2006) An osteopontin splice variant induces anchorage independence in human breast cancer cells. Oncogene 25:2192-2202.

214. Liapis H, Flath A, Kitazawa S (1996) Integrin alpha V beta 3 expression by bone-residing breast cancer metastases. Diag Mol Pathol 5:127-135.

215. Rudzki Z, Jothy S (1997) CD44 and the adhesion of neoplastic cells. Mol Pathol 50:7-71.

216. Furger KA, Allan AL, Wilson SM, Hota C, Vantyghem SA, Postenka CO, Al-Katib W, Chambers AF, Tuck AB (2003) Beta(3) integrin expression increases breast carcinoma cell responsiveness to the malignancy-enhancing effects of osteopontin. Mol Cancer Res 1:810-819.

217. Senger DR, Brown LF, Perruzzi CA, Papadopoulos-Sergiou A, Van de Water L (1995) Osteopontin at the tumor/host interface. Functional regulation by thrombin-cleavage and consequences for cell adhesion. Ann N Y Acad Sci 760:83-100.

218. Fedarko NS, Jain A, Karadaq A, Fisher LW (2004) Three small integrin binding ligand N-linked glycoproteins (SIBLINGs) bind and activate specific matrix metalloproteinases. FASEB J 18:734-736.

219. Agnihotri R, Crawford HC, Haro H, Matrisian LM, Havrda MC, Liaw L (2001) Osteopontin, a novel substrate for matrix metalloproteinase-3 (stromelysin-1) and matrix metalloproteinase-7 (matrilysin). J Biol Chem 276:28261-28267.

220. Philip S, Bulbule A, Kundu GC (2001) Osteopontin stimulates tumor growth and activation of promatrix metalloproteinase-2 through nuclear factor-kappa B-mediated induction of membrane type 1 matrix metalloproteinase in murine melanoma cells. J Biol Chem 276:44926-44935.

221. Das R, Mahabeleshwar GH, Kundu GC (2003) Osteopontin stimulates cell motility and nuclear factor kappaB-mediated secretion of urokinase type plasminogen activator through phosphatidylinositol 3-kinase/Akt signaling pathways in breast cancer cells. J Biol Chem 278:28593-28606.

222. Oates AJ, Barraclough R, Rudland PS (1996) The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. Oncogene 13;97-104.

223. Tuck AB, Hota C, Chambers AF (2001) Osteopontin(OPN)-induced increase in human mammary epithelial cell invasiveness is urokinase (uPA)-dependent. Breast Cancer Res Treat 70:197-204.

224. Nemoto H, Rittling SR, Yoshitake H, Furuya K, Amagasa T, Tsuji K, Nifuji A, Denhardt DT, Noda M (2001) Osteopontin deficiency reduces experimental tumor cell metastasis to bone and soft tissues. J Bone Miner Res 16:652-659.

225. Fong YC, Liu SC, Huang CY, Li TM, Hsu SF, Kao ST, Tsai FJ, Chen WC, Chen CY, Tang CH (2008) Osteopontin increases lung cancer cells migration via activation of the alphavbeta3 integrin/FAK/Akt and NF-kappaB-dependent pathway. Lung Cancer 64:263-270.

226. Shevde LA, Das S, Clark DW, Samant RS (2010) Osteopontin: an effector and an effect of tumor metastasis. Curr Mol Med 10:71-81.

227. Karapanagiotou EM, Terpos E, Dilana KD, Almara C, Gkioyos I, Polyzos A, Syrigos KN (2010) Serum bone turnover markers may be involved in the metastatic potential of lung cancer patients. Med Oncol 27:332-338.

228. Li T, Zhou J, Deng Z, Fu C, Jiang H, Gao Z, Wang J, Ren H, Wang P (2009) [Expression of FGF-2 and osteopontin in non-small cell lung cancer]. Zhong Nan Da Xue Xue Bao Yi Xue Ban 34:1114-1119.

229. Pan HW, Ou YH, Peng SY, Liu SH, Lai PL, Lee PH, Sheu JC, Chen CL; Hsu HC (2003) Overexpression of osteopontin is associated with intrahepatic metastasis, early recurrence,

and poorer prognosis of surgically resected hepatocellular carcinoma. Cancer 98:119-127.

230. Denhardt DT, Chambers AF (1994) Overcoming obstacles to metastasis--defenses against host defenses: osteopontin (OPN) as a shield against attack by cytotoxic host cells. J Cell Biochem 56:48-51.

231. Crawford HC, Matrisian LM, Liaw L (1998) Distinct roles of osteopontin in host defense activity and tumor survival during squamous cell carcinoma progression in vivo. Cancer Res 5206-5215.

232. Zhang J, Takahashi K, Takahashi F, Shimizu K, Ohshita F, Kameda Y, Maeda K, Nishio K, Fukuchi Y (2001) Differential osteopontin expression in lung cancer. Cancer Letters 171:215-222.

233. Han SS, Lee SJ, Kim WJ, Ryu DR, Won JY, Park S, Cheon MJ (2013) Plasma osteopontin is a useful diagnostic biomarker for advanced non-small cell lung cancer. Tuberc Respir Dis (Seoul). 75:104-110.

234. Jin Y, Tong DY, Tang LY, Chen JN, Zhou J, Feng ZY, Shao CK (2012) Expressions of Osteopontin (OPN), αvβ3 and Pim-1 Associated with Poor Prognosis in Non-small Cell Lung Cancer (NSCLC). Chin J Cancer Res 24:103-108.

235. Rud AK, Boye K, Oijordsbakken M, Lund-Iversen M, Halvorsen AR, Solberg SK, Berge G, Helland A, Brustugun OT, Mælandsmo GM (2013) Osteopontin is a prognostic biomarker in non-small cell lung cancer. BMC Cancer 13:540.

236. Chang YS, Kim HJ, Chang J, Ahn CM, Kim SK, Kim SK (2007) Elevated circulating level of osteopontin is associated with advanced disease state of non-small cell lung cancer. Lung Cancer 57:373-380.

237. Gu T, Ohashi R, Cui R, Tajima K, Yoshioka M, Iwakami S, Sasaki S, Shinohara A, Matsukawa T, Kobayashi J, Inaba Y, Takahashi K (2009) Osteopontin is involved in the development of acquired chemo-resistance of cisplatin in small cell lung cancer. Lung Cancer 66:176-183.

238. Snitcovsky I, Leitao GM, Pasini FS, Brunialti KC, Maistro S, de Casto G Jr, Villar RC (2009) Plasma osteopontin levels in patients with head and neck cancer undergoing chemoradiotherapy. Arch. Otolaryngol. Head Neck Surg 135:807-811.

239. Mack PC, Redman MW, Chansky K, Williamson SK, Farneth NC, Lara PN Jr, Franklin WA, Le QT, Crowley JJ, Gandara DR SWOG (2008) Lower osteopontin plasma levels are associated with superior outcomes in advanced non-small-cell lung cancer patients receiving platinum-based chemotherapy: SWOG Study S0003. J Clin Oncol 26:4771-4776.

240. Isa S, Kawaguchi T, Teramukai S, Minato K, Ohsaki Y, Yonei T, Hayashibara K Fukishima M, Kawahara M, Furuse K, Mack PC (2009) Serum osteopontin levels are highly prognostic for survival in advanced non-small cell lung cancer: results from JMTO LC 0004. J Thorac Oncol 4:1104-1110.

241. Brizel DM, Dodge RK, Clough RW, Dewhirst MW (1999) Oxygenation of head and neck cancer: changes during radiotherapy and impact on treatment outcome. Radiother Oncol 53:113-117.

242. Sodhi CP, Phadke SA, Batlle D, Sahai A (2001) Hypoxia stimulates osteopontin expression and proliferation of cultured vascular smooth muscle cells: potentiation by high glucose. Diabetes 50:1482-1490.

243. Zips D, Böke S, Kroeber T, Meinzer A, Brüchner K, Thames HD, Baumann M, Yaromina A (2011) Prognostic value of radiobiological hypoxia during fractionated irradiation for local tumor control. Strahlenther Onkol 187:306-310.

244. Lukacova S, Overgaard J, Alsner J, Horsman MR (2006) Strain and tumour specific variations in the effect of hypoxia on osteopontin levels in experimental models. Radiother Oncol 80:165-171.

245. Hui EP, Sung FL, Yu BK, Wong CS, Ma BB, Lin X, Chan A, Wong WL, Chan AT (2008) Plasma osteopontin, hypoxia, and response to radiotherapy in nasopharyngeal cancer. Clin

Cancer Res 14:7080-7087.

246. Le QT, Sutphin PD, Raychaudhuri S, Yu SC, Terris DJ, Lin HS, Lum B, Pinto HA Koong AC, Giaccia AJ (2003) Identification of osteopontin as a prognostic plasma marker for head and neck squamous cell carcinomas. Clin Cancer Res 9:59-67.

247. Le QT, Kong C, Lavori PW, O'Byrne K, Erler JT, Huang X, Chan Y, Cao H, Tibshirani R, Denko N, Giaccia AJ, Koong AC (2007) Expression and prognostic significance of a panel of tissue hypoxia markers in head-and-neck squamous cell carcinomas. Int J Radiat Oncol Biol Phys 69:167-175.

248. Zhu Y, Denhardt DT, Cao H, Sutphin PD, Koong AC, Giaccia AJ, Le QT (2005) Hypoxia upregulates osteopontin expression in NIH-3T3 cells via a Ras-activated enhancer. Oncogene 24:6555-6563.

249. Said HM, Katzer A, Flentje M (2005) Response of the plasma hypoxia marker osteopontin to in vitro hypoxia in human tumor cells. Radiother Oncol 76:200-205.

250. Said HM, Hagemann C, Staab A, Stoiic, Kühnel S, Vince GH, Flentje M, Roosen K, Vordermark D (2007) Expression patterns of the hypoxia-related genes osteopontin, CA9, erythropoietin, VEGF and HIF-1alpha in human glioma in vitro and in vivo. Radiother Oncol 83:398-405.

251. Bache M, Reddemann R, Said HM (2006) Immunohistochemical detection of osteopontin in advanced head-and-neck cancer: prognostic role and correlation with oxygen electrode measurements, hypoxia-inducible-factor-1alpha-related markers, and hemoglobin levels. Int J Radiat Oncol Biol Phys 66:1481-1487.

252. Overgaard J, Eriksen JG, Nordsmark M, Alsner J, Horsman MR; Danish Head and Neck Cancer Study Group (2005) Plasma osteopontin, hypoxia, and response to the hypoxia sensitiser nimorazole in radiotherapy of head and neck cancer: results from the DAHANCA 5 randomised double-blind placebo-controlled trial. Lancet Oncol 6:757-764.

253. Overgaard J, Hansen HS, Overgaard M, Bastholt L, Berthelsen A, Specht L, Lindeløv B, Jørgensen K (1998) A randomized double-blind phase III study of nimorazole as a hypoxic radiosensitizer of primary radiotherapy in supraglottic larynx and pharynx carcinoma. Results of the Danish Head and Neck Cancer Study (DAHANCA) Protocol 5-85. Radiother Oncol 46:135-46.

254. Durand RE, Olive PL (1997) Physiologic and cytotoxic effects of tirapazamine in tumor-bearing mice. Radiat Oncol Investig 5:213-219.

255. Lim AM, Rischin D, Fisher R, Cao H, Kwok K, Truong D, McArthur GA, Young RJ, Giaccia A, Peters L, Le QT (2012) Prognostic significance of plasma osteopontin in patients with locoregionally advanced head and neck squamous cell carcinoma treated on TROG 02.02 phase III trial. Clin Cancer Res 18:301-307.

256. Blasberg JD, Pass HI, Goparaju CM, Flores RM, Lee S, Donington JS (2010) Reduction of elevated plasma osteopontin levels with resection of non-small-cell lung cancer. J Clin Oncol 28:936-941.

257. Bautista DS, Saad Z, Chambers AF, Tonkin KS; O'Malley FB, Singhal H, Tokmakejian S, Bramwell V, Harris JF (1996) Quantification of osteopontin in human plasma with an ELISA: basal levels in pre- and postmenopausal women. Clin. Biochem 29:231-239.

258. Gutierrez J, Konecny GE, Hong K, Burges A, Henry TD, Lambiase PD, Lee Wong W, Meng YG (2008) A new ELISA for use in a 3-ELISA system to assess concentrations of VEGF splice variants and VEGF(110) in ovarian cancer tumors. Clin Chem 54:597-601.

259. Závada J, Závadová Z, Zat'ovicová M, Hyrsl L, Kawaciuk I (2003) Soluble form of carbonic anhydrase IX (CA IX) in the serum and urine of renal carcinoma patients. Br J Cancer 89:1067-1071.

260. Creaney J, Yeoman D, Musk AW (2011) Plasma versus serum levels of osteopontin and mesothelin in patients with malignant mesothelioma--which is best? Lung Cancer 74:55-60.

261. Cristaudo A, Foddis R, Bonotti A, Simonini S, Vivaldi A, Guglielmi G, Ambrosino N, Canessa PA, Chella A, Lucchi M, Mussi A, Mutti L (2010) Comparison between plasma and serum osteopontin levels: usefulness in diagnosis of epithelial malignant pleural mesothelioma. Int J Biol Markers 25:164-170.

262. Zhou GX, Ireland J, Rayman P, Finke J, Zhou M (2010) Quantification of carbonic anhydrase IX expression in serum and tissue of renal cell carcinoma patients using enzyme-linked immunosorbent assay: prognostic and diagnostic potentials. Urology 75:257-261.

263. Wind TC1, Messenger MP, Thompson D, Selby PJ, Banks RE (2011) Measuring carbonic anhydrase IX as a hypoxia biomarker: differences in concentrations in serum and plasma using a commercial enzyme-linked immunosorbent assay due to influences of metal ions. Ann Clin Biochem 48:112-120.

264. Vordermark D, Said HM, Katzer A, Kuhnt T, Hängsen G, Dunst J, Flentje M, Bache M (2006) Plasma osteopontin levels in patients with head and neck cancer and cervix cancer are critically dependent on the choice of ELISA system. BMC Cancer 6:207.

265. Anborgh PH, Wilson SM, Tuck AB, Winquist E, Schmidt N, Hart R, Kon S, Maeda M, Uede T, Stitt LW, Chambers AF (2009) New dual monoclonal ELISA for measuring plasma osteopontin as a biomarker associated with survival in prostate cancer: clinical validation and comparison of multiple ELISAs. Clin Chem 55:895-903.

266. Jain S, Chakraborty G, Bulbule A, Kaur R, Kundu GC (2007) Osteopontin: an emerging therapeutic target for anticancer therapy. Expert Opin Ther Targets 11:81-90.

267. Hoskin PJ Saunders MI, Dische S (1999) Hypoxic radiosensitizers in radical radiotherapy for patients with bladder carcinoma: hyperbaric oxygen, misonidazole, and accelerated radiotherapy, carbogen, and nicotinamide. Cancer 86:1322-1328.

268. Bache M, Kappler M, Said HM, Staab A, Vordermark D (2008) Detection and specific targeting of hypoxic regions within solid tumors: current preclinical and clinical strategies. Curr Med Chem 15:322-338.

269. Weber GF (2001) The metastasis gene osteopontin: a candidate target for cancer therapy. Biochim Biophys Acta 1552:61-85.

270. Hahnel A, Wichmann H, Kappler M, Kotzsch M, Vordermark D, Taubert H, Bache M (2010) Effects of osteopontin inhibition on radiosensitivity of MDA-MB-231 breast cancer cells. Radiat Oncol 5:82.

271. Ohno K, Nishimori H, Yasoshima T, Kamiguchi K, Hata F, Fujui R, Okuya K, Kimura Y, Denno R, Kon S, Uede T, Sato N, Hirtata K (2010) Inhibition of osteopontin reduces liver metastasis of human pancreatic cancer xenografts injected into the spleen in a mouse model. Surg Today 40:347-356.

272. Zhang A, Liu Y, Shen Y, Xu Y, Li X (2009) Osteopontin silencing by small interfering RNA induces apoptosis and suppresses invasion in human renal carcinoma Caki-1 cells. Med Oncol 27:1179-1184.

273. Wai PY, Mi Z, Guo H, Sarraf-Yazdi S, Gao C, Wei J, Marroquin CE, Clary B, Kuo PC (2005) Osteopontin silencing by small interfering RNA suppresses in vitro and in vivo CT26 murine colon adenocarcinoma metastasis. Carcinogenesis 26:741-751.

274. Kou G, Shi J, Chen L, Zhang D, Hou S, Zhao L, Fang C, Zheng L, Thang X, Liang X, Zhang X, Li B, Guo Y (2010) A bispecific antibody effectively inhibits tumor growth and metastasis by simultaneous blocking vascular endothelial growth factor A and osteopontin. Cancer Lett 299:130-136.

275. Likui W, Hong W, Shuwen Z, Yuangang Y, Yan W (2011) The potential of osteopontin as a therapeutic target for human colorectal cancer. J. Gastrointest Surg 15:652-659.

276. Adwan H, Baeuerle TJ, Berger MR (2004) Downregulation of osteopontin and bone sialoprotein II is related to reduced colony formation and metastasis formation of MDA-MB-231 human breast cancer cells. Cancer Gene Ther 11:109-120.

277. Muramatsu T, Shima K, Ohta K, Kizaki H, Ro Y, Kohno Y, Abiko Y, Shimono M (2005) Inhibition of osteopontin expression and function in oral cancer cell lines by antisense oligonucleotides. Cancer Lett 217:87-95.

278. Potter C, Harris AL (2004) Hypoxia inducible carbonic anhydrase IX, marker of tumour hypoxia, survival pathway and therapy target. Cell Cycle 3:164-167.

279. Rami M, Dubois L, Parvathaneni NK, Alterio V, van Kuijk SJ, Monti SM, Lambin P, De Simone G, Supuran CT, Winum JY (2013) Hypoxia-targeting carbonic anhydrase IX inhibitors by a new series of nitroimidazole-sulfonamides/sulfamides/sulfamates. J Med Chem 56:8512-8520.

280. McDonald PC, Winum JY, Supuran CT, Dedhar S (2012) Recent developments in targeting carbonic anhydrase IX for cancer therapeutics. Oncotarget 3:84-97.

281. McIntyre A, Patiar S, Wigfield S, Li JL, Ledaki I, Turley H, Leek R, Snell C, Gatter K, Sly WS, Vaughan-Jones RD, Swietach P, Harris AL (2012) Carbonic anhydrase IX promotes tumor growth and necrosis in vivo and inhibition enhances anti-VEGF therapy. Clin Cancer Res 18:3100-3111.

282. Nieder C, Wiedenmann N, Andratschke N, Molls M (2006) Current status of angiogenesis inhibitors combined with radiation therapy. Cancer Treat Rev 32:348-364.

283. Willett CG, Duda DG, Ancukiewicz M, Shah M, Czito BG, Bentley R, Poleski M, Fujita H, Lauwers GY, Carroll M, Tyler D, Mantyh C, Shellito P, Chung DC, Clark JW, Jain RK (2010) A safety and survival analysis of neoadjuvant bevacizumab with standard chemoradiation in a phase I/II study compared with standard chemoradiation in locally advanced rectal cancer. Oncologist 15:845-851.

284. Willett CG, Kozin SV, Duda DG, di Tomaso E, Kozak KR, Boucher Y, Jain RK (2006) Combined vascular endothelial growth factor-targeted therapy and radiotherapy for rectal cancer: theory and clinical practice. Semin Oncol 33:S35-40.

285. Liu D, Cao G, Cen Y, Liu T, Peng W, Sun J, Li X, Zhou H (2013) The radiosensitizing effect of CpG ODN107 on human glioma cells is tightly related to its antiangiogenic activity via suppression of HIF-1α/VEGF pathway. Int Immunopharmacol 17:237-244.

286. Zhuang HQ, Yuan ZY, Wang P (2014) Research progress on the mechanisms of combined bevacizumab and radiotherapy. Recent Pat Anticancer Drug Discov 9:129-134.

287. http://www.ibl-japan.co.jp/files/topics/1607_ext_02_en_0.pdf, 2009-08-21.

288. http://www.rndsystems.com/pdf/dve00.pdf, 2009-08-25.

289. http://www.rndsystems.com/pdf/DCA900.pdf, 2013, 01-09.

290. Wheatley-Price P, Yang B, Patsios D, Patel D, Ma C, Xu W, Leighl N, Feld R, Cho BC, O'Sullivan B, Roberts H, Tsao MS, Tammemagi M, Anraku M, Chen Z, de Perrot M, Liu G (2010) Soluble mesothelin-related Peptide and osteopontin as markers of response in malignant mesothelioma. J Clin Oncol 28:3316-3322.

291. Bramwell VH, Doig GS, Tuck AB, Wilson SM, Tonkin KS, Tomiak A, Perera F, Vandenberg TA, Chambers AF (2006) Serial plasma osteopontin levels have prognostic value in metastatic breast cancer. Clin Cancer Res 12:3337-3343.

292. Wai PY, Kuo PC (2004) The role of Osteopontin in tumor metastasis. J Surg Res 121:228-241.

293. Singhal H, Bautista DS, Tonkin KS, O'Malley FP, Tuck AB, Chambers AF, Harris JF (1997) Elevated plasma osteopontin in metastatic breast cancer associated with increased tumor burden and decreased survival. Clin Cancer Res 3:605-611.

294. Fortis S, Khadaroo RG, Haitsma JJ, Zhang H (2015) Osteopontin is associated with inflammation and mortality in a mouse model of polymicrobial sepsis. Acta Anaesthesiol Scand 59:170-175.

295. Chiba S, Rashid MM, Okamoto H, Shiraiwa H, Kon S, Maeda M, Murakami M, Inobe M, Kitabatake A, Chambers AF, Uede T (2000) The role of osteopontin in the development of granulomatous lesions in lung. Microbiol Immunol 44:319-332.

296. O'Regan A (2003) The role of osteopontin in lung disease. Cytokine Growth Factor Rev 14:479-488.

297. Patarca R, Saavedra RA, Cantor H (1994) Molecular and cellular basis of genetic resistance to bacterial infection: the role of the early T-lymphocyte activation-1/osteopontin gene. Crit Rev Immunol 13:225-246.

298. Wolak T (2014) Osteopontin - a multi-modal marker and mediator in atherosclerotic vascular disease. Atherosclerosis 236:327-337.

299. Rangaswami H, Bulbule A, Kundu GC. Osteopontin: role in cell signaling and cancer progression. Trends Cell Biol 16:79-87.

300. Brown LF, Papadopoulos-Sergiou A, Berse B, Manseau EJ, Tognazzi K, Perruzzi CA, Dvorak HF, Senger DR (1994) Osteopontin expression and distribution in human carcinomas. Am J Pathol 145:610-623.

301. Kaya A, Ciledag A, Gulbay BE, Poyraz BM, Celik G, Sen E, Savas H, Savas I (2004) The prognostic significance of vascular endothelial growth factor levels in sera of non-small cell lung cancer patients. Respir Med 98:632-636.

302. Cheng F, Wang X, Zhong D, Sun L, Wang Q, Liu C (2015) [Significance of detection of serum carbonic anhydrase IX in the diagnosis of lung cancer]. Zhongguo Fei Ai Za Zhi 18:29-33.

303. Zhao L, Wang Y, Qu N, Huang C, Chen L (2012) Significance of plasma osteopontin levels in patients with bladder urothelial carcinomas. Mol Diagn Ther 16:311-316.

304. Nabih MI, Aref WM, Fathy MM (2014) Significance of plasma osteopontin in diagnosis of hepatitis C virus-related hepatocellular carcinoma. Arab J Gastroenterol 215:103-107.

305. Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW, Mok SC (2002) Osteopontin as a potential diagnostic biomarker for ovarian cancer. JAMA 287:1671-1679.

306. Chambers AF, Wilson SM, Kerkvliet N, O'Malley FP, Harris JF, Casson AG. Osteopontin expression in lung cancer. Lung Cancer 15:311-323.

307. Wu CY, Wu MS, Chiang EP, Wu CC, Chen YJ, Chen CJ, Chi NH, Chen GH, Lin JT (2007) Elevated plasma osteopontin associated with gastric cancer development, invasion and survival. Gut 56:782-789.

308. Phuoc NB, Ehara H, Gotoh T, Nakano M, Kamei S, Deguchi T, Hirose Y (2008) Prognostic value of the co-expression of carbonic anhydrase IX and vascular endothelial growth factor in patients with clear cell renal cell carcinoma. Oncol Rep 20:525-530.

309. Becker A, Stadler P, Krause U, Utzig D, Hänsgen G, Lautenschläger C, Rath FW, Molls M, Dunst J (2001) [Association between elevated serum VEGF and polarographically measured tumor hypoxia in head and neck carcinomas]. Strahlenther Onkol 177:182-188.

310. Rudat V, Stadler P, Becker A, Vanselow B, Dietz A, Wannenmacher M, Molls M, Dunst J, Feldmann HJ (2001) Predictive value of the tumor oxygenation by means of pO2 histography in patients with advanced head and neck cancer. Strahlenther Onkol 177:462-468.

311. Shijubo N, Uede T, Kon S, Maeda M, Segawa T, Imada A, Hirasawa M, Abe S (1999) Vascular endothelial growth factor and osteopontin in stage I lung adenocarcinoma. Am J respire Crit Care Med 160:1269-1273.

312. Shijubo N, Uede T, Kon S, Nagata M, Abe S (2000) Vascular endothelial growth factor and osteopontin in tumor biology. Crit Rev Oncog 11:135-146.

313. Solberg TD, Nearman J, Mullins J, Li S, Baranowska-Kortylewicz J (2008) Correlation between tumor growth delay and expression of cancer and host VEGF, VEGFR2, and osteopontin in response to radiotherapy. Int J Radiat Oncol Biol Phys 72:918-926.

314. Raja R, Kale S, Thorat D, Soundararajan G, Lohite K, Mane A, Karnik S, Kundu GC (2014) Hypoxia-driven osteopontin contributes to breast tumor growth through modulation of HIF1α-mediated VEGF-dependent angiogenesis. Oncogene 33:2053-2064.

315. Paliwal P, Pishesha N, Wijaya D, Conboy IM (2012) Age dependent increase in the levels of osteopontin inhibits skeletal muscle regeneration. Aging (Albany NY) 4:553-566.

316. Fuhrmann-Benzakein E, Ma MN, Rubbia-Brandt L, Mentha G, Ruefenacht D, Sappino AP, Pepper MS (2000) Elevated levels of angiogenic cytokines in the plasma of cancer patients. Int J Cancer 85:40-45.

317. Rittling SR, Chambers AF (2004) Role of osteopontin in tumour progression. Br J Cancer 90:1877-1881.

318. Karadag F, Gulen ST, Karul AB, Kilicarslan N, Ceylan E, Kuman NK, Cildag O (2011) Osteopontin as a marker of weight loss in lung cancer. Scand J Clin Lab Invest 71:690-694.

319. Hu Z, Lin D, Yuan J, Xiao T, Zhang H, Sun W, Han N, Ma Y, Di X, Gao M, Ma J, Zhang J, Cheng S, Gao Y (2005) Overexpression of osteopontin is associated with more aggressive phenotypes in human non-small cell lung cancer. Clin Cancer Res 11:4646-4652.

320. Zhao B, Sun T, Meng F, Qu A, Li C, Shen H, Jin Y, Li W (2011) Osteopontin as a potential biomarker of proliferation and invasiveness for lung cancer. J Cancer Res Clin Oncol 137:1061-1070.

321. Johns N, Stephens NA, Fearon KC. Muscle wasting in cancer. Int J Biochem Cell Biol 45:2215-2229.

322. Bache M, Kappler M, Wichmann H, Rot S, Hahnel A, Greither T, Said HM, Kotzsch M, Würl P, Taubert H, Vordermark D (2010) Elevated tumor and serum levels of the hypoxia-associated protein osteopontin are associated with prognosis for soft tissue sarcoma patients. BMC Cancer 10:132.

323. Weber GF, Lett GS, Haubein NC (2010) Osteopontin is a marker for cancer aggressiveness and patient survival. Br J Cancer 103:861-869.

324. Mountzios G, Ramfidis V, Terpos E, Syrigos KN (2011) Prognostic significance of bone markers in patients with lung cancer metastatic to the skeleton: a review of published data. Clin Lung Cancer 12:341-349.

325. Liang Y, Li H, Hu B, Chen X, Miao JB, Li T, You B, Chen QR, Fu YL, Wang Y, Hou SC (2011) Elevated circulating levels of osteopontin are associated with metastasis in advanced non-small cell lung cancer. Chin J Cancer Res 23:64-68.

326. Urquidi V, Sloan D, Kawai K, Agarwal D, Woodman AC, Tarin D, Goodison S (202) Contrasting expression of thrombospondin-1 and osteopontin correlates with absence or presence of metastatic phenotype in an isogenic model of spontaneous human breast cancer metastasis. Clin Cancer Res 8:61-74.

327. Hotte SJ, Winquist EW, Stitt L, Wilson SM, Chambers AF (2002) Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. Cancer 95:506-512.

328. Furger KA, Menon RK, Tuck AB, Bramwell VH, Chambers AF (2001) The functional and clinical roles of osteopontin in cancer and metastasis. Curr Mol Med 1:621-632.

329. Kumar S, Sharma P, Kumar D, Chakraborty G, Gorain M, Kundu GC. Functional characterization of stromal osteopontin in melanoma progression and metastasis. PLoS One 8:e69116.

330. Poon RT, Fan ST, Wong J (2001) Clinical implications of circulating angiogenic factors in cancer patients. J Clin Oncol 19:1207-1225.

331. Park MG, Oh MM, Yoon JH, Park JY, Park HS, Moon DG, Yoon DK (2012) The value of plasma osteopontin levels as a predictive factor of disease stage and recurrence in patients with bladder urothelial carcinoma: a prospective study. Kaohsiung J Med Sci 28:526-530.

332. Takenaka M, Hanagiri T, Shinohara S, Yasuda M, Chikaishi Y, Oka S, Shimokawa H, Nagata Y, Nakagawa M, Uramoto H, So T, Yamada S, Tanaka F (2013) Serum level of osteopontin as a prognostic factor in patients who underwent surgical resection for non-small-cell lung cancer. Clin Lung Cancer 14:288-294.

333. Filia A, Elliott F, Wind T, Field S, Davies J, Kukalizch K, Randerson-Moor J, Harland M, Bishop DT, Banks RE, Newton-Bishop JA (2013) Plasma osteopontin concentrations in patients with cutaneous melanoma. Oncol Rep 30:1575-1580.

334. Baisi A, Raveglia F, De Simone M, Cioffi U (2013) Micrometastasis and skip metastasis as predictive factors in non-small-cell lung cancer staging. Eur J Cardiothorac Surg 43:1075.

335. Soliman M, Yaromina A, Appold S, Zips D, Reiffenstuhl C, Schreiber A, Thames HD, Krause M, Baumann M (2013) GTV differentially impacts locoregional control of non-small cell lung cancer (NSCLC) after different fractionation schedules: subgroup analysis of the prospective randomized CHARTWEL trial. Radiother Oncol 106:299-304.

336. Ball DL, Fisher RJ, Burmeister BH, Poulsen MG, Graham PH, Penniment MG, Vinod SK, Krawitz HE, Joseph DJ, Wheeler GC, McClure BE (2013) The complex relationship between lung tumor volume and survival in patients with non-small cell lung cancer treated by definitive radiotherapy: a prospective, observational prognostic factor study of the Trans-Tasman Radiation Oncology Group (TROG 99.05). Radiother Oncol 106:305-311.

337. Carlinfante G, Vassiliou D, Svensson O, Wendel M, Heinegård D, Andersson G (2003) Differential expression of osteopontin and bone sialoprotein in bone metastasis of breast and prostate carcinoma. Clin Exp Metastasis 20:437-444.

338. Wong CC, Kai AK, Ng IO (2014) The impact of hypoxia in hepatocellular carcinoma metastasis. Front Med 8:33-41.

339. Anborgh PH, Mutrie JC, Tuck AB, Chambers AF. Role of the metastasis-promoting protein osteopontin in the tumour microenvironment. J Cell Mol Med 14:2037-2044.

340. Rodrigues LR, Teixeira JA, Schmitt FL, Paulsson M, Lindmark-Mänsson H (2007) The role of osteopontin in tumor progression and metastasis in breast cancer. Cancer Epidemiol Biomarkers Prev 16:1087-1097.

341. El-Tanani MK (2008) Role of osteopontin in cellular signaling and metastatic phenotype. Front Biosci 13:4276-4284.

342. Shojaei F, Scott N, Kang X, Lappin PB, Fitzgerald AA, Karlicek S, Simmons BH, Wu A, Lee JH, Bergqvist S, Kraynov E (2012) Osteopontin induces growth of metastatic tumors in a preclinical model of non-small lung cancer. J Exp Clin Cancer Res 31:26.

343. Blasberg JD, Goparaju CM, Pass HI, Donington JS (2010) Lung cancer osteopontin isoforms exhibit angiogenic functional heterogeneity. J Thorac Cardiovasc Surg 139:1587-1593.

344. Erpolat OP, Gocun PU, Akmansu M, Ozgun G, Akyol G. Hypoxia-related molecules HIF-1α, CA9, and osteopontin: predictors of survival in patients with high-grade glioma. Strahlenther Onkol 189:147-154.

345. Donati V, Boldrini L, Dell'Omodarme M, Prati MC, Faviana P, Camacci T, Lucchi M, Mussi A, Santoro M, Basolo F, Fontanini G (2005) Osteopontin expression and prognostic significance in non-small cell lung cancer. Clin Cancer Res 11:6459-6465.

346. Hasegawa Y, Takanashi S, Okudera K, Kumagai M, Hayashi A, Morimoto T, Okumura K (2005) Vascular endothelial growth factor level as a prognostic determinant of small cell lung cancer in Japanese patients. Intern Med 244:26-34.

347. Zhan P, Qian Q, Yu LK (2013) Serum VEGF level is associated with the outcome of patients with hepatocellular carcinoma: a meta-analysis. Hepatobiliary Surg Nutr 2:209-215.

348. De Schutter H, Landuyt W, Verbeken E, Goethals L, Hermans R, Nuyts S (2005) The prognostic value of the hypoxia markers CA IX and GLUT 1 and the cytokines VEGF and IL 6 in head and neck squamous cell carcinoma treated by radiotherapy +/- chemotherapy. BMC Cancer 5:42.

349. Senger DR, Ledbetter SR, Claffey KP, Padapopoulos-Serqiou A, Peruzzi CA, Detmar M (1996) Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol 149:293-305.

350. Adams A, van Brussel AS, Vermeulen JF, Mali WP, van der Wall E, van Diest PJ, Elias SG (2013) The potential of hypoxia markers as target for breast molecular imaging--a systematic review and meta-analysis of human marker expression. BMC Cancer 13:538.

351. Ilie M, Hofman V, Zangari J, Chiche J, Mouroux J, Mazure NM, Pouysségur J, Brest P, Hofman P (2013) Response of CAIX and CAXII to in vitro re-oxygenation and clinical significance of the combined expression in NSCLC patients. Lung Cancer 82:16-23.

352. Dehing-Oberije C, Aerts H, Yu S, De Ruysscher D, Menheere P, Hilvo M, van der Weide H, Rao B, Lambin P (2011) Development and validation of a prognostic model using blood biomarker information for prediction of survival of non-small-cell lung cancer patients treated with combined chemotherapy and radiation or radiotherapy alone (NCT00181519, NCT00573040, and NCT00572325). Int J Radiat Oncol Biol Phys 81:360-368.

353. Yaromina A, Quennet V, Zips D, Meyer S, Shakirin G, Walenta S, Mueller-Klieser W, Baumann M (2009) Co-localisation of hypoxia and perfusion markers with parameters of glucose metabolism in human squamous cell carcinoma (hSCC) xenografts. Int J Radiat Biol 85:972-980.

354. Zips D, Zöphel K, Abolmaali N, Perrin R, Abramyuk A, Haase R, Appold S, Steinbach J, Kotzerke J, Baumann M (2012) Exploratory prospective trial of hypoxia-specific PET imaging during radiochemotherapy in patients with locally advanced head-and-neck cancer. Radiother Oncol 105:21-28.

355. Hu M, Kong L, Zhao SQ, Yang GR, Yang WF, Han AQ, Fu Z, Ma L, Zheng JS, Yu JM (2010) [Value of 18F-FETNIM PET-CT for detection of tumor hypoxia in non-small-cell lung cancer]. Zhonghua Zhong Liu Za Zhi 32:463-466.

356. Arabi M, Piert M (2010) Hypoxia PET/CT imaging: implications for radiation oncology. Q J Nucl Med Mol. 54:500-509.

357. Tachibana I, Nishimura Y, Shibata T, Kanamori S, Nakamatsu K, Koike R, Nishikawa T, Ishikawa K, Tamura M, Hosono M (2013) A prospective clinical trial of tumor hypoxia imaging with 18F-fluoromisonidazole positron emission tomography and computed tomography (F-MISO PET/CT) before and during radiation therapy. J Radiat Res 54:1078-1084.

358. Vera P, Bohn P, Edet-Sanson A, Salles A, Hapdey S, Gardin I, Ménard JF, Modzelewski R, Thiberville L, Dubray B (2011) Simultaneous positron emission tomography (PET) assessment of metabolism with ^{18}F-fluoro-2-deoxy-d-glucose (FDG), proliferation with ^{18}F-fluoro-thymidine (FLT), and hypoxia with ^{18}fluoro-misonidazole (F-miso) before and during radiotherapy in patients with non-small-cell lung cancer (NSCLC): a pilot study. Radiother Oncol 98:109-116.

359. Graves EE, Maity A, Le QT (2010) The tumor microenvironment in non-small-cell lung cancer. Semin Radiat Oncol 20:156-163.

360. Kessler J, Hahnel A, Wichmann H, Rot S, Kappler M, Bache M, Vordermark D (2010) HIF-1α inhibition by siRNA or chetomin in human malignant glioma cells: effects on hypoxic radioresistance and monitoring via CA9 expression. BMC Cancer 10:605.

361. Bandopadhyay M, Bulbule A, Butti R, Chakraborty G, Ghorpade P, Ghosh P, Gorain M, Kale S, Kumar D, Kumar S, Totakura KV, Roy G, Sharma P, Shetti D, Soundararajan G, Thorat D, Tomar D, Nalukurthi R, Raja R, Mishra R, Yadav AS, Kundu GC (2014) Osteopontin as a therapeutic target for cancer. Expert Opin Ther Targets 18:883-895.

362. Joseph S, Harrington R, Walter D, Goldberg JD, Li X, Beck A, Litton T, Hirsch N, Blasberg J, Slomiany M, Rom W, Pass H, Donington J (2012) Plasma osteopontin velocity differentiates lung cancers from controls in a CT screening population. Cancer Biomark12:177-184.

Theses

1. OPN plasma levels do not significantly change during radiotherapy, but notably (even though not significantly) decrease after radical radiotherapy of NSCLC.
2. OPN plasma levels before, during and after radiotherapy are interrelated, OPN baseline plasma levels are positively correlated with CAIX and VEGF in NSCLC M0 patients and baseline VEGF and OPN inversely correlate with hemoglobin.
3. Baseline OPN plasma levels are associated with age, gender, poor patient oxygenation (FeV1, hemoglobin) and clinical parameters indicating advanced / aggressive tumor disease (T-stage, weight loss); OPN plasma levels at all measurement time points are significantly elevated in metastasized NSCLC patients (compared to M0-stage patients).
4. Baseline OPN, VEGF and CAIX plasma levels are related to tumor burden (i.e. metastasis, tumor volume/GTV, N-stage) and absolute OPN (but not CAIX and VEGF) plasma levels before, at the end and 4 weeks after radiotherapy predict MFS as do OPN plasma level changes after radical radiotherapy of NSCLC.
5. Baseline biomarker plasma levels are not associated with tumor control and therapy response after radiotherapy but end-of-treatment OPN plasma levels predict tumor response and time to progression in NSCLC M0 patients.
6. Decreasing OPN plasma levels during radiotherapy predict superior therapy response and time to progression, post-treatment OPN plasma level changes are related to tumor control (freedom from local relapse) in NSCLC M0 patients.
7. Absolute OPN plasma levels before, 4 weeks after radiotherapy and relative OPN plasma level changes after therapy remain independent predictors for OS after radical radiotherapy of NSCLC.
8. Baseline VEGF, CAIX plasma levels and OPN plasma level changes during radiotherapy are not associated with OS or PFS (univariate analysis).
9. OPN plasma levels at the end, 4 weeks after radiotherapy and post-treatment OPN plasma level changes predict PFS after radical radiotherapy of NSCLC.
10. The prognostic effect of a single biomarker is augmented by combining biomarkers with the triple marker combination OPN-VEGF-CAIX showing the most prominent impact on prognosis (OS / PFS) in both univariate and multivariate analysis.

Curriculum Vitae

Personal Data:

Name, prename:	Ostheimer, Christian, Emil, Arthur
Adress:	Carl-von-Ossietzky-Strasse 11, 06114 Halle (Saale)
Telephone:	+49-345-5239813
Mobile:	+49-152-03372793
Fax:	+49-9621-308941
Email:	christian.ostheimer@uk-halle.de
Birthdate:	04.12.1984
Birthplace:	Munich
Nationality:	German

Pirmary education:

1991 – 1996	Poppenricht elementary school Poppenricht, Germany
1996 – 2005	Herzog-Christian-August-High School High school diploma (German „Abitur“) Sulzbach-Rosenberg, Germany
2005 – 2012	Medical school Martin-Luther-University Halle-Wittenberg Halle/Saale, Germany

Nursing clerkships:

29.08. – 27.09.2005	St. Anna Hospital (Internal Medicine) Sulzbach-Rosenberg, Germany
20.02. – 23.03.2006	St. Anna Hospital (Surgery) Sulzbach-Rosenberg, Germany
24.02. – 25.03.2007	St. Anna Hospital (Intensive care unit) Sulzbach-Rosenberg, Germany

Medical clerkships:

18.02. – 18.03.2008	St. Anna Hospital Internal Medicine (Gastroenterology) Sulzbach-Rosenberg, Germany
01.08. – 01.09.2008	General Hospital Vienna (AKH) Anaesthesiology Vienna, Austria
08.02. – 22.02.2009	Martin-Luther-University Halle-Wittenberg

	Nuclear Medicine Halle/Saale, Germany
23.02. – 24.03.2009	Private Practice for Cardiology Prof. Dr. Osterziel Amberg, Germany
18.07. – 01.08.2009	Martin-Luther-University Halle-Wittenberg Radiation Oncology Halle/Saale, Germany
12.02. – 12.03.2010	National Defense Medical Center Tri-Service-General-Hospital Radiology Taipei, Taiwan R.O.C.

Internships:

21.02. – 12.06.2011	National Defense Medical Center Tri-Service-General-Hospital Chest Surgery (Lung Cancer Division) General Surgery (Liver Cancer Division) Taipei, Taiwan R.O.C.
13.06. – 02.10.2011	Martin-Luther-University Halle-Wittenberg Radiation Oncology Halle/Saale, Germany
03.10. – 22.12.2011	Elisabeth-Hospital Halle Internal Medicine / Oncology Halle/Saale, Germany
03.01. – 27.01.2012	Memorial-Sloan-Kettering-Cancer Center Dept. for Gastroenterologic Malignancies (Pancreatic Cancer Division) New York, USA

Medical licensing exam:

12.09.2007	First German medical licensing exam
06.11.2012	Secong German medical licensing exam
07.11.2012	Medical license (German „Approbation“)

Residency:

Since 01.12.2012	Resident in Radiation Oncology Martin-Luther-University Halle-Wittenberg Halle/Saale, Germany

Awards:

2011	Young Researchers Award & Scholarship of the 20th symposium experimental radiation oncology and clinical radiation biology of the national research center for radiation oncology (OncoRay), Dresden, Germany
2013	Hasik-Seide-Award 2013 of the Karol-Marcinkowski Medical University Poznan, Poland
2014	Scholarship of the German Cancer Aid Fund and the German Cancer Society for the 31st annual German Cancer Congress 2014, Berlin, Germany

Memberships:

- German Society for Radiation Oncology (Deutsche Gesellschaft für Radioonkologie, DEGRO), *full member*
- European Society of Radiation Oncology (ESTRO), *in-training member*

Languages:

German (native speaker)
English (C2-level, CAE, TOEFL)
French (B2-level)
Chinese (B1-level, HSK 1 und 2)

Christian Ostheimer

Statement of autonomy

I hereby declare that I created the presented work without non-permitted help of third parties and without use of other than the herein mentioned resources. Concepts and data directly or indirectly taken from other sources are marked by stating the sources. The rules of assurance of good scientific practice have been respected (Amtsblatt der MLU Nr. 5, 02.07.09).

I further declare that I did not make use of nongratuitous help from consultation services (counselors for conferral of a doctorate or other persons) for the creation of this work with regards to content. Nobody has been given direct or indirect monetary values by me which are related to the content of the presented work.

This work in its present or a similar form has not been submitted to any other examination authority neither domestic nor abroad.

Christian Ostheimer

Statement of prior conferral of a doctorate

I hereby testify that no prior attempt of conferral of a doctorate has been made by me and that there is no current attempt of conferral of a doctorate made by me at another university.

Christian Ostheimer

Acknowledgment

I want to express my sincerest gratitude to my doctoral advisor, Prof. Dr. med. Dirk Vordermark for granting me the opportunity for this work and his highly appreciated counseling.

In addition, I would like to thank especially Dr. Matthias Bache for providing the research environment and equipment for this work.

I am deeply indebted to my parents, Mr. Dieter and Ms. Rita Ostheimer, for their continuous support.