Identification of prognostic biomarkers in patients undergoing pulmonary metastasectomy from primary colorectal cancer

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Doctor of Philosophy

Submitted by

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“When a plant goes to seed, its seeds are carried in all directions; but they can only live and grow if they fall on congenial soil.”

Stephen Paget, English surgeon (1855 - 1926)
Declaration

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Abstract

Pulmonary metastases (PM) contribute to the high cancer-related morbidity and mortality in patients with solid malignancies including colorectal cancer (CRC). Despite advances in the diagnosis and treatment of patients with early-stage CRC, the optimal treatment of patients with distant metastases from CRC - especially PM - is currently unclear. To improve the clinical outcome of these patients, the biology of metastatic CRC needs to be further elucidated.

In this work we aimed to describe clinical as well as histopathological and genetic factors contributing to the diminished prognosis in patients with an unfavorable tumor biology. On DNA level, we assessed the mutation status of KRAS and BRAF, which are thought to play an important role in CRC progression. Moreover, the expression of EGFR in PM was described. In the second part of this work we focused on the tumor-surrounding stroma. To the best of our knowledge, we could describe for the first time that Hsp27 is highly expressed by activated fibroblasts adjacent to the disseminated tumor cells. Moreover, these activated fibroblasts are a prognostic marker after pulmonary metastasectomy.

In this work we demonstrated that patients with PM from CRC are a highly heterogenous group regarding the tumor biology and the outcome after pulmonary metastasectomy can be prognosticated by the underlying tumor biology. The accurate description of PM based on molecular markers might lead to an improved treatment of patients with metastatic CRC.
Zusammenfassung

Publications arising from this thesis


Abbreviations

95%CI... 95% confidence interval
alpha-SMA... alpha smooth muscle actin
CEA... carcinoembryonic antigen
COX-2... cyclooxygenase-2
CRC... colorectal cancer
CT... computed tomography
CTC... circulating tumor cells
CXCL... C-X-C motif ligand
DFI... disease-free interval
EGFR... epidermal growth factor receptor (EGFR).
EMT... epithelial-mesenchymal transition
FAP... fibroblast-activation protein alpha
Hsp27... heat shock protein 27
KRAS... Kirsten rat sarcoma viral oncogene homolog
MVD... microvessel density
Nd:YAG... neodymium-doped yttrium aluminium garnet
NFkappa-B... nuclear factor kappa-light-chain-enhancer of activated B-cells
NRAS... Neuroblastoma RAS viral oncogene homolog
OR... Odd’s ratio
PI3K... phosphatidylinositol 3-kinase
TGF beta... transforming growth factor beta
TKR... tyrosine kinase receptors
TRAIL-R2... Tumor necrosis factor-related apoptosis-inducing ligand receptor 2
VEGF... vascular endothelial growth factor
Wnt... Wingless-related integration site
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CHAPTER ONE: INTRODUCTION

1. General introduction

In Europe, colorectal cancer (CRC) is the most commonly diagnosed type of cancer. In 2012, the age-standardized incidence in male was 55.7/100,000 and in female 34.7/100,000, resulting in an overall incidence of 43.5/100,000. Regarding the mortality, CRC is the second most common cause of cancer-related deaths, following lung cancer (Ferlay et al, 2013). Similar to other malignancies, distant metastasis is the cause of the high mortality of patients with CRC (Vatandoust et al, 2015).

1.1 Epidemiology of CRC pulmonary metastases

Second to the liver, the lungs are the most common site of distant spreading of CRC. During the course of disease, about 10-15% of all patients diagnosed with CRC will develop pulmonary metastasis (Mitry et al, 2010; Pihl et al, 1987). Metastasis can occur either synchronously, i.e. metastases are present at the time of diagnosis of the primary tumor, or metachronously, which means that the metastases are diagnosed during follow-up examinations. The median time between diagnosis of primary CRC and lung metastasis is 24.6 months, which is about 7 months longer than the average time to occurrence of liver metastases (Manfredi et al, 2006). This reflects the model of a stepwise metastatic spread of CRC, usually starting with the liver as first site of metastasis, afterwards involving the lung and finally further distant organs like the brain or the bones. Compared to colon cancer, patients with rectal cancer have a significantly higher risk of pulmonary metastases which is true for both, synchronous (OR (95%CI) 2.80 (1.65 to 4.76)) and metachronous (OR (95%CI) 2.63 (1.69 to 4.08)) metastatic spreading. This can be explained by tumor cells bypassing the portal system through rectal veins and thus reaching the lungs as first filter organ. Patients with isolated lung metastasis have a significantly better overall survival than patients with at least one more site of metastatic spread, independently whether the lung metastases are synchronous or metachronous (Mitry et al, 2010).
1.2 Pathobiology of CRC lung metastases

The metastatic cascade comprises (1) local invasion at the primary tumor site, (2) intravasation into blood or lymphatic vessels, (3) circulating to the target organ, (4) arrest at the metastatic site, (5) extravasation and (6) outgrowth at the secondary organ (Valastyan & Weinberg, 2011).

Local invasion is characterized by the disintegration of the basement membrane, the degradation of the surrounding extracellular matrix and the detachment of the tumor cells. The process of detachment is called epithelial-mesenchymal-transition (EMT), which occurs physiologically during embryogenesis (Kalluri & Weinberg, 2009). Mainly by downregulation of E-cadherin by the transcription factor SNAIL, tumor cells can detach from the cell sheets and invade in the surrounding parenchyma and get in close contact to vessels (Oda et al, 1998; Thiery et al, 2009).

The intravasation into lymphatic vessels and blood vessels is a further keystep in the metastatic process. Evidence of lymphatic vessel invasion in the primary tumor is an important clinical prognosticator in various malignancies including CRC (Ishii et al, 2009). Our group could demonstrate that lymphatic invasion is of prognostic value in metastatic CRC (Schweiger et al, 2015). The intravasation of tumor cells in tumor-adjacent vessels is facilitated by the poor quality of intratumoral vessels, formed by excessive angiogenesis (Carmeliet & Jain, 2011).

As soon as the tumor cells reach the lumen of vessels, these tumor cells en route to metastatic organ sites are detectable in the peripheral blood and are termed circulating-tumor cells (CTC). The presence of these cells, which are a pre-requisite for metastatic disease, are also an negative prognostic marker in CRC (Romiti et al, 2014). CTC have to overcome several factors to form distant metastases. First of all, normal cells detached from their surrounding undergo anoikis. This programmed cell-death due to the loss of anchorage needs to be suppressed (Guo & Giancotti, 2004). Additionally, CTC have to be resistant to shear stress. Interestingly this seems to be mediated by coverage of the CTC with thrombocytes. By the same mechanism CTC can escape cytotoxic immune cells until they reach a distant capillary bed (Joyce & Pollard, 2009).

Whether the arrest of the tumor cell at the target site is a passive process (tumor microemboli) or merely a site-specific process is currently under discussion. Hematogenous dissemination is believed to be main route of metastasis to the lung, which is especially true for CRC (Woodard et al, 1998). As previously mentioned, in patients with rectal cancer metastases can be found more often in the lungs as initial metastatic site, compared to colon cancer in general and especially compared to right-sided colon cancer (Mitry et al, 2010). Thus, the first capillary bed encountered by a circulating tumor cell determined by vascular
anatomy is a key determinant of the metastatic process. However, there are limitations to this theory, which were firstly described by the English surgeon Stephen Paget (1855 - 1926) and the Viennese ophthalmologist Ernst Fuchs (1851 – 1930). Based on the observation that the frequency of metastases at different target organ sites can not be explained solely by anatomical considerations, the “seed and soil”-theory was proposed by Paget based on his findings in patients with breast cancer and a work published seven years before by Fuchs on patients with “uveal sarcoma” (Fuchs, 1882; Paget, 1889). According to this theory, the target organ site is also determined by characteristics of the tumor cell (“seed”) and the microenvironment of the secondary organ site (“soil”). Based on this hypothesis, the so-called metastatic niche, corresponding to the “soil”, has gained the attention of today’s oncological research (Psaila & Lyden, 2009). According to this model, the target organ forms a conductive environment by various mechanisms. For instance, by up-regulation of matrix-metalloproteinases, the extracellular matrix at the niche is degraded and pro-inflammatory cytokines are released, facilitating the extravasation and outgrowth of metastatic cells (Hiratsuka et al, 2002). Moreover, a site-specific colonization mediated by stromal-derived factor-1 (released by the target organ) and CXC-chemokine receptor 4 (expressed by the tumor cell) has been described (Feys et al, 2015).

The extravasation of intra-luminal CTC is the next step in the metastatic cascade. By the disintegration of endothelial cells and pericytes, tumor cells can advance into the parenchyma of the metastatic organ site and can therefore be seen as the opposite of intravasation. The underlying mechanisms are a matter of current research. Proteins increasing the permeability of capillaries like COX-2, VEGF and matrix-metalloproteinases were demonstrated to be secreted by tumors (Gupta et al, 2007). Interestingly, by releasing mediators capable to increase the permeability of the capillary bed, already the primary tumor can prime the secondary organ site (“pre-metastatic niche”) to facilitate the extravasation as soon as CTC arrive (Huang et al, 2009).

Finally, the metastatic tumor cells need to survive and grow at the distant organ site, despite the distinct environment. It was proposed that also the microenvironment is already modulated at an earlier stage of cancer progression (Psaila & Lyden, 2009). Cell proliferation needs to outnumber cell death at the distant site to grow out to a micro- and further on to a macro-metastasis. Increased neoangiogenesis is thought to be the prerequisite for the outgrowth as it confers the supply with oxygen and nutrients (Klauber-DeMore et al, 2001).
1.3 Clinical prognostic factors in pulmonary metastasectomy

The pre-existing literature describes mainly clinical risk factors for a decreased outcome after pulmonary metastasectomy. The most frequently discussed clinical prognostic factors are summarized in the following.

**Carcinoembryonic antigen**
The blood level of the tumor marker carcinoembryonic antigen (CEA) is thought to reflect the total tumor burden in CRC patients (Choi et al., 2005). Moreover, it was reported that an increased serum CEA level before pulmonary metastasectomy prognosticates a worse outcome after pulmonary metastasectomy. Suzuki et al. found a significantly decreased disease-specific survival for patients with elevated pre-metastasectomy CEA levels (5-year survival 57.0% vs. 30.9%) (Suzuki et al., 2015), without describing the used cut-off. In a cohort of 199 patients undergoing PM, CEA levels > 5ng/mL were associated with a significantly reduced overall survival (5-year survival 55% vs. 28%) (Zampino et al., 2014). In another study cohort Pfannschmidt et al. also applied a 5 ng/mL cut-off and found a 5-year survival of 48.3% for patients with low CEA levels and a 5-year survival rate of 22.7% for patient with elevated serum levels (Pfannschmidt et al., 2003). Additionally, a large multicenter study in Japan including 1030 patients confirmed these findings in univariate and multivariate survival analysis (Iida et al., 2013). 5-year survival was 60.4% in the group with normal CEA levels, compared to 43.4% in patients with increased serum levels.

**Completeness of resection**
A microscopic (R1) or even macroscopic (R2) incomplete resection is the most important and most widely accepted clinical prognosticator for decreased outcome after pulmonary metastasectomy. In an international survey of the European Society of Thoracic Surgeons, 98% of the participating surgeons consider an expected incomplete resection of pulmonary metastases as absolute (74%) or relative (24%) contraindication for pulmonary metastasectomy (Internullo et al., 2008). The reported 5-year survival drops to 8% -14% in patients with incomplete resection compared to 5-year survival rates in the respective studies of 47% - 55.8% for macroscopically and microscopically complete resection (Iida et al., 2013; Zampino et al., 2014). Moreover, an international registry (International Registry of Lung Metastases) including 5206 patients undergoing pulmonary metastasectomy confirmed the completeness of resection as a valuable prognostic marker (Pastorino et al., 1997). Thus, pulmonary metastasectomy should only be offered to patients if the pre-operative work-up
suggests that a complete resection of all pulmonary metastases is technically (and functionally) feasible.

Disease-free survival
The disease-free interval (DFI), which describes the time between the diagnosis of the primary tumor and distant metastases, is thought to reflect an aggressive tumor biology and thus is another proposed prognostic factor in various malignancies. The International Registry of Lung Metastases identified a DFI less than 36 months as prognosticator for decreased overall survival after metastasectomy. In other studies on pulmonary metastasectomy for metastatic CRC, the reported cut-off varies between 6 months and 60 months. Significant differences in survival dependent on the DFI could be demonstrated by some of the studies (Hwang et al., 2010; Onaitis et al., 2009; Yedibela et al., 2006). Although it appears to be legitimate to use the DFI as a measurable parameter for rapidly spreading tumors, most of the studies assessing clinical prognostic markers in pulmonary metastasectomy for CRC could not confirm the DFI as statistically significant (Iizasa et al., 2006; Kanemitsu et al., 2004; Melloni et al., 2006; Pfannschmidt et al., 2003; Saito et al., 2002; Suzuki et al., 2015; Welter et al., 2007a; Zampino et al., 2014). This might be due to various confounders like time of diagnosis of the primary tumor, different follow-up intervals, patients’ compliance, etc. Thus, patients with a short DFI should not be excluded from surgery. Excellent long-term outcome is achievable despite early evidence of pulmonary recurrence.

Lymph node involvement
In primary CRC, the evidence of lymph node metastases is an important prognostic factor and thus part of the staging system (TNM staging) of primary CRC (Gertler et al., 2009). Interestingly, in patients with pulmonary metastasis from CRC, the involvement of thoracic, i.e. hilar and mediastinal lymph nodes, has gained special attention in the scientific community as relatively novel prognostic factor. Patients with pulmonary CRC metastases and evidence for thoracic LN metastases have a dramatically decreased prognosis. For instance, in the Japanese multicenter study including 1030 patients, 5-year survival for patients with pathologically proven thoracic lymph node metastasis was 37.3%, compared to 59.4% in patients without evidence for lymph node involvement. In the vast majority of other studies, the 5-year survival drops to <20% as soon as thoracic lymph node metastases are detected (Iizasa et al., 2006; Inoue et al., 2004; Pfannschmidt et al., 2003; Renaud et al., 2014; Welter et al., 2007b; Zampino et al., 2014). Interestingly, the presence of lymph node metastases has only prognostic implications, the resection of these lymph node metastases does not add any survival benefit (Bolukbas et al., 2014; Renaud et al., 2014). These findings
suggest, that patients with CRC lung metastases and thoracic lymph node involvement, which corresponds to “metastasis-from-metastasis”, do not benefit from surgery. Our group could demonstrate in another recent study that evidence for infiltration to the lymphatic system in the pulmonary metastases is capable to predict the occurrence of lymph node metastases (Schweiger et al, 2015). Moreover, presence of lymphovascular invasion was associated with a diminished prognosis after pulmonary metastasectomy (see Figure 1).

Figure 1: Overall survival of patients with CRC pulmonary metastases exhibiting sustained lymphangiogenesis (left) or with evidence of lymphatic invasion (right) have a diminished prognosis after pulmonary metastasectomy. The outcome is comparable to patients with pathologically verified thoracic lymph node metastasis (Group B; dashed line). Adopted from „Increased lymphangiogenesis in lung metastases from colorectal cancer is associated with early lymph node recurrence and decreased overall survival“ (Schweiger et al, 2015).

Number of nodules
Another proposed prognostic factor in patients undergoing pulmonary metastasectomy is the number of resected metastases (see Figure 2). Most studies differentiate between singular versus multiple metastases. Iida et al. described multiple metastases as significant poor prognosticator regarding the overall survival after pulmonary metastasectomy in univariate and multivariate analysis (5-year survival 42.0% versus 61.5%) (Iida et al, 2013). Also Zampino et al. found a significantly different survival between patients with single and multiple pulmonary metastases. 5-year survival was 51% for singular metastases compared to 44% for multiple metastases. After 10-year almost twice as many patients with single metastases were alive compared to the group of patients with multiple nodules (40% versus 23%) (Zampino et al, 2014). These recent works confirmed the prognostic impact of the number of pulmonary metastases, which was also described by several works on pulmonary metastasectomy for metastatic CRC in the past (Chen et al, 2009; Inoue et al, 2004; Kanemitsu et al, 2004; Onaitis et al, 2009; Pfannschmidt et al, 2003; Rama et al, 2009; Saito et al, 2002; Welter et al, 2007b; Yedibela et al, 2006). Moreover, the number of metastases
was also described as prognostic factor in the International Registry of Lung Metastases, which found a 5-year survival for patients with single metastasis of 43%, compared to 34% for patients with 2-3 metastases and 27% for patients with 4 or more metastases.

Figure 2: CT scan (A), PET scan (B) and merged PET-CT (C) showing strong 18F-FDG uptake of two lung metastases in a patient with a history of colorectal cancer.
1.4 Molecular prognostic factors in pulmonary metastasectomy

Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers (published PDF)

Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers

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INTRODUCTION

Since the first case report on a surgically removed pulmonary nodule by Weinlechner [1] in 1882, pulmonary metastasectomy has evolved into a standard procedure in patients with lung metastases. Widely accepted selection criteria are (i) a controlled or controllable primary tumour, (ii) the absence of extrathoracic metastases, (iii) the patient must be able to tolerate the degree of the planned lung resection and (iv) the non-availability of another superior effective therapy [2].

Although resection of pulmonary metastases is routinely performed in a variety of primary tumours, little is known on prognostic factors influencing the outcome of pulmonary metastasectomy. In times of individualized patient treatment, this knowledge is of great importance. Patients with a high risk of early tumour recurrence might benefit from adjuvant chemotherapy regimens. On the contrary, surveillance strategies may be sufficient for intermediate or low-risk patients. Moreover, a mapping of prognostic factors might help in the decision-making process when offering patients a resection. A localized removal of pulmonary nodules is not recommended if a recurrence within weeks is expected. Important clinical prognostic factors have been proposed by the International Registry of Lung Metastases (IRLM) including a short disease-free interval, multiple pulmonary nodules and an incomplete surgical resection [3]. However, contradicting studies can be found for other well-established clinical markers in regard to their prognostic impact after pulmonary metastasectomy [4, 5]. Thus, factors reflecting the tumour biology might be more accurate in defining the prognosis of patients with pulmonary tumour spread [6]. This review focuses on the prognostic markers predicting the clinical outcome in patients undergoing pulmonary metastasectomy that can be identified by molecular, biological and radiological methods.

Using tissue or body fluid samples of patients to predict the individual risk of a certain disease or the success of a therapeutic approach has nowadays become routine in many diseases. In addition, biological markers are also of prognostic value in the course of some diseases [7]. The quest for reliable biomarkers is an important field of today’s research in general and cancer research in particular. Compared with the bulk of literature regarding markers in primary tumours, little data are available on biological markers in pulmonary metastasectomy. Currently, evidence in literature is mostly based on small series of patients with primary colorectal carcinoma (CRC), melanoma and sarcoma.

In order to provide a clear overview, reviewed markers will be grouped into soluble tumour markers, cell adhesion, tumour growth, tumour suppressor genes/proto-oncogenes, cell metabolism and tumour angiogenesis (Fig. 1).

Soluble tumour markers

Soluble forms of tumour-associated proteins can be measured in blood samples to substantiate a diagnosis and for surveillance...
purposes in the follow-up of patients with malignant disease. Some soluble factors have also been proposed to be capable of predicting the outcome of patients undergoing pulmonary metastasectomy.

Carcinoembryonic antigen. A heightened level of carcinoembryonic antigen (CEA) can be detected in patients with several types of cancer, inter alia CRC. Regarding its prognostic value in pulmonary metastasectomy, several contradicting studies with small sample sizes have been performed [4, 5]. Recently, two large studies including 1030 and 1112 patients confirmed the prognostic value of increased prethoracotomy CEA level (cut-off value >5 ng/ml) for 5-year survival in multivariate analysis [8, 9]. CEA expression persists during the metastatic process and has been identified in 93.3% of pulmonary metastases of CRC by immunohistochemistry [10]. Hence, it poses a helpful parameter in detecting the recurrence of disease.

CA19-9, alpha-fetoprotein and human chorionic gonadotropin. CA19-9 could be identified in 75% of pulmonary metastases and in 73.3% of paired primary CRC with high concordance in a work by Kayser et al. However, it did not influence the outcome [10]. Increased levels of alpha-fetoprotein (AFP, cut-off value ≥500 ng/ml), a marker associated with hepatocellular carcinoma (HCC), have been described as a negative prognostic factor for survival in patients undergoing resection for pulmonary nodules from HCC [11, 12]. These findings were confirmed by Kuo et al. [13], who found a significant association of high AFP and worse disease-free survival applying a cut-off value of 100 ng/ml. Although sample sizes of these three studies are small, they emphasize the potential value of serum markers to predict the postoperative outcome of patients following pulmonary metastasectomy. Besides AFP, des-gamma-carboxy prothrombin (DCP) is an established serum marker to monitor tumour burden and recurrence in patients with primary HCC. Kitano et al. [14] demonstrated a prognostic effect of DCP in their study population of 45 patients undergoing pulmonary metastasectomy from primary HCC (cut-off value: 40 mAU/ml) in univariate analyses regarding the disease-free survival and the overall survival. In germ cell tumours AFP and human chorionic gonadotropin (hCG) are accepted tumour markers. Elevated prethoracotomy serum levels of either AFP (cut-off value ≥15 μg/l) or hCG (cut-off value ≥5 Units/l) were significantly associated with reduced 5-year survival (50 vs 87.2 months) in patients with pulmonary spread of primary germ cell tumours [15].

Cell adhesion

The role of cell adhesion in cancer was first described in 1914 by Boveri. Since that time, the attention of metastasis research was drawn to molecules mediating cell-cell and cell-matrix adhesion [16]. Cell-adhesion molecules (CAMs) are capable of initiating

Figure 1: Potential prognostic biomarkers and radiological (*) markers in pulmonary metastases according to the classification chosen by the authors. Predictive markers for chemoresistance ($) have also been described in patients with pulmonary spreading of malignant disease.
intracellular signalling and play a key role in epithelial-mesenchymal transition, which is a key mechanism in the metastatic cascade [17]. Loss of cell adhesion can be determined by demonstrating the presence of invasion of other structures with light microscopy. Shiono et al. demonstrated in pulmonary metastases from primary CRC that vascular invasion as well as intra-alveolar cancer cell clusters was associated with diminished prognosis after pulmonary metastasectomy in multivariate analysis (39.9 vs 85.8%; 46.9 vs 88.2%, respectively), and the presence of 10 or more of such infiltrative cell clusters predicted early local recurrence. Supporting this, lymphatic and pleural invasion were further variables with significant impact on 5-year survival in univariate analysis (47.0 vs 78.4 months; 49.5 vs 63.5 months) [18, 19].

Expression of membrane-bound adhesion molecules like cadherins, immunoglobulin-like CAMs (Ig-CAMs), CD44 and integrins can be determined by the utilization of immunohistochemistry.

Expression of cadherins. Epithelial-cadherin is one of the most extensively studied member of the cadherin-family. It mediates cell–cell adhesion, and thus its down-regulation plays a pivotal role in tumour dissemination [20]. In patients undergoing pulmonary metastasectomy subsequent to primary CRC, reduced expression of E-cadherin in resected nodules was an independent predictor for poor survival (5-year survival 74.5 vs 23.9 months). Loss of beta-catenin, which binds to E-cadherin at the intracellular side of the cell membrane, was also associated with worse 5-year survival in univariate analysis [21].

Expression of immunoglobulin-like cell-adhesion molecules. The expression of members of the Ig-CAM family was investigated in hepatoblastoma patients. Up-regulation of CEA-related CAM 1 (CEACAM1, also known as CD66a) predicted early metastasis to the lung and was associated with a decreased 5-year survival (92 vs 29%) [22]. CEACAM1 is also down-regulated in invasive HCC, however, data on expression levels in thoracic nodules and a possible prognostic value need to be elucidated [23].

Expression of CD44. The standard isoform of CD44 is expressed by many cell types and mediates cell–matrix adherence by binding hyaluronic acid. As it is also a molecular marker for cancer stem cells, extensive research has been undertaken to understand the role of this family of proteins in tumour progression and metastasis [24, 25]. Different CD44 variants, which are the result of alternative splicing of the CD44 gene, are expressed in tumour tissue. The up-regulation of CD44 seems to play an important role in the homing of tumour cells to metastatic sites [25]. An increased expression of the CD44 gene in pulmonary metastases compared with corresponding primary CRC was found in a study by Kim et al. [26]. In a subsequent study, CD44 variant 9 expression was found in 88% of primary CRC with consecutive pulmonary dissemination, compared with 42% in patients without pulmonary dissemination. However, specimens from pulmonary nodules were not examined and follow-up after pulmonary dissemination was not described [27]. Additionally, an increased expression level of CD44 variant 6 in patients with primary CRC was related to a poor 2-year survival after pulmonary metastasectomy [28].

Expression of integrins. Another group of proteins relevant to cell–matrix interaction are integrins. They consist of an alpha- and a beta-subunit and mediate their adhesive effect by binding fibronectin and laminin [29]. Integrin alpha v was found to be expressed in 85.7% of lung metastases from primary CRC and in 52.4% of the paired primary tumours [30]. Yet, no data on the post-metastasectomy follow-up were provided by this study.

Tumour growth

Sustained cell proliferation is the key characteristic of malignant tumours. It is a consequence of attributes described as the hallmarks of cancer by Hanahan and Weinberg [31], such as resistance to cell death and up-regulation of proliferative signals. Proliferation can either be quantified by estimating the tumour growth radiologically or ex post by histological evaluation of the resected specimen.

Proliferation markers. Ki-67 (also known as MIB-1) is routinely used in histopathology to determine the proliferation rate of tumours. A high expression level at the primary tumour site was associated with pulmonary recurrence in osteosarcoma patients and mortality was increased in the strongly positive sub-group compared with patients with less proliferating osteosarcomas [32]. In contrast to that, a study on pulmonary metastases and matched primary osteosarcomas showed that Ki-67 expression did not correlate with the time to first pulmonary spreading. Follow-up until pulmonary recurrence after metastasectomy was not given and survival analysis dependent on Ki-67 was not described [33]. Besides, in patients with primary melanoma, the number of Ki-67 cells in the pulmonary nodules did not correlate with overall survival [34]. In patients with primary renal cell carcinoma (RCC) another marker for proliferation, the proliferating cell nuclear antigen (PCNA), was significantly higher in pulmonary metastases compared with primary tumours of patients with or without metastases. Moreover, mean PCNA expression was higher in patients with poor survival [35]. Though highly proliferating primary CRC was linked to tumour grade, lymphnode involvement, distant metastasis and an independent prognostic marker for 5-year survival in multivariate analysis, specific data after pulmonary metastasectomy are still lacking [36, 37].

Tumour doubling time. Rapid tumour proliferation can also be estimated in vivo by imaging techniques, e.g. computed tomography (CT). The tumour doubling time (TDT), indicating aggressive tumour biology, can vary between different types of tumours, within the same histological entities and even within the same patient (e.g. with multiple metastases) [38]. In patients with pulmonary metastases from CRC, a TDT <100 days was predictive for early pulmonary recurrence. Additionally, the overall survival was decreased in this sub-group with rapidly growing metastases; however, statistical significance was not reached [39, 40]. In patients with primary soft-tissue sarcoma, the TDT examined by chest X-ray was not associated with the patients’ outcome [41]. Using CT for surveillance of patients with primary soft-tissue sarcoma, two studies identified the TDT of pulmonary nodules as a poor prognostic marker for overall survival [42, 43]. Moreover, in a series of 39 patients with primary osteosarcoma, the TDT of the pulmonary nodule was also predictive of poor overall survival, whereas this finding was not supported by Nakamura et al. in a small series of 11 patients [42, 43]. Ollila et al. [44] could identify a TDT <60 days as a significant prognostic marker for poor survival after pulmonary metastasectomy from primary melanoma using chest X-ray. Subsequently, the prognostic value and the cut-off value at 60 days were confirmed by Lee et al. It is noteworthy that the proliferative index determined by immunohistochemical staining for Ki-67 did not correlate with the radiologically determined TDT in their patient cohort [34].

Markers of apoptosis. As tumour growth is the result of cell division minus cell loss, antiapoptotic markers like bcl-2 or
proteins involved in apoptosis (e.g. caspases) might provide additional information on the cell turnover. The presence of bcl-2 in pulmonary metastases correlated with worse overall survival in patients with primary CRC and was divergent from the expression in the corresponding primary tumour [10]. This negative correlation between bcl-2 expression and patients’ prognosis could not be confirmed in a series of osteosarcoma patients. Neither the expression level in the primary nor in the pulmonary metastases had influence on the recurrence-free survival and overall survival [45]. Caspase-3, a marker for cell apoptosis, was assessed in pulmonary metastases from primary melanoma, but was shown to have no effect on the clinical outcome after pulmonary metastasectomy [34] (Tables 1 and 2).

**Tumour suppressor genes/proto-oncogenes**

In general, tumour suppressor genes encode proteins, which have repressive functions on the cell-cycle. Proto-oncogenes are cellular genes, which are titled as oncogenes as soon they are mutated, amplified or rearranged, leading to up-regulated cell growth [46]. Genes are indicated by italic letters, whereas proteins are denoted by normal letters. Alterations of DNA or protein levels might have a diverging clinical impact.

**Expression of p53.** One of the most extensively studied tumour suppressors is the TP53 gene, coding for the p53 protein, which plays an essential role in the cell-cycle arrest and apoptosis of damaged cells [47]. Kayser et al. found positive p53 protein staining in 26/60 pulmonary metastases and in 17/60 paired primary CRC, with a total of 45/60 concordant positive or negative stainings. Nevertheless, p53 expression was not associated with post-metastasectomy outcome [10]. Danner et al. compared chromosomal aberrations in the TP53 gene region in paired primary and metastatic tissue specimens from CRC. Gain mutations at 20q, which correlates with altered TP53, were evident in 70 and 54% of primary tumours and metastases, respectively. Furthermore, they could confirm the finding by Kayser et al., who found no significant correlation with recurrence-free survival after pulmonary metastasectomy or overall survival [48]. In patients with primary osteosarcoma, expression of p53 in the primary tissue failed to predict tumour progression [49]. However, in a more recent study including patients with pulmonary nodules from primary osteosarcoma, negative expression of p53 was significantly associated with poor survival after pulmonary metastasectomy in univariate analysis (3-year post-metastasectomy survival rate 17 vs 34%). It was concluded that the p53 might have a role as prognostic marker only in the subset of patients presenting with pulmonary dissemination [45].

**Chromosomal aberrations.** SMAD2, SMAD4/DPC4 and DCC, other well-investigated tumour suppressor genes, are clustered on the chromosome arm 18q, and a loss of 18q is associated with tumourigenesis [50]. In CRC patients, a loss of 18q was evident in 86% of pulmonary metastases, compared with 70% in corresponding primary tumours. However, a significant relationship between the loss of 18q and the clinical outcome could not be established [48].

**K-ras mutation status.** In CRC, mutations of the proto-oncogene k-ras, which encodes a small guanosine triphosphate-binding molecule involved in growth signalling pathways, are thought to be an early event in the progression from adenoma to carcinoma. However, only one-third of primary carcinomas have detectable alterations in this gene [46, 51]. K-ras mutation can more often be found in lung metastases (60%) than in liver metastases (33.3%). Additionally, in patients with k-ras mutation in the primary CRC, the lung is more often the initial site of metastasis compared with k-ras wild-type, thus k-ras might play an important role in metastatic dissemination, particularly to the lung [52–54]. These findings were corroborated by data from the VICTOR study revealing that patients with k-ras mutation in the primary tumour have a significantly higher risk of lung relapse in multivariate analysis (hazard ratio: 1.9, 95% confidence interval: 1.1–3.1), whereas this effect could not be observed regarding liver metastases [53]. In support of this, Cejas et al. found a significantly decreased post-metastasectomy disease-free survival in patients with k-ras mutation (12.0 vs 18.0 months) without differentiating between lung and liver metastases.

**Expression of Her2/neu.** Another proto-oncogene, c-erbB2, encodes for the epidermal growth factor (EGF) homologon Her2/neu (alternatively named human EGF 2). Her2/neu is mostly known for its prognostic role and implications on treatment regimens in primary breast cancer. It could be shown that expression of Her2/neu in pulmonary metastases of primary breast cancer was associated with poor 5-year survival after metastasectomy (22 vs 74%). Additionally, loss of oestrogen receptor expression in the pulmonary metastases is associated with a worse post-metastasectomy 5-year survival in these patients (77 vs 12%) [55]. Her2/neu expression status is also strongly associated with pulmonary-specific metastasis in osteosarcoma patients [49, 56, 57]. Her2/neu could be detected immunohistochemically in 32% of primary osteosarcomas and in 53% of corresponding pulmonary metastases. Moreover, Her2/neu correlated with early pulmonary spreading (17.2 vs 31.8 months), but did not affect the post-metastasectomy survival [45]. Zhou et al. [58] confirmed these results and provided additional evidence of c-erbB2 gene amplifications in some cases.

**Expression of FOS-B and Ets-1.** Addressing the prognostic value of proto-oncogenes in pulmonary metastasectomy, Finkel-Biskis-Jinkins murine osteosarcoma viral oncogene homologue B (FOS-B) has been investigated. Initially, FOS-B has been associated with tumour progression in primary CRC [59]. When detected in pulmonary metastases of CRC, FOS-B significantly affected the recurrence-free survival as well as the overall survival in univariate analysis, although this could not be confirmed in multivariate analysis [60]. Ets-1, an oncprotein and transcription factor involved in matrix degradation is expressed in tumour cells as well as in the tumour-surrounding stroma, facilitating tumour progression. The expression level in pulmonary metastases correlated with corresponding primary CRC. Furthermore, stromal expression of Ets-1 significantly and independently predicted pulmonary recurrence of primary CRC [61]. Evaluating the prognostic value of Ets-1 regarding the post-metastasectomy outcome was not an aim of this study.

**Cell metabolism**

**Glucose processing.** An altered cell metabolism is common in various cancerous tissues. It is necessary for a tumour to be provided with sufficient amounts of carbohydrates, proteins, lipids and nucleic acids in order to meet the increased demand. One of the most investigated metabolic phenotype in cancer is the excessive glucose uptake of tumour tissue, the so-called Warburg
Table 1: Potential biomarkers in pulmonary metastases sorted by the entity of the corresponding primary tumour

<table>
<thead>
<tr>
<th>Primary tumour</th>
<th>Marker</th>
<th>No. of patients</th>
<th>Outcome/result</th>
<th>First author</th>
<th>Year</th>
<th>Reference</th>
</tr>
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<tr>
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<tr>
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<td>31</td>
<td>OS</td>
<td>Shiono</td>
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<tr>
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<td>ASFC</td>
<td>87</td>
<td>OS</td>
<td>Shiono</td>
<td>2005</td>
<td>[19]</td>
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<tr>
<td></td>
<td>bcl-2</td>
<td>60</td>
<td>OS Marker for OS (univariate)</td>
<td>Kayser</td>
<td>2002</td>
<td>[10]</td>
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<td></td>
<td>BI-catenin</td>
<td>86</td>
<td>OS</td>
<td>Shiono</td>
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<td>[21]</td>
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<td></td>
<td>BRCA1</td>
<td>80</td>
<td>OS Outcome after OS and adjuvant chemotherapy</td>
<td>Kaira</td>
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<td>[70]</td>
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<td>[21]</td>
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<tr>
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<td>n.s.</td>
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<td>OS</td>
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<td>AFP (≥100 ng/ml)</td>
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<td>OS (univariate)</td>
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<td>OS</td>
<td>Obba</td>
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<td>DCP (&gt;40 mAU/ml)</td>
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<td>Glut-1</td>
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<td>OS</td>
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<td></td>
<td>Ki-67</td>
<td>20</td>
<td>n.s.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MVD (CD31)</td>
<td>20</td>
<td>OS</td>
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<tr>
<td></td>
<td>Testis</td>
<td>Serum AFP (&gt;15 μg/l)/hCG (&gt;5 U/l)</td>
<td>41</td>
<td>OS</td>
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<td>No follow-up</td>
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<td>p53 (HC)</td>
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<td>3-year survival (univariate)</td>
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<td>Lung-specific recurrence-free survival</td>
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<td>Lung-specific recurrence-free survival</td>
<td>Zhou</td>
<td>2003</td>
<td>[58]</td>
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<td></td>
<td>P-glycoprotein</td>
<td>19</td>
<td>n.s.</td>
<td>Ferrari</td>
<td>2004</td>
<td>[45]</td>
</tr>
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<td></td>
<td>COX-2</td>
<td>36</td>
<td>Disease-specific survival</td>
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<td>Renal cell</td>
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<td>Increased in patients with survival &lt;3 years</td>
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<tr>
<td>Various</td>
<td>EGFR</td>
<td>35</td>
<td>No follow-up</td>
<td>Muelling</td>
<td>2010</td>
<td>[77]</td>
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</table>
Table 1: Potential radiological markers in patients with pulmonary metastases sorted by the entity of the corresponding primary tumour

<table>
<thead>
<tr>
<th>Primary tumour</th>
<th>Modality</th>
<th>Parameter</th>
<th>No. of patients</th>
<th>Outcome/result</th>
<th>First author Year Reference</th>
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<tbody>
<tr>
<td>Colorectal</td>
<td>CT</td>
<td>TDT</td>
<td>65</td>
<td>5-year intrapulmonary recurrence-free survival</td>
<td>Tomimaru 2008 [40]</td>
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<tr>
<td>Melanoma</td>
<td>CXR</td>
<td>TDT</td>
<td>20</td>
<td>OS</td>
<td>Lee 2009 [34]</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>CT</td>
<td>TDT</td>
<td>129</td>
<td>OS</td>
<td>Ollila 1998 [44]</td>
</tr>
<tr>
<td>Soft-tissue sarcoma</td>
<td>CT</td>
<td>TDT</td>
<td>39</td>
<td>OS</td>
<td>Roth 1985 [42]</td>
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<tr>
<td>Various</td>
<td>PET-CT</td>
<td>FDG uptake</td>
<td>146</td>
<td>OS, correlates with GLUT1, hexokinase 1, HIF-1, VEGF and MVD</td>
<td>Kaira 2011 [65]</td>
</tr>
</tbody>
</table>

CT: computed tomography; CXR: chest X-ray; FDG: 18F-fluorodeoxyglucose; GLUT1: glucose transporter 1; HIF: hypoxia-inducible factor 1; MVD: microvasculature density; n.s.: not significant; OS: 5-year overall survival; PET: positron emission tomography; TDT: tumour doubling time; VEGF: vascular endothelial growth factor.

Table 2: Continued

<table>
<thead>
<tr>
<th>Primary tumour</th>
<th>Marker</th>
<th>No. of patients</th>
<th>Outcome/result</th>
<th>First author Year Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGFR</td>
<td>VEGFR</td>
<td>146</td>
<td>Correlation with 18F-FDG uptake</td>
<td>Kaira 2011 [65]</td>
</tr>
<tr>
<td>GLUT1</td>
<td>Hexokinase 1</td>
<td>146</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIF-1</td>
<td>VEGF</td>
<td>MVD (CD34)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AF: Alpha-fetoprotein; ASFC: aerogenous spread with floating cancer cell clusters; bcl-2: B-cell lymphoma 2; BRCA1: breast cancer susceptibility gene 1; CA: carbohydrate antigen; CD: cluster of differentiation; CEA: carcinoembryonic antigen; CEACAM: carcinoembryonic antigen-related cell-adhesion molecule 1 (CD66a); COX-2: cyclooxygenase 2; DCP: des-gamma-carboxy prothrombin; DFS: disease-free survival; DPD: dihydropirimidine dehydrogenase; EGRF: epidermal growth factor receptor; ER: oestrogen receptor; ERCC1: excision repair cross-complementation group 1; FDG: 18F-fluorodeoxyglucose; FOS-B: Finkel-Biskis-Jinkins murine osteosarcoma viral oncogene homologue B; GLUT: glucose transporter; hCG: human chorionic gonadotropin; HER1/neu: human epidermal growth factor receptor 2/erb-B; HIF-1: hypoxia-inducible factor 1; IGF1R: insulin-like growth factor 1 receptor; KRAS: Kirsten rat sarcoma viral oncogene homologue; LMP5: lung metastasis-free survival; MVD: microvasculature density; n.s.: not significant; OPRT: orotate phosphoribosyltransferase; OS: 5-year overall survival; PCNA: proliferating cell nuclear antigen; PDGFR: platelet-derived growth factor receptor; PM: pulmonary metastasectomy; TS: thymidylate synthase; VEGF: vascular endothelial growth factor; VEGFR: vascular endothelial growth factor receptor.

effect [62]. Tumour cells generate adenosine triphosphate (ATP) to a high degree through aerobic glycolysis. Aerobic glycolysis is less efficient in generating ATP, therefore resulting in an increased glucose demand and consecutive uptake of glucose in tumour tissue. However, it has been speculated that aerobic glycolysis is superior to oxidative phosphorylation in hypoxic tumour tissue and leads to clonal selection of these cells [63]. The increased uptake of glucose via glucose transporters (GLUT) is also the basis for clinical tumour imaging by 18F-fluorodeoxyglucose–positron emission tomography (FDG-PET). Hence, FDG uptake correlates with the expression of GLUT1 [64]. Kaira et al. published a work comparing 18F-FDG uptake and histological markers associated with glucose metabolism in patients with either NSCLC or pulmonary metastases from extrapulmonary malignancy and a control group with benign nodules. A 18F-FDG uptake beyond the calculated cut-off value was evident in 60, 29, 36, 44, 66, 66, 71 and 50% of pulmonary nodules of primary CRC (n = 80), sarcoma (n = 17), head and neck cancer (n = 14), breast cancer (n = 9), genital cancers (n = 12), oesophageal cancer (n = 3), gastrointestinal cancer (n = 7) and others (n = 4), respectively. Furthermore, 18F-FDG uptake correlated with expression of GLUT1, hexokinase 1, HIF-1alpha vascular endothelial growth factor (VEGF) and microvascular density in the pulmonary metastases and the size of the pulmonary nodules. The 5-year survival for all patients with confirmed metastases from an extrathoracic primary was 75.6 months. Assigning them to groups according the 18F-FDG uptake, patients with highly positive pulmonary nodules were shown to have a significantly shorter median survival time. This finding was, however, limited by the fact that patients with different primary tumours were included in this analysis [65]. GLUT1 overexpression was also identified as a prognostic factor for poor overall survival after pulmonary metastasectomy in patients with primary melanoma. A potential correlation with 18F-FDG uptake was not assessed in this work [34]. Further studies are needed to elucidate the glucose metabolism imaged by 18F-FDG uptake as a prognostic tool.
**Predictive markers indicating chemoresistance.** Another important aspect with regard to cell metabolism lies in the changes leading to resistance to chemotherapeutics. In patients with pulmonary dissemination receiving an inter-disciplinary treatment, surgical removal of lung metastases is often accompanied by adjuvant chemotherapy. For that reason, markers predicting the response of patients to cytotoxic agents might affect the prognosis after metastasectomy. In the following, we discuss interesting findings regarding predictive markers in patients with pulmonary metastases. Thymidylate synthase (TS) is an enzyme that is involved in the metabolism of 5-fluorouracil (5-FU), pemetrexed and raltitrexed. An increased expression level of TS in primary CRC has been described as predictive for 5-FU response and patients’ prognosis. Jensen et al. [66] underlined the importance of differentiating between tumour tissues from the primary tumour site or the metastasis, as they possess divergent characteristics regarding their pattern of chemoresistance. Supporting this, an increased expression of TS was detected in the pulmonary metastases of primary CRC, compared with the corresponding primary tumour and/or liver metastases [67–69]. Patients with CRC receiving 5-FU-based adjuvant chemotherapy after pulmonary metastasectomy and sufficient expression of TS in lung metastases benefited most from adjuvant chemotherapy. In the same study, this could also been shown for other proteins, which are involved in chemoresistance (orotate phosphoribosyltransferase (OPRT), excision repair cross-complementation group 1 (ERCC1) and breast cancer susceptibility gene 1 (BRCA1)) [70]. In patients with primary osteosarcoma, the expression of P-glycoprotein is significantly increased in pulmonary metastases compared with the primary tumours [45]. P-glycoprotein is a product of the multidrug resistance gene 1 and mediates the resistance to doxorubicin [71]. Expression of P-glycoprotein in primary tumours or pulmonary metastases is not associated with recurrence-free survival or survival after metastasectomy [45]. Cyclooxygenase-2 (COX-2) is involved in metastasis and resistance to chemoradiotherapy, particularly in patients with osteosarcoma [72, 73]. Increased expression of COX-2 in lung metastases predicts a worse outcome of patients with primary osteosarcoma and lung metastases, whereas the expression level of COX-2 seems not to be capable to prognosticate the outcome [74, 75]. Therefore, COX-2 seems to be a valuable prognostic marker in the sub-population of patients undergoing pulmonary metastasectomy.

In summary, existing data on the prognostic and predictive effect of cell metabolism markers is contradictory, and the contribution of these enzymes to the treatment outcome of patients with metastatic disease needs to be further elucidated.

**Tumour angiogenesis**

Sufficient blood supply is crucial for the growth of a tumour. Pivotal proangiogenic mediators are VEGF, EGF, platelet-derived growth factor (PDGF) and their receptors (VEGFR, EGFR and EGFRII, respectively) [76]. The increased activity of the proangiogenic pathways leads to strongly vascularized tumours. This hypervascularization can be determined histologically and/or radiologically.

**Receptor tyrosine kinases and ligands.** The expression level of receptor tyrosine kinases has been described in various primary tumours and paired pulmonary metastases, irrespective of their potential role as biomarkers [77, 78]. An increased expression of VEGFα in pulmonary nodules compared with corresponding primary CRC could be determined by reverse transcription polymerase chain reaction [26]. Additionally, this increased expression of VEGF in CRC pulmonary metastases was found be an independent prognostic marker for poor 5-year survival in multivariate analysis (18 vs 46.1%) [79]. In metastatic osteosarcoma, the expression of VEGF in the metastatic lesion was comparable to that of the primary tumour. Nevertheless, the high expression level of VEGF in the primary tumour was associated with decreased survival in univariate analysis, whereas expression of VEGF in the metastatic lesion did not affect the outcome [33]. In a further work assessing pulmonary metastases from primary CRC, expression of VEG-F-D, a VEGF sub-type that mediates lymphangiogenesis, was not associated with recurrence-free survival or overall survival [60]. Interestingly, VEGF expression was shown to correlate with 18F-FDG uptake in pulmonary metastases from primary epithelial cancers [65]. This finding emphasizes the potential of PET-imaging to reflect tumour biology. Another growth factor involved in angiogenesis is the insulin-like growth factor. The increased expression of the corresponding receptor (IGF1R) seems not have prognostic value in metastatic CRC [21].

**Microvessel density.** Microvessel density can be determined immunohistochemically by staining of e.g. CD31 or CD34 and calculating the number of vessels per area. Pulmonary metastases from primary CRC with low microvessel density are associated with better outcomes after pulmonary metastasectomy and 5-FU-based adjuvant chemotherapy, whereas this is unaffected by the expression of VEGF [70]. As mentioned above, microvessel density correlates with 18F-FDG uptake in various tumours [65]. Hyper-vascularized pulmonary metastases from primary melanoma are associated with worse survival after pulmonary metastasectomy [34].

**CONCLUSION**

Currently, the prognosis of cancer patients is based on the TNM classification. As this system is of limited value for patients undergoing pulmonary metastasectomy [4], further prognostic markers are warranted. The most prominent literature on prognostic factors following pulmonary metastasectomy was published 1997, reviewing over 5000 data sets from the IRLM [3]. The risk factors described in this publication are solely based on clinical observations. However, these factors correspond to tumour aggressiveness, characterized by a rapid, invasive and diffuse pattern of metastasis (short disease-free interval, incomplete resection and number of nodules). Each molecular and radiological marker of our review can be linked to the tumour phenotype described by the IRLM. Two examples illustrate this relationship: E-cadherin expression and markers of tumour angiogenesis. A loss of the epithelial marker E-cadherin is a key factor in the process of a diffuse tumour spreading. It enables the disaggregation of cancer cells from one another, which is the first step of the metastatic cascade. Thus, E-cadherin expression may be directly linked to the number of nodules and the disease-free interval. Supporting this, decreased levels of E-cadherin are still evident in metastatic tissue. On the other hand, angiogenesis is considered an essential component of tumour metastasis. Most of the disseminated tumour cells undergo apoptosis or remain in a silenced state on the secondary site, because sufficient vascularization limits the outgrowth of micrometastases to clinically detectable macrometastases. Therefore, the up-regulation of TRK-pathways as described in this
review may contribute to the number of metastasis—one of the IRLM risk factors for poor survival.

Methods in molecular biology and radiological imaging techniques have provided clinicians with a myriad of novel prognostic markers in cancer research. The majority of the findings reviewed here underline the divergent biology of primary tumour and corresponding pulmonary metastases. Compared with the existing literature on prognostic markers in primary tumours, only a few studies have focused on the subset of patients with pulmonary metastases, which widely consists of a small series of highly selected patients. The best evidence for prognostic significance that is already clinically applicable are the findings on CEA (pre-metastasectomy serum level ≥5 ng/ml) in patients with primary CRC. Detection of CEA is easy and reproducible by commercially available assays and has pioneered the use of novel biomarkers in pulmonary metastasectomy. Besides this promising serum marker, non-invasive methods in radiology/nuclear medicine, e.g. PET-CT might be an important prognostic tool in the future. All other suggested markers of this review are still in a preclinical or even experimental phase and therefore a transition to a clinical application is currently not possible. It is important to notice that identifying poor prognostic markers in patients with pulmonary metastases remains a desirable aim, but should not lead to an increase in the long-term survival of our patients solely by stronger stratification.

Conflict of interest: none declared.

REFERENCES


**KRAS in metastatic colorectal cancer**

A key-characteristic of cancer is the autonomous, dysregulated growth. This is achieved by enhanced signaling via tumor oncogenes or loss of tumor suppressors (Hanahan & Weinberg, 2011). A well-described tumor oncogene in CRC is V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS). KRAS is a GTPase located at the cell membrane and exhibits its function by transducing signals from the cell surface downstream to other intracellular mediators (Schmick et al, 2014). Most extensively described downstream signaling pathways are the PI3K/Akt pathway and the MAPK pathway (Normanno et al, 2009). Activated, GTP-bound KRAS activates these pathways and thus contributes to cancer progression by anti-apoptotic, proliferative and cell transforming effects (Bryant et al, 2014; Velho et al, 2008; Yang et al, 2015). KRAS itself is activated by tyrosine kinase receptors (TKR), e.g. the epidermal growth factor receptor (EGFR). These TKR are valuable therapeutic targets in a variety of cancer types, especially CRC. The monoclonal anti-EGFR antibodies cetuximab and panitumumab were shown to be effective anti-tumor drugs in CRC. As activating mutations in the KRAS gene, especially codon 12 and 13 of exon 2, lead to an EGFR- independent activation of the downstream signaling cascades, KRAS mutation is a predictive marker for the treatment response to anti-EGFR therapy (Bokemeyer et al, 2011; Van Cutsem et al, 2009).

Beyond the predictive value of KRAS mutations, KRAS mutant CRC might exhibit a different metastatic behavior compared to KRAS wild-type tumors. Tie et al. found KRAS mutations in 62.0% of CRC lung metastases, compared to 56.5% of CRC brain metastases and 32.3% CRC liver metastases. Furthermore, patients with KRAS mutant CRC had a higher risk for developing pulmonary metastases (HR 2.2; 95%CI 1.2-3.5; P = 0.007) (Tie et al, 2011). Cejas et al. found a significantly higher prevalence of KRAS mutations in CRC patients with lung metastases compared to patients with liver metastases (Cejas et al, 2009).
Heat shock protein 27 and fibroblasts

Extensive scarring, i.e. fibrosis is a common feature of various benign and malignant diseases. On a cellular level, fibrosis consists of activated fibroblasts, also called myofibroblasts as they express high levels of alpha-smooth muscle actin (alpha-SMA), extracellular matrix (mainly produced by myofibroblasts) and inflammatory cells (Wynn & Ramalingam, 2012). Physiologically, myofibroblasts play an essential role during wound healing. As they can also be found in the tumor stroma of solid cancer, the role of these cells in malignant disease is of increasing interest.

Beyond the over-expression of alpha-SMA, myofibroblasts are considered positive for fibroblast-activation protein alpha (FAP), palladin, osteopontin and vimentin (Brentnall et al, 2012; Henry et al, 2007; Nakagawa et al, 2004; Ngan et al, 2007; Pardo et al, 2005; Tsujino et al, 2007). Moreover, the small heat shock protein Hsp27 has been described to be over-
expressed and functional relevant in myofibroblasts in the context of benign disease (Hirano et al, 2004; Suarez et al, 2013). Moreover, our group – amongst others – recently demonstrated a systemic elevation of Hsp27 in benign and malignant lung disease (Hacker et al, 2009; Zimmermann et al, 2012). Using FAP as marker for myofibroblasts, Henry et al demonstrated that high density of FAP+ fibroblasts was associated with a decreased survival in patients with metastastic CRC (median 671 versus 428 days, \( P = 0.042 \)). To the best of our knowledge, the prognostic value of Hsp27+ fibroblasts was never assessed in the context of CRC.

1.7 Treatment of pulmonary metastases

The first report on pulmonary metastaectomy was published by the Viennese surgeon Josef Weinlechner in 1882 (Weinlechner, 1882). The prerequisites for the surgical removal of pulmonary metastases were defined by Thomford in 1965 and basically remained valid until today (Thomford et al, 1965):

I) Functional resectability, which means that the patient needs to be in general condition to tolerate surgery and the expected remaining lung function after metastaectomy needs to be sufficient
II) The primary tumor site must be under control
III) No extra-thoracic metastatic tumor site or resectability of these extra-thoracic lesions
IV) A complete resection of all pulmonary metastases is technically feasible

Indication for pulmonary metastaectomy should only be set, if all of these criteria are met. The aim of pulmonary metastaectomy is a complete resection of all nodules together with the preservation of as much lung parenchyma as possible. As pulmonary metastases might recur after the first metastaectomy, the remaining functional lung parenchyma after the first procedure is crucial for further pulmonary metastaectomies.

There are several surgical devices and techniques available to resect lung metastases. Enucleation of the nodule can be performed using either electrocautery or a Nd:YAG laser device. Compared to other resection techniques, enucleation with either electrocaautery or laser allows the preservation of as much lung parenchyma as possible. This makes enucleation the resection technique of choice especially for multiple metastases (e.g. complete resection of >100 metastases during one procedure can be performed) (Rolle et al, 2006). The second application of enucleation techniques are centrally located nodules. These could theoretically also be resected extra-anatomically, but with a concomitant
substantial loss of lung parenchyma. Thus, enucleation is preferred for deeply intraparenchymal and/or multiple lesions. The most commonly applied technique to resect pulmonary metastases is an extra-anatomical (“wedge”) resection using stapler devices with an integrated blade. These devices allow an optimal sealing of the lung parenchyma and have widely replaced the traditional technique requiring multiple steps, including clamping of an extra-anatomical segment, resecting it with a surgical blade and oversewing the resection line afterwards with a running suture. These steps are all integrated in mechanical stapling devices. Depending on the location of the pulmonary metastases and the functional capacity of the patient, anatomical resection of metastases are possible to achieve a complete resection of all nodules. Lung segments can be resected following the inter-segmental, anatomical border (“segmentectomy”). Moreover, lobectomy or even pneumonectomy can be indicated in selected cases (Migliore et al, 2010). Even repeated metastasectomy is possible in some patients. A low peri-operative morbidity and mortality and a reasonable long-term survival makes re-metastasectomy a valid option for recurrent lung metastases (Jarabo et al, 2011; Kandioler et al, 1998; Migliore et al, 2010; Park et al, 2010; Salah et al, 2013).
1.8 Aims of this thesis

a. Description of the EGFR expression, KRAS and BRAF mutations in CRC lung metastases
b. Correlation of EGFR, KRAS and BRAF alterations with clinical characteristics
c. Detection and quantification of myofibroblasts in CRC primary tumors, liver metastases and lung metastases
d. Description of the microvessel density dependent on the expression of Hsp27 in tumor-associated fibroblasts
e. Measurement of systemic Hsp27 before and after pulmonary metastasectomy
f. Evaluation of the assessed histological characteristics as prognostic markers in patients receiving pulmonary metastasectomy
CHAPTER TWO: RESULTS

2. Prologue

Pulmonary metastases are a significant contributor to cancer-associated morbidity and mortality in patients with CRC (La Vecchia et al, 2010; Malvezzi et al, 2011). Despite pulmonary metastasectomy is commonly performed, the selection of patients in which long-term survival can be achieved remains an obstacle in thoracic surgery. The underlying tumor biology, which determines the fate of the patient, has rarely been assessed in the context of pulmonary metastases.

After assessing lung, liver and brain metastases of CRC, Tie et al. found that KRAS mutations were significantly more frequent in patients who presented with lung recurrence (HR 2.1; 95% CI 1.2-3.5) (Tie et al, 2011). Similar to this work, Cejas et al. described the increased propensity of KRAS mutant CRC to spread to the lungs by assessing tissue samples from primary CRC (Cejas et al, 2009). In our work “EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study” we investigated the time and pattern of disease recurrence after pulmonary metastasectomy in patients with metastatic CRC dependent on the KRAS and BRAF mutation status and the expression level of EGFR. We could describe that KRAS mutant tumors were significantly associated with recurrent lung metastasis after the first pulmonary metastasectomy and had a significantly shorter time to pulmonary recurrence (Schweiger et al, 2014). Thus, we could show that KRAS is - specifically in patients with CRC pulmonary metastases undergoing surgery – a valuable prognostic marker.
2.1 EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study (published PDF)

**ABSTRACT**

**Background.** Pulmonary metastasectomy is an integral part of the interdisciplinary treatment of patients with pulmonary metastases (PMs) from colorectal carcinoma (CRC). Although alterations in the epidermal growth factor receptor (EGFR) pathway are common in CRC, there is still insufficient data regarding PM. We hypothesized that EGFR expression and Kirsten rat sarcoma viral oncogene homolog (**KRAS**) and **BRAF** mutations (Mts) might be associated with clinicopathological variables and the outcome in patients undergoing pulmonary metastasectomy.

**Methods.** In this single-center study, 44 patients undergoing pulmonary metastasectomy from primary CRC were included and prospectively followed up. Tissue specimens of resected PMs were assessed. Restriction fragment length analysis was used for **BRAF** V600E and **KRAS** codons 12 and 13 Mt analyses. EGFR expression was evaluated by immunohistochemistry. Patients were followed up in 3–6-month intervals.

**Results.** EGFR expression was evident in 49% of the PMs, whereas Mts in **KRAS** and **BRAF** were detected in 48 and 0%, respectively. Time to lung-specific recurrence after metastasectomy was significantly decreased in patients with **KRAS** mutated PMs in univariate (\( p = 0.013 \)) and multivariate analysis (\( p = 0.035 \)), whereas EGFR expression had no impact on recurrence free survival. Moreover, **KRAS** Mts were associated with the number of PMs (\( p = 0.037 \)) and with the lung as first site of recurrence after metastasectomy (\( p = 0.047 \)).

**Discussion.** This is the first evaluation of EGFR pathway alterations in the setting of pulmonary metastasectomy. Our data suggest that patients with **KRAS** Mts are at high risk for early pulmonary recurrence and have a more diffuse pattern of metastasis. These findings may have impact on the therapeutic management of CRC patients with pulmonary spreading.

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the world. Despite advances in the treatment of the disease, CRC remains one of the leading causes of cancer-related death. One of six patients develops pulmonary metastases (PMs) during the course of the disease, whereas the identification of patients with increased risk for PMs is a matter of current research. Pulmonary metastasectomy is an integral part of the interdisciplinary treatment of these patients. Although randomized, controlled trials on pulmonary metastasectomy are still missing, the resection of metastases embedded in a multidisciplinary treatment plan is a widely accepted concept for selected patients. In addition to the concept of re-metastasectomy for recurrent disease is associated with good long-term survival in metastatic CRC.
Prediction of the outcome of patients undergoing pulmonary (re-)metastasectomy remains an obstacle in surgical oncology. An a priori identification of patients with high risk for pulmonary recurrence may affect the treatment strategy. Thus, a quest for accurate biomarkers in pulmonary metastasectomy has evolved.

The epidermal growth factor receptor (EGFR) and its downstream signalling cascade are key regulators of cellular growth, proliferation, and survival. Mutations (Mts), gene amplifications, or protein overexpression in this pathway are a frequent finding in many neoplasms. EGFR expression can be found in high frequency in primary CRC. Although 25–90 % of tumors present with a positive staining pattern, the prognostic value of this finding is still controversial. However, the therapeutic application of anti-EGFR antibodies led to promising results.

The BRAF protein, a serin–threonin protein kinase, is a downstream signal transductor in the EGFR–MAPK (mitogen-activated protein kinase) pathway. BRAF gene Mts are less frequent than Kirsten rat sarcoma viral oncogene homolog (KRAS) Mts. They can only be found in less than 10 % of all primary CRCs. In contrast to KRAS Mts, alterations in the BRAF oncogene strongly correlate with shorter progression-free and overall survival.

The V-Ki-ras2 KRAS is part of the EGFR–MAPK pathway. As most patients with KRAS Mt are resistant to anti-EGFR therapy, KRAS Mt has to be excluded in patients potentially receiving such a therapy. Therefore, KRAS is especially useful as a predictive biomarker, whereas its prognostic value is still under discussion. Interestingly, recent studies have shown that KRAS Mts interfere with other relevant pathways promoting metastasis and that KRAS Mts occur in a high percentage of primary tumors with subsequent pulmonary spreading.

Therefore, we hypothesized that alterations in the EGFR-pathway might be relevant in the subset of patients undergoing pulmonary metastasectomy.

**MATERIALS AND METHODS**

**Study Population**

From April 2009 to October 2012, 44 patients who underwent pulmonary metastasectomy after primary CRC were included in this prospective, single center study (Fig. 1). Inclusion criteria were the first pulmonary metastasectomy, the absence of any extrapulmonary spreading, a controlled primary tumor site, and an expected complete resection based on preoperative CT images. The study was approved by the Ethics Committee of the Medical University of Vienna (EK1044/2012) and was conducted according to the Helsinki Declaration and the guidelines for good scientific practice of the Medical University of Vienna. All patients were treated according to current guidelines for pulmonary metastasectomy with curative intent. The majority of chemotherapy regimens before or after metastasectomy were based on 5-fluorouracil/oxaliplatin ± bevacizumab. For singular subpleural metastases, a VATS approach was applied; all other patients underwent a muscle-sparing anterior or posterior thoracotomy with a bimanual palpation of the lung. Complete resection was achieved in all patients, and a systematic lymph node sampling was performed if enlarged lymph nodes were found intraoperatively. Formalin-fixed, paraffin-embedded (FFPE) specimens and pathological data were provided by our pathology department. The mean diameters of the histologically confirmed metastatic nodules were measured, and the estimated tumor volume was calculated by assuming a spherical shape \(4/3 \times \pi \times r^3\) based on the radius \(r\) of the nodule given in the pathological report. Patients were

![FIG. 1 Study scheme of the patients included in the analysis](image-url)
followed-up in the outpatient ward with a CT scan every 3 months within the first year. If no recurrence was detected within the first 12 months, follow-up intervals were changed to biannual controls. Thirty-nine patients were included in the final analysis. Lung metastasis-free survival (LMFS) was defined as the time between the diagnoses of the primary and metastatic tumors. Time to recurrence was defined as the time from pulmonary metastasectomy to the evidence of tumor recurrence irrespective of the recurrence site. Time to recurrence was calculated from the time of pulmonary metastasectomy to pulmonary recurrence of the disease identified by CT scan.

**Immunohistochemistry and Scoring**

Immunohistochemical staining was performed following a standard protocol. Briefly, FFPE specimens were assessed using a Benchmark Ultra Immunostainer (Ventana, Tucson, AZ). The binding of rabbit anti-EGFR antibody (ready to use; Ventana) was visualized with DAB substrate, and the tissue slides were counterstained with hematoxylin. In negative controls, the primary antibody was omitted. Staining intensity was scored as described previously. In brief, sections showing membranous staining in ≥1% of the tumor cells were scored as positive. Two independent, blinded observers rated the staining intensity. In case of a disagreement, a consensus score was determined.

**KRAS and BRAF Mt Analysis**

Restriction fragment length analysis was performed to detect KRAS codons 12 and 13 and BRAF codon 600 Mt as described previously. Briefly, macrodissection of paraffin-embedded tumors was performed following the pathologists’ assessment of tumor enriched area and tumor cell content. Next, genomic DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). PCR was performed with AmpliTaqGold PCR Master Mix (Applied Biosystems, Foster City, CA) using primers designed to introduce restriction sites in the products generated from the wild-type (Wt) allele. The primer sequences were as follows: for KRAS codon 12 forward 5'-GAATATTACCTTGTGGTACTTGAGCCCT-3' and reverse 5'-GGTCTCTGGCACCAGTAAATAG-3', for KRAS codon 13 forward 5'-GAATATAACCTTGTGGTACTTGAGCCCT-3' and reverse 5'-GGTCTCTGGCACCAGTAAATAG-3'. The products obtained were then digested with BsmI and BglII, respectively. BRAF codon 600 Mt was analysed in a two-stage PCR but with Mt-F and -R primer (5'-AAAAATTAAGCAGTGGAATAATAGC-3'). The PCR products were then digested with BstI (New England Biolabs, Beverly, MA). All products were visualized on 3% agarose gels stained with ethidium bromide.

**Statistical Analysis**

All data collected in this study were evaluated statistically using SPSS 19 (SPSS, Inc., Chicago, IL) and GraphPad Prism 6 (GraphPad Software, Inc., California) software. Nonparametric data were expressed as median and range. Mann–Whitney U test was used to compare medians between two groups. The Kaplan–Meier method, log-rank test, and Cox regression were used to compare survival functions. χ² test and Fisher’s exact test were used to compare binominal variables. All tests were two-sided. p ≤ 0.05 was considered statistically significant.

**RESULTS**

**Clinicopathologic Characteristics**

Twenty male and 19 female patients with a median age of 64 (range 37–79) years were included in the final analyses. Three patients were excluded because the low tumor DNA content of their samples impeded reliable mutational analyses; two additional patients were excluded because they refused to participate in the follow-up program. Tumor stage and grading of the primary tumor is depicted in detail in Table 1. The mean follow-up period was 27 months; the median follow-up was 23 months and did not differ in the assessed subgroups. Five patients had already stage M1 disease at the time of diagnosis. Mean LMFS was 27 (range 0–115) months. Twelve patients had already undergone liver metastasectomy when presenting with their first pulmonary metastases. An average number of 1.85 ± 0.28 (mean ± SEM) histologically confirmed pulmonary nodules were resected per patient, ranging from one to eight per patient. Complete resection could be achieved in all of the included patients. Forty-one enucleations (20 Nd:YAG laser enucleations, 21 electrocautery enucleations), 25 wedge resections, and 6 lobectomies were performed. Nine patients had bilateral metastases. Forty-two of the 48 accesses (30 patients had unilateral and 9 had patients bilateral nodules) were anterolateral or posterior muscle-sparing thoracotomies; in 6 patients a VATS approach was applied. In eight patients, a repeated pulmonary metastasectomy was performed due to tumor recurrence within the study period. Two patients died during follow-up (15 and 39 months after metastasectomy.
TABLE 1 Correlation of clinicopathologic characteristics and KRAS mutational status in patients with CRC metastasizing to the lungs (N = 39)

<table>
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<tr>
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<th>Total</th>
<th>KRAS Wt</th>
<th>KRAS Mt</th>
<th>( \chi^2 ) test</th>
<th>EGFR-</th>
<th>EGFR+</th>
<th>( \chi^2 ) test</th>
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<td></td>
<td>N = 39</td>
<td>%</td>
<td>N = 21</td>
<td>%</td>
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<tr>
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<td>20</td>
<td>51.3</td>
<td>14</td>
<td>70</td>
<td>6</td>
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<td>48.7</td>
<td>17</td>
<td>36.8</td>
<td>12</td>
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<td>Age (years)</td>
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<tr>
<td>Median</td>
<td>64</td>
<td>68</td>
<td>57</td>
<td>0.013**</td>
<td>58.5</td>
<td>68</td>
<td>0.079a</td>
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<td>37–76</td>
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<td>37–78</td>
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<tr>
<td>Median (range)</td>
<td>23 (4–42)</td>
<td>26 (5–37)</td>
<td>22 (4–42)</td>
<td>0.621*</td>
<td>21 (4–41)</td>
<td>27 (5–42)</td>
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<td>Mean ± SD</td>
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<td>21 ± 11</td>
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<td>0.87</td>
<td>22 ± 13</td>
<td>24 ± 13</td>
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<td>Right-sided</td>
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<td>0</td>
<td>4</td>
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<td>5</td>
<td>45.5</td>
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because of end-stage disease). Tissue samples of repeated metastasectomies also were evaluated according the EGFR, KRAS, and BRAF statuses, but these data were not included in the analysis.

**TABLE 1 continued**

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LMFS lung-metastasis-free survival after primary tumor

*a* Mann–Whitney test  
*b* Log-rank test  
*c* Student’s *t* test

**FIG. 2** Pulmonary metastases showed negative (a) or positive membranous staining for EGFR (brown; DAB substrate), which varied from moderate to highly positive intensity (b and c, respectively; ×40 magnification; black bars indicate 50 μm) EGFR expression was not associated with time to any recurrence (d) or time to lung-specific recurrence after pulmonary metastasectomy (e).

EGFR Pathway and Lung-Specific Metastasis

EGFR overexpression was a frequent finding in resected PMs (Fig. 2). Nineteen of 39 patients had positive staining.
for EGFR (49%). EGFR expression was not associated with clinicopathologic characteristics (Table 1). Regarding the follow-up, Kaplan–Meier analysis revealed no impact of the EGFR expression levels on time to any recurrence (Fig. 2d) or time to pulmonary recurrence (Fig. 2e). Moreover, EGFR expression was not associated with Mts in the \( \textit{KRAS} \) gene (Fisher’s exact test, \( p = 0.341 \)).

**KRAS and BRAF Mt Status**

Forty-six percent of resected metastases had a \( \textit{KRAS} \) gene Mt. In 89% of these cases, codon 12 was mutated, whereas codon 13 Mt was found in only two cases (11%). Significant correlations between \( \textit{KRAS} \) Mt status and clinicopathological parameters could be found regarding the age and the site of primary tumor (Table 1). This is in line with published data, showing that the worse prognosis of right sided CRC might be due to an unfavorable genetic profile.\(^{28}\) Mucinous differentiation could not be observed in the right-sided tumors. In one patient, hereditary nonpolyposis CRC was suspected and ruled out by immunohistochemical staining for MSH2, MSH6, and MLH1. The time between the diagnoses of the primary tumor and first PM was shorter in patients harboring a \( \textit{KRAS} \) Mt than in those with Wt tumors (23 vs. 31 months, respectively) without reaching the level of significance (\( p = 0.279 \)). Moreover, in patients presenting with multiple metastases at the first metastasectomy \( \textit{KRAS} \) was more likely to be mutated than in patients with a singular node at initial presentation (73 vs. 27%; \( p = 0.037 \); Fig. 3a). In absolute numbers, patients with \( \textit{KRAS} \) Mt in the tumor tissue had significantly more metastases than the \( \textit{KRAS} \) Wt group (\( p = 0.029 \)), whereas no significant
difference could be found regarding the estimated total tumor volume ($p = 0.272$; see Supplementary material). Twenty-five patients evidenced a disease recurrence within the study period. Within this subgroup, KRAS Mt was associated with the lung as initial site of recurrence ($p = 0.047$; Fig. 3b).

All KRAS Wt samples were further evaluated for their BRAF V600E status. No BRAF Mts were found in the assessed PMs. This finding is in line with published evidence on primary CRC.  

**Table 2** Clinicopathological variables and time to recurrence and time to pulmonary recurrence of patients ($N = 39$) with CRC metastasizing to the lungs in the log-rank and Cox proportional hazards models

<table>
<thead>
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<th>Time to pulmonary recurrence</th>
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**Univariate and Multivariate Analysis**

We evaluated the impact of clinicopathological characteristics of the primary tumor and EGFR and KRAS status on time to tumor recurrence in univariate and multivariate analyses (Table 2). Neither clinicopathological characteristics nor EGFR protein expression or KRAS status were associated with the time to tumor recurrence irrespective of the recurrence site in univariate analysis. Likewise, we assessed the impact of clinical and molecular factors on
time to lung-specific recurrence. In univariate analysis, we found that patients receiving adjuvant chemotherapy after pulmonary metastasectomy had longer recurrence-free survival than those treated by surgery alone (25 vs. 17 months, respectively). However, this difference remained nonsignificant ($p = 0.071$). Mt in KRAS resulted in a significantly decreased time to pulmonary recurrence ($p = 0.013$; Fig. 3d). We further investigated all factors reaching $p < 0.1$ in log-rank tests in multivariate analysis (chemotherapy postmetastasectomy and KRAS Mt status). In multivariate analysis, the KRAS Mt status remained a poor prognostic factor associated with significantly decreased time to pulmonary recurrence after the first metastasectomy ($p = 0.035$).

**DISCUSSION**

Pulmonary metastasectomy has become an accepted approach in the treatment algorithm of CRC. However, there is a lack of well-designed prospective studies in this field. The two major obstacles are the low number of cases per year—even in high-volume centers—and the heterogeneity of the patient population in question. Thus, evidence is limited to retrospective analyses; most of the studies represent single-center experiences with an observation period of two to three decades. To overcome this problem, we established a prospective follow-up study with the goal to evaluate various histological and clinical characteristics on patients’ outcome after metastasectomy. Within an observation period of 4 years, 44 patients underwent metastasectomy.

This study represents the first systematic evaluation of EGFR expression and KRAS/BRAF Mt status in the setting of pulmonary metastasectomy with curative intent. We evaluated resected metastases for EGFR expression and KRAS and BRAF Mts.

Up to date, only two descriptive studies on EGFR expression in PMs from CRC are available. In our patient cohort, we found no impact of EGFR expression on tumor recurrence. This confirms studies on the primary tumor site, where prognosis was independent from EGFR expression.

We found a mutated KRAS gene in nearly half of our patients. This is in line with already published evidence from genetic analysis of the primary and metastatic tumor sites, with high occurrence of KRAS Mts in patients with PMs. However, to date no evidence on the impact of KRAS status on metastasis-free survival after first pulmonary metastasectomy has been published. We were able to show that patients with KRAS mutated tumors had a higher likelihood to develop pulmonary recurrence compared with the KRAS Wt group. Moreover, KRAS Mt was associated with multiple pulmonary nodules, which supports the idea of an aggressive, lung-specific behavior of primary cancers harboring a KRAS Mt. Because only two patients died during follow-up, determining a potential impact of EGFR pathway alterations on overall survival was not possible. Noteworthy, in a cohort of patients undergoing liver or lung metastasectomy, KRAS did not affect the overall survival. As KRAS and BRAF Mts are, in general, mutually exclusive, we further assessed KRAS Wt specimens regarding the BRAF V600E Mt. In contrast to moderately frequent Mts in the KRAS gene, the BRAF V600E Mt could not be detected in any tissue specimen.

Both KRAS and BRAF Mts are thought to be highly concordant during the progression of the disease. For this reason, we did not routinely examine tissue from the primary tumor. When more than one nodule was resected, genotyping revealed a consistent KRAS and BRAF status in all metastases. Furthermore, we analysed resected metastases of eight patients who underwent re-metastasectomy within the study period. Mt status also was highly conserved in all of these patients.

It is generally agreed that the identification of patients who will benefit at the utmost from pulmonary metastasectomy is difficult. This is especially true when patients present with pulmonary recurrence for a second, third, or fourth metastasectomy. Thus, the identification of risk factors for early recurrence might help the surgeon to weight a local surgical approach against a systemic chemotherapeutic regime.

Our study is limited by the fact that there was a variation in the received chemotherapy schemes. Due to the typically complex and heterogeneous history of patients with metastatic disease, this limitation can only be avoided by conducting large collaborative studies. In our series, patients with adjuvant chemotherapy after metastasectomy showed a tendency toward increased recurrence-free survival. However, we believe that our findings on altered EGFR signaling in PMs might have an impact on the individualized therapeutic approach and, moreover, on the postoperative tumor surveillance of patients with metastatic spread to the lungs.

In summary, our work underlines the association of KRAS Mt with lung-specific metastasis in a closely followed up study cohort. Additional large international collaborative studies are required to define the precise and optimal role of EGFR expression measurements and KRAS mutational testing in the diagnostic and therapeutic algorithm of CRC metastasizing to the lungs.

**ACKNOWLEDGMENT** This study was funded by the Christian Doppler Research Association and the Medical University of Vienna. Further support: TÁMOP 424A/1-11-1-2012-0001 (Dome B), OTKA MOB 80325 (Hegedus B), KTIA AIK 12-1-2013-0041 (Dome B), OTKA K109626 and OTKA K108465 (Dome B).
CONFLICT OF INTEREST  None declared.

REFERENCES

2.1.1. Interlude

After assessing molecular markers in tumor cells themselves, we focused in the work “Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases” on the tumor surrounding stroma. The tumor-surrounding stroma consisting of immune cells, fibroblasts and vessels gained growing attention in oncology. Activated fibroblasts are believed to contribute to disease progression and resistance to chemotherapy in solid malignancies, including CRC (Tsujino et al., 2007) (Lotti et al., 2013). The prognostic value of myofibroblasts, characterized by strong expression of alpha-smooth muscle actin (alpha-SMA) and vimentin, in CRC lung metastases remained elusive. Therefore we assessed occurrence of alpha-SMA\textsuperscript{+}, vimentin\textsuperscript{+} fibroblasts in CRC lung metastases, liver metastases and paired primary tumors. We could demonstrate that the small heat shock protein Hsp27 is co-expressed in these activated fibroblasts, introducing a novel and targetable marker for myofibroblasts. Hsp27\textsuperscript{+} fibroblasts were more common in lung metastases than in liver metastases, supporting the theory of a site-specific composition of the tumor microenvironment. Tumor vascularization, measured by CD31\textsuperscript{+} vessels, was increased in patients with strong stromal reaction. Moreover, patients with lung metastases harboring a highly active, Hsp27\textsuperscript{+} tumor stroma had a worse outcome after pulmonary metastasectomy.
2.2 Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases (published PDF)

Stromal Expression of Heat-Shock Protein 27 Is Associated with Worse Clinical Outcome in Patients with Colorectal Cancer Lung Metastases

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* hendrik.ankersmit@meduniwien.ac.at

Abstract

Background
Pulmonary metastases are common in patients with primary colorectal cancer (CRC). Heat-shock protein 27 (Hsp27) is upregulated in activated fibroblasts during wound healing and systemically elevated in various diseases. Cancer-associated fibroblasts (CAFs) are also thought to play a role as prognostic and predictive markers in various malignancies including CRC. Surprisingly, the expression of Hsp27 has never been assessed in CAFs. Therefore we aimed to investigate the expression level of Hsp27 in CAFs and its clinical implications in patients with CRC lung metastases.

Methods
FFPE tissue samples from 51 pulmonary metastases (PMs) and 33 paired primary tumors were evaluated for alpha-SMA, CD31, Hsp27 and vimentin expression by immunohistochemistry and correlated with clinicopathological variables. 25 liver metastases served as control group. Moreover, serum samples (n=10) before and after pulmonary metastasectomy were assessed for circulating phospho-Hsp27 and total Hsp27 by ELISA.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration and by the Medical University of Vienna. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: This study was supported by the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration and by the
Results
Stromal expression of Hsp27 was observed in all PM and showed strong correlation with alpha-SMA ($P < 0.001$) and vimentin ($P < 0.001$). Strong stromal Hsp27 was associated with higher microvessel density in primary CRC and PM. Moreover, high stromal Hsp27 and $\alpha$SMA expression were associated with decreased recurrence-free survival after pulmonary metastasectomy ($P=0.018$ and $P=0.008$, respectively) and overall survival ($P=0.031$ and $P=0.017$, respectively). Serum levels of phospho- and total Hsp27 dropped after metastasectomy to levels comparable to healthy controls.

Conclusions
Herein we describe for the first time that Hsp27 is highly expressed in tumor stroma of CRC. Stromal $\alpha$-SMA and Hsp27 expressions correlate with the clinical outcome after pulmonary metastasectomy. Moreover, serum Hsp27 might pose a future marker for metastatic disease in CRC.

Introduction
Colorectal cancer (CRC) is, after lung cancer, the second most common cause of cancer-related death in Europe [1]. More than one fifth of the patients with CRC present with metastases already at time of diagnosis of the primary cancer and the same proportion will develop metastases during the course of disease [1, 2]. The lungs are the second most common site of distant metastasis, making pulmonary metastases (PM) an essential contributor to the high mortality of CRC.

Besides cancer cells themselves, a tumor comprises stromal cells. The interactions of stromal and cancer cells is thought to be a major determinant of the tumor behavior and response to therapy [3, 4]. Cellular components of the stroma are fibroblasts, endothelial cells, immune cells and pericytes [5]. Cancer-associated fibroblasts (CAF), and especially activated fibroblasts, play a major role in the tumor-stroma network, similar to dermal fibroblasts in wound healing. This contributed to the description of tumors as "wounds that do not heal" by Dvorak et al. in the late 80’s [6]. CAF contribute to various tumor-promoting characteristics like extra-cellular matrix turnover, tumor growth, angiogenesis and metastasis [7, 8]. Due to the expression of $\alpha$-smooth muscle actin ($\alpha$-SMA), activated CAF are often described as myofibroblasts. They have also been shown to be positive for fibroblast-activation protein-$\alpha$/seprase, palladin and vimentin [9–12]. Recently, efforts have been made to characterize the "signature" of these fibroblasts by proteome and gene expression profiling [13, 14].

In the context of benign diseases, wound healing and keloid formation it is well known that activated fibroblasts express high levels of heat-shock protein 27 (Hsp27), which is crucial for fibroblast adhesion, contractility and motility [15–17]. The TGF-beta induced p38-MAPK pathway is the key regulator in the induction of Hsp27 in smooth muscle cells and myofibroblasts [18, 19]. Once synthesized, two main functions of Hsp27 are critical in wound healing process: promoting myofibroblast motility and angiogenesis. Hsp27 is involved in the stabilization of actin filaments and of SNAIL, an inducer of epithelial-mesenchymal transition. Both mechanisms contribute to the induction of the myofibroblastic phenotype [20, 21]. Another function of Hsp27 executed in a paracrine manner is enhancing angiogenesis. It was demonstrated that extracellular Hsp27 leads to Nf$k$B activation and subsequent expression of the
proangiogenic factors VEGF and interleukin-8 (IL-8) in endothelial cells [22]. Together with others, our group could show that an overexpression of Hsp27 is strongly linked to several benign and malignant pathologies of the lung associated with fibroblast activation, including emphysema/chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis and non-small cell lung cancer [21, 23–26].

Given the fact that activated fibroblasts play an important role in the progression of malignant disease as well as in various non-malignant diseases of the lung, we aimed to investigate the prevalence of Hsp27 positive tumor stroma in CRC lung metastases and corresponding primary tumors. Furthermore, we sought to describe the implications on the clinical outcome after metastasectomy and the presence of cellular and secreted Hsp27 in these patients.

Materials and Methods

Study population

From April 2009 to November 2013, all consecutive cases of pulmonary metastasectomy from primary CRC and appropriate tissue samples were included in this study. Patients received diagnostic work-up including thoracic and abdominal computed tomography (CT). If patients had undergone pulmonary metastasectomy before, specimen of the first metastasectomy was also examined and the date of the first metastasectomy was used for outcome calculation. Lung metastasis free survival (LMFS) was defined as the time between diagnosis of the primary tumor and diagnosis of the metastatic spreading to the lung. R0 resection was achieved in all patients. Of 33 patients, specimens of the corresponding primary tumor could be obtained. Follow-up examinations were carried out in 3 to 6 months intervals. Recurrence-free survival (RFS) was defined as the period from the first pulmonary metastasectomy to evidence of recurrent disease at any site verified by CT scans. In 10 consecutive cases, serum samples obtained before metastasectomy and during follow-up were available. Follow-up serum samples were collected 3 to 6 months after surgery. Additionally, serum samples from age-, gender- and smoking status- matched healthy volunteers were collected. All patients gave their written informed consent prior to blood collection and participation. A study cohort of 25 consecutive patients with resected CRC liver metastases served as additional control group. The study was approved by the ethics committee of the Medical University of Vienna (EK 91/2006, EK1194/2011 and EK1044/2012) and was conducted according to the declaration of Helsinki. The current study cohort is based on previous published works by our group.[27, 28]

Immunohistochemistry and immunofluorescence

Immunohistochemical staining was performed according to a standard protocol. Shortly, formalin-fixed, paraffin-embedded tissue specimens were cut in 4-μm thick sections and deparaffinized. Heat-mediated antigen retrieval was performed. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Sections were incubated with the appropriate primary antibody for 1h at room temperature. For immunohistochemistry, as secondary step, the polymer-based ImmPRESS kit (Vector Laboratories, Burlingame, California) was used according to the manufacturer’s protocol. 3,3’-Diaminobenzidine (DAB) was used as substrate (Vector Laboratories, Burlingame, California). Finally, the sections were counterstained with hematoxylin. Vimentin staining was performed in a Ventana ES Immunostainer System (Ventana Medical Systems Inc., Tucson, AZ, USA). For immunofluorescence, appropriate fluorescent secondary antibodies were used and cell nuclei were counterstained with DAPI (Sigma Aldrich, St. Louis, MO, USA). As negative controls, the primary antibody was omitted. Positive controls were tissue samples with known presence of the respective target protein. A detailed list of antibodies and dilutions is provided as supplementary information (S1 Table).
Scoring of stained tumor cells. Immunohistochemistry staining score (IHC score) was calculated as described previously [29]. The percentage of positive tumor cells could reach values between 0 and 100%, and were multiplied by the staining intensity (0 to 3). Thus, IHC scores could range from 0 to 300. Two blinded observers rated the staining. In case that the two ratings differed, the slide was discussed and re-evaluated. The continuous IHC score was dichotomized by applying the median score of metastases or primary tumors as cut-off value.

Scoring of stained stromal cells. The sections were scored semiquantitatively as described previously [10]. Briefly, the slides were screened at low magnification and evaluated for their staining intensity in the stromal cells in the tumor center. The stromal staining was assessed as grade 0 (negative, <1% positive stromal cells), grade 1+ (low, 1–10% positive stromal cells), grade 2+ (intermediate, >10–50% positive stromal cells) and grade 3+ (strong, >50% positive stromal cells) by two blinded observers. In case that the two ratings differed, the slide was discussed, re-evaluated and a consensus was reached on all slides.

Determination of microvessel density. Microvessel density (MVD) was measured using the “hotspot” method, as published elsewhere [30]. In brief, the slides were screened at low magnification to identify the area with the greatest number of CD31-positive microvessels (“hotspot”). MVD was determined by counting all microvessels at 200x magnification (corresponding to 0.95 mm²). Mean values were calculated from two independently counted densities. In case of strong inter-observer discrepancy, the slide was reevaluated.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were assessed by commercially available human Hsp27, phospho-Hsp27 and interleukin-8 ELISA kits (all R&D Systems, Minneapolis, USA). Measurement was conducted according to the manufacturer’s instructions. The samples were assayed in duplicates. The absorbance was measured at 450nm using a plate reader (PerkinElmer, Waltham, USA), compared to a standard curve with known protein content and converted to pg/mL.

Statistical analysis

All obtained data was evaluated statistically using SPSS 19 (SPSS Inc., Chicago, USA) and GraphPad Prism 6 (GraphPad Software Inc., California, USA). Student’s t-test was used to compare means of two independent groups, paired t-test for dependent groups and expressed as mean±standard deviation (SD). Kaplan-Meier curves and log-rank test were used to compare survival functions. Chi-square test and Fisher’s exact test were used to compare binominal variables. All tests were two-sided. P-values equal or below 0.05 were considered as statistically significant.

Results

Tissue specimens of PM from a total of 51 consecutive patients were available. Median age at metastasectomy was 63 years (range 33–83). 29 (56.9%) male and 22 (43.1%) female patients were included. The clinicopathological characteristics of the included patients are summarized in Table 1.

Hsp27 is highly expressed in cancer-associated stroma of lung metastases

To assess the expression level of Hsp27 in cancer-associated stroma, tissue sections were stained for Hsp27 and vimentin (Fig. 1). The Hsp27 expression of tumor stroma in PM was scored in 0 (0%), 17 (33%), 17 (33%), 17 (33%) cases as 0, 1+, 2+ and 3+, respectively. The
Table 1. Descriptive data on patient, primary tumor and metastases characteristics stratified by stromal Hsp27 and α-SMA expression (N = 51).

| Patients | Sex | Male | 29 | 56.9 | 10 | 19.6 | 8 | 15.7 | 11 | 21.6 | 0.571 | 8 | 15.7 | 12 | 23.5 | 9 | 17.6 | 0.771 |
| | | Female | 22 | 43.1 | 7 | 13.7 | 9 | 17.6 | 6 | 11.8 | | 6 | 11.8 | 11 | 21.6 | 5 | 9.8 | |
| Age (years) | Median | 63 | 66 | 62 | 63 | 0.618 | 67 | 62 | 61.5 | 0.383 | 50 | 38 | 37–78 | 33–74 |
| | Range | 33–83 | 47–83 | 37–78 | 33–74 | | |
| Primary tumor | Location | Colon | 27 | 52.9 | 9 | 17.6 | 8 | 15.7 | 10 | 19.6 | 0.790 | 6 | 11.8 | 14 | 27.5 | 7 | 13.7 | 0.549 |
| | Rectum | 24 | 47.1 | 8 | 15.7 | 9 | 17.6 | 7 | 13.7 | | 6 | 11.8 | 14 | 27.5 | 7 | 13.7 | |
| | T stage | pT1 | 1 | 2.1 | 1 | 2.1 | 0 | 0.0 | 0 | 0.0 | 1 | 2.1 | 0 | 0.0 | 0 | 0.0 | |
| | | pT2 | 7 | 14.6 | 1 | 2.1 | 3 | 6.3 | 3 | 6.3 | 0.109 | 1 | 2.1 | 3 | 6.3 | 3 | 6.3 | 0.032 |
| | | pT3 | 34 | 70.8 | 11 | 22.9 | 11 | 22.9 | 12 | 25.0 | | 8 | 16.7 | 16 | 33.3 | 10 | 20.8 | |
| | | pT4 | 6 | 12.5 | 2 | 4.2 | 3 | 6.3 | 1 | 2.1 | | 3 | 6.3 | 3 | 6.3 | 0 | 0.0 | |
| | N/A | 3 | - | | | | | | | | | | | | | | |
| N stage | pN0 | 21 | 43.8 | 9 | 18.8 | 6 | 12.5 | 6 | 12.5 | 0.169 | 7 | 14.6 | 9 | 18.8 | 5 | 10.4 | 0.299 |
| | pN1 | 11 | 22.9 | 2 | 4.2 | 6 | 12.5 | 3 | 6.3 | | 2 | 4.2 | 7 | 14.6 | 2 | 4.2 | |
| | pN2 | 16 | 33.3 | 4 | 8.3 | 5 | 10.4 | 7 | 14.6 | | 4 | 8.3 | 6 | 12.5 | 6 | 12.5 | |
| | N/A | 3 | - | | | | | | | | | | | | | | |
| Grading | G1 | 2 | 3.9 | 1 | 2.0 | 1 | 2.0 | 0 | 0.0 | 1 | 2.0 | 1 | 2.0 | 0 | 0.0 | |
| | G2 | 42 | 82.4 | 13 | 25.5 | 14 | 27.5 | 15 | 29.4 | 0.487 | 11 | 21.6 | 19 | 37.3 | 12 | 23.5 | 0.713 |
| | G3 | 7 | 13.7 | 3 | 5.9 | 2 | 3.9 | 2 | 3.9 | | 2 | 3.9 | 3 | 5.9 | 2 | 3.9 | |
| Stromal HSP27 | Low | 6 | 12.5 | 0 | 0.0 | 2 | 6.1 | 4 | 12.1 | | 6 | 12.5 | 0 | 0.0 | 2 | 3.9 | 3 | 12.1 |
| | Intermediate | 15 | 45.5 | 6 | 18.2 | 6 | 18.2 | 3 | 9.1 | 0.053 | 5 | 15.2 | 8 | 24.2 | 2 | 6.1 | 0.081 |
| | High | 12 | 36.4 | 4 | 12.1 | 4 | 12.1 | 4 | 12.1 | | 4 | 12.1 | 5 | 15.2 | 3 | 9.1 | |
| | N/A | 3 | - | | | | | | | | | | | | | | |
| Stromal α-SMA | Low | 8 | 24.2 | 2 | 6.1 | 3 | 9.1 | 3 | 9.1 | 3 | 9.1 | 3 | 9.1 | 2 | 6.1 | |
| | Intermediate | 14 | 42.4 | 4 | 12.1 | 6 | 18.2 | 4 | 12.1 | 0.711 | 3 | 9.1 | 7 | 21.1 | 4 | 12.1 | 0.779 |
| | High | 11 | 33.3 | 4 | 12.1 | 3 | 9.1 | 4 | 12.1 | | 3 | 9.1 | 6 | 18.2 | 2 | 6.1 | |
| | N/A | 18 | - | | | | | | | | | | | | | | |
| Pulmonary metastasis | Microvessel density | Mean | 39.0 | 33.0 | 40.9 | 43.0 | 0.169 | 32.4 | 35.9 | 44.6 | 0.136 |
| | Range | 12–96 | 12–56 | 16–76 | 19–96 | | 12–76 | 18–85 | 19–96 | |
| | Previous liver metastasis | Yes | 16 | 31.4 | 5 | 9.8 | 6 | 11.8 | 5 | 9.8 | 0.913 | 1 | 2.0 | 12 | 23.5 | 3 | 5.9 | 0.004 |
| | No | 35 | 68.6 | 12 | 23.5 | 11 | 21.6 | 12 | 23.5 | | 13 | 25.5 | 11 | 21.6 | 11 | 21.6 | |
| | No. of nodules | 1 | 33 | 64.7 | 12 | 23.5 | 9 | 17.6 | 12 | 23.5 | 0.462 | 8 | 15.7 | 14 | 27.5 | 11 | 21.6 | 0.236 |

*Kruskal-Wallis test;
 Fisher’s exact test;
 One-way-ANOVA; LMFS: Lung-metastasis free survival after primary tumor
correlation of the stromal Hsp27 staining with patient and tumor characteristics is depicted in Table 1. Staining intensity of the tumor cells was determined separately (S1A Fig). 23 (45%) metastases were scored as Hsp27 highly positive and 28 (55%) metastases as low/negative (IHC score range 0–160; median/cut off 30). There was no correlation between tumor and stromal Hsp27 expression in pulmonary metastases (Chi square test; \( P = 0.237 \)).

33 corresponding primary tumors were available. The stromal Hsp27 expression in the primary tumors 0 (0%), 6 (18%), 15 (46%) and 12 (36%) were scored as 0, 1+, 2+ and 3+, respectively. Determining the Hsp27 staining in the tumor cells in the primary tumor tissue, 14 (42%) of the cases were scored as Hsp27 positive and 19 (58%) as low/negative (IHC score range 5–100; median/cut off 70).

Hsp27 is co-expressed with \( \alpha \)-SMA and vimentin in the stroma of PMs

Activated tumor stroma has been described as highly \( \alpha \)-SMA positive in primary and metastatic CRC [12, 31]. Therefore, the specimens were stained for \( \alpha \)-SMA. The expression of \( \alpha \)-SMA in the stroma was rated in the same semiquantitative manner as the Hsp27 staining. 0 (0%), 13 (25.5%), 24 (51%) and 13 (25.5%) cases were scored as 0, 1+, 2+ and 3+, respectively. The staining score distribution according to the clinicopathological variables is depicted in Table 1.
Expression levels of α-SMA correlated significantly with Hsp27 expression in PMs (P < 0.001, Fig. 2A). This was also observed in liver metastases (S2 Fig). To further assess the stromal co-expression of α-SMA and Hsp27, representative slides of primary and metastatic tumors were co-labeled for α-SMA and Hsp27 by immunofluorescence. A strong co-expression could be observed especially in PMs (Fig. 2D). Tumor stroma with strong Hsp27 and α-SMA staining was also highly positive for vimentin (S3 Table).

MVD is increased in tissue samples with Hsp27-positive tumor stroma

Recently it was shown that Hsp27 mediates angiogenesis [22]. We therefore determined the MVD by CD31 staining and correlated it with the Hsp27 expressions (Fig. 2B and 2C). In PM, we found a trend towards higher MVD in samples with high Hsp27 levels. MVD was 33.0±2.9, 40.1±3.8 and 43.0±4.7 for 1+, 2+ and 3+ Hsp27 intensity (mean±SD). However, this trend did not reach the level of significance (P = 0.081). Similarly, in primary CRC with high stroma levels of Hsp27, significantly more microvessels could be found (27.7±3.2, 37.2±4.0 and 46.4±5.2 for 1+, 2+ and 3+ Hsp27 intensity, respectively; P = 0.031). A detailed description of MVD stratified by clinicopathological characteristics is provided in S2 Table.
Stromal Hsp27 is associated with poor outcome after pulmonary metastasectomy

We assessed the association of relevant clinicopathological variables with LMFS, RFS and OS after metastasectomy (Table 2). No variable had significant impact on the LMFS. Female patients had a strong trend towards a shorter time to PMs ($P=0.091$). The stromal expression of neither α-SMA nor Hsp27 was associated with a significantly decreased LMFS. However, both histological markers had a significant prognostic impact on the RFS after pulmonary metastasectomy ($P=0.018$ and $P=0.008$ for stromal Hsp27 and α-SMA, respectively) (Fig. 3). Again, female patients showed a strong trend towards decreased RFS. Moreover, patients with a history of previous liver metastases had a decreased RFS compared to patients without liver metastases in the history. However, both variables, sex and the history of previous liver metastases, had no significant influence on the RFS. Additionally to that, extensive stromal Hsp27 and α-SMA were associated with a decreased overall survival after metastasectomy ($P=0.031$ and $P=0.017$ for stromal Hsp27 and α-SMA, respectively). Due to the high rate of concordance, stromal Hsp27 and α-SMA were no independent factors in the outcome analysis when adding both variables into a multivariate analysis (data not shown).

Table 2. Univariate analysis assessing clinicopathological variables and lung metastasis free survival, recurrence free survival after metastasectomy and overall survival of patients (N = 51) with CRC metastasizing to the lung.

<table>
<thead>
<tr>
<th>Total</th>
<th>Lung metastasis free survival</th>
<th>Recurrence free survival</th>
<th>Overall survival</th>
</tr>
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<td>Univariate analysis (log-rank)</td>
<td>Univariate analysis (log-rank)</td>
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<tr>
<td>N = 51</td>
<td>Months</td>
<td>HR (95% CI)</td>
<td>p-value</td>
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<tr>
<td>Sex</td>
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<td>43.1</td>
</tr>
<tr>
<td></td>
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<td>56.9</td>
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<tr>
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<tr>
<td></td>
<td>&gt; 64 yrs</td>
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<td>31.4</td>
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<tr>
<td></td>
<td>high</td>
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Stromal Hsp27 in CRC Lung Metastases
Soluble Hsp27 is systemically increased in patients before pulmonary metastasectomy

Elevated levels of soluble Hsp27 can be detected systemically in various malignant and non-malignant diseases. In a subset of 10 of our patients paired serum samples (pre- and post-metastasectomy) were available (Fig. 4). We compared the serum level of total Hsp27 and phospho-Hsp27 to matched healthy volunteers (S3 Table). Serum total Hsp27 levels were 2276±905, 3245±1684 and 2064±1226 pg/mL (mean±SD) for control, pre- and post-metastasectomy samples, respectively. Total Hsp27 decreased significantly after metastasectomy (P = 0.016). Serum phospho-Hsp27 levels were 84±63, 156±82 and 88±114 pg/mL (mean±SD) for control, pre- and post-metastasectomy samples, respectively. A significant difference was found between healthy controls and pre-metastasectomy samples (P = 0.041). Although the levels dropped after metastasectomy, the difference was not significant. IL-8, a proangiogenic cytokine involved in the Hsp27 signaling, was detected at very low concentrations of 15±8, 45±58 and 9±8 pg/mL (mean±SD) in the serum samples. The differences of systemic IL-8 levels between the three groups did not differ significantly from each other. No correlation was found between the expression level of stromal Hsp27, tumor Hsp27 and systemic levels (data not shown). Moreover, no significant correlation between total or phospho-Hsp27 and standard inflammatory markers (CRP and fibrinogen) was found (S1B and S1C Fig).

Fig 4. Kaplan-Meier plots showing the lung-metastasis free survival, recurrence free survival and overall survival after metastasectomy dependent on stromal Hsp27 (A), stromal α-SMA (B) and tumor Hsp27 (C) scoring.

Figure 3

Soluble Hsp27 is systemically increased in patients before pulmonary metastasectomy
Stromal Hsp27 expression in pulmonary metastases is significantly increased compared to liver metastases

We further evaluated the stromal expression of stromal Hsp27 and α-SMA in liver metastases of 25 patients. Regarding the stromal Hsp27 expression 0 (0%), 12 (48.0%), 11 (44.0%) and 2 (8.0%) cases were scored as 0, 1+, 2+ and 3+, respectively. Stromal α-SMA expression was rated as expression 0 (0%), 8 (32.0%), 11 (44%) and 6 (24.0%) cases were scored as 0, 1+, 2+ and 3+, respectively. We found a strong correlation between stromal Hsp27 and α-SMA (P < 0.001). Furthermore, stromal Hsp27 positivity was significantly more often found in pulmonary metastases compared to liver metastases (P = 0.014), whereas this difference was not significant for α-SMA (P = 0.625). Moreover, expression of Hsp27 in tumor cells (median 120 (range 0–270)) was significantly increased in liver metastases compared to pulmonary metastases (P = 0.037). We further analyzed the overall survival of the patients with liver metastases depending on their stromal Hsp27, α-SMA and tumor Hsp27 expression, but significant differences were not found (S3 Fig).

Discussion

Distant metastases are the main cause of cancer-related mortality. Today, the tumor microenvironment and especially fibroblasts become of growing interest, providing additional information on tumor behavior and potential therapeutic targets [32]. Myofibroblasts can be found in...
the microenvironment of various tumors and extensive fibroblast-activation could be linked to rapid disease progression in malignant disease [32].

Herein we could demonstrate in a well-defined study cohort that vimentin-αSMA+ stromal cells are highly present in pulmonary metastases of primary CRC. Wikberg et al. outlined that myofibroblasts in the tumor center are a better prognosticator for poor prognosis than fibroblasts at the tumor margin [33]. We, therefore, restricted the scoring of the putative fibroblast activation to the central portion of the tumor. Compared to Henry et al., who identified myofibroblasts by FAP-staining in primary colon cancer, we found a similar distribution of the stromal scoring (0%, 20%, 38% and 35% for negative, low, intermediate and strong staining, respectively). Additionally, they described the association of high amounts of activated fibroblasts with a decreased overall survival in patients with colon cancer, especially in the metastasized situation [10]. This goes in line with our findings showing a decreased RFS after pulmonary metastasectomy.

Interestingly, α-SMA positive phenotype of tumor-associated stroma was accompanied by the expression of Hsp27. On the one hand, this small heat-shock protein has been examined in CRC tumor cells themselves [34–36]. An increased expression of Hsp27 in tumor cells of primary CRC was described as negative prognosticator for survival [37, 38]. On the other hand, Hsp27 expressing fibroblasts were examined in the context of wound healing and fibrosis. To the best of our knowledge, stromal Hsp27 has not been described as a potential prognostic marker in CRC. Interestingly, strong stromal Hsp27 expression was associated with high MVD in the primary tumors (P = 0.031) and in PM (P = 0.081). Due to the strong correlation of Hsp27 with α-SMA as an established marker for fibroblast activation, the over-expression of Hsp27 was associated with significantly worse clinical outcome after pulmonary metastasectomy (median RFS 19, 17 and 8 months for 1+, 2+ and 3+, respectively), comparable to the prognostic impact of stromal α-SMA expression. The decrease RFS was also translated in a significantly different OS between the low, intermediate and high stromal Hsp27 or α-SMA, respectively (Fig. 3). Similarly, Tsujino et al. described α-SMA positive fibroblasts as being capable to predict disease recurrence in a cohort of patients with stage II and III primary CRC [12]. Kahlert et al. demonstrated by microdissection of primary CRC, lung and liver metastases that a panel of pro-angiogenic factors are differentially expressed in tumor cells and the stromal compartment. The stromal expression of angiopoietin-2 in pulmonary metastases was an independent prognosticator for poor survival after surgery in multivariate analysis (P = 0.044), even in a rather small study cohort (n = 25) [39]. These findings support the results of our work, in which we could describe Hsp27 as a further proangiogenic, stromal marker with prognostic impact after metastasectomy. It is also important to mention, that Sato et al. described high stromal expression of the description factors ETS1 as a predictor of CRC lung metastases [40]. Interestingly, ETS1 is strongly interacting with the small heat-shock protein network [41].

The analysis of blood samples in a subgroup of patients revealed that metastatic CRC is a relevant inducer even of systemic Hsp27 expressions. The level of soluble total Hsp27 and phospho-Hsp27, a polymerized form of Hsp27, significantly decreased after complete removal of the PMs. Moreover, compared to a matched control group of healthy volunteers, pre-operative total Hsp27 and phospho-Hsp27 levels were increased and dropped after surgery to comparable low levels. Similar to our findings, Zhao et al. could demonstrate increased serum levels of Hsp27 in ovarian cancer with peritoneal metastases. In the same work it was also shown that the levels dropped after chemotherapy [42]. IL-8, which is thought to mediate locally the proangiogenic effect of Hsp27, was also elevated before metastasectomy without reaching significance [22]. Nonetheless, the circulating levels of Hsp27 did not correlate with the stromal or tumor expression. Furthermore it did not correlate with the systemic levels of CRP or
fibrinogen. Thus, the circulating Hsp27 might be influenced by other factors like secretion, degradation and elimination. Based on these preliminary results, further studies with adequate cohort sizes will be necessary to further clarify the possible prognostic and predictive role of circulating Hsp27 levels in patients with CRC.

In patients with CRC liver metastases the tumor stroma Hsp27+ vimentin+αSMA+ fibroblasts were less evident than in pulmonary metastases. In contrast to this, the Hsp27 expression in tumor cells was significantly higher in resected liver metastases compared to pulmonary metastases. This goes in line with the observation that the p38 MAPK signaling, a pivotal kinase for Hsp27 phosphorylation and activation, is downregulated in CRC pulmonary metastases compared to liver metastases [43].

The findings of this work are of translational relevance because of two aspects: first, Hsp27, systemically, or confined to the tumor stroma, might possess potential prognostic and predictive value. Pulmonary metastasectomy is a widely offered treatment option in patients with CRC. However, the identification of patients who will benefit from surgery alone or an optional adjuvant chemotherapy is a matter of current research [44]. Biomarkers like Hsp27 might help to identify patients with high risk of early recurrence of disease after surgery. Adjuvant chemotherapy and a stringent follow-up could be offered to these patients. During the follow-up of patients with malignant disease, serum markers, e.g. CEA, CA19–9 and beta-HCG, are routinely used nowadays and additional markers might potentiate the accuracy of these established markers. A limitation to this study is, that CEA and CA19–9 levels were not routinely determined before metastasectomy. Correlation of these established tumor markers with serum Hsp27 will be addressed in a future study.

Secondly, Hsp27 is a promising drug target. Apatorsen (OGX-427, Oncogenex, Bothell, Washington, USA), a modified antisense oligonucleotide, binds to the Hsp27 mRNA transcript and therefore inhibits the Hsp27 expression [45]. Currently ongoing Phase II studies are recruiting patients with advanced prostate, bladder, pancreatic and non-small cell lung cancer [46]. The elevated expression levels of Hsp27 in the tumor-associated stroma provide a rationale for the use of Hsp27 also in patients with primary CRC. Given that not only CRC lung metastases, but also liver and lymph node metastases exhibit strong myofibroblast recruitment, especially patients with metastatic disease might benefit from Hsp27 inhibition.

In conclusion, we could demonstrate that Hsp27 is co-expressed with αSMA in the tumor stroma of CRC lung metastases and, moreover, that its over-expression is associated with worse clinical outcome after metastasectomy. Of note, soluble Hsp27 was also systemically measurable, making serum Hsp27 a potential future serum marker in CRC.

Supporting Information

S1 Table. Antibodies and dilutions used for immunohistochemistry and immunofluorescence.

S2 Table. Descriptive data on patient, primary tumor and metastases characteristics stratified by MVD and vimentin (N = 51).

S3 Table. Characteristics of matched patients and healthy controls.

S1 Fig. Hsp27 expression was also found in tumor cells itself (anti-Hsp27; DAB substrate; 200x magnification) (A). Correlation of pre-operative Hsp27 and C-reactive protein (CRP) or
Neither total Hsp27 (B), nor phospho-Hsp27 (C) correlated significantly with CRP or fibrinogen.

S2 Fig. In CRC liver metastases stromal Hsp27 and α-SMA scoring correlated significantly (n = 25) (A).

(TIF)

S3 Fig. The distribution of stromal Hsp27 (A) differed significantly in liver metastases (n = 25) compared to lung metastases (n = 51). This difference did not reach significance for stromal α-SMA (B). Hsp27 expression in tumor cells was significantly higher in liver metastases compared to lung metastases (C). No significant differences were observed between the subgroups regarding overall survival (D-F).

(TIF)

Author Contributions
Conceived and designed the experiments: MM PB BD WK KH HJA. Performed the experiments: TS CN DT MZ BH. Analyzed the data: TS DT KH HJA. Contributed reagents/materials/analysis tools: PS MB MZ PB BH BD MM WK KH HJA. Wrote the paper: TS PS MB MZ PB BH BD MM WK KH HJA.

References


CHAPTER THREE: DISCUSSION

3.1 General discussion

In this thesis we could describe multiple clinical and tissue-based prognostic markers in a well-defined cohort of patients with CRC lung metastases. We demonstrated that various alterations on DNA and protein level - which can be present in tumor cells or in the tumor-surrounding stroma – are capable of prognosticating the outcome after pulmonary metastasectomy. The herein described findings might contribute to a better understanding of pulmonary metastasis in CRC.

In the first work included in this thesis, patients with KRAS mutant metastases were demonstrated to have a worse clinical outcome after pulmonary metastasectomy. This finding was confirmed in various other study cohorts. Renaud et al. described in a retrospective cohort of 180 patients receiving CRC pulmonary metastasectomy a similar high incidence of KRAS mutations (51.7%). In multivariate analysis, KRAS wild-type tumors had a significantly better overall survival after metastasectomy than KRAS mutant tumors (HR 0.04; 95% CI 0.02–0.1, \( P < 0.0001 \)). In accordance to our study, time to lung-recurrence was significantly longer in patients with KRAS wild-type tumors (HR 0.16; 95%CI 0.07–0.33, \( P < 0.0001 \)). Interestingly, if the recurrent metastases were resected during a second metastasectomy in KRAS mutant patients, the overall survival was not inferior to patients with KRAS wild-type tumors (Renaud et al, 2015). In a study conducted at the MD Anderson Cancer Center (Houston, Texas, USA) including patients with metastatic CRC, patients with KRAS mutant tumors had a significantly shorter time to occurrence of lung metastases (22.4 versus 15.2 months; HR 1.40; 95% CI 1.12–1.75; \( P = 0.002 \)). The overall survival did not differ between the two groups (Pereira et al, 2015). In contrast to this, Ghidini et al. found a significantly decreased overall survival in patients with KRAS mutant after pulmonary metastasectomy (60.9 versus 36.6 months, HR 2.17, 95% CI 1.19-3.96, \( P = 0.012 \)) (Ghidini et al, 2016). The heterogeneity in the management of recurrent disease (chemotherapy plus surgery versus chemotherapy or surgery alone) might contribute to a substantial difference in the outcome analysis in these studies.

Interestingly, also in CRC with isolated liver metastasis and liver metastasectomy, KRAS mutant tumors have an increased propensity to form lung metastases subsequently. Shindoh et al. showed in a patient cohort with 163 CRC liver metastases that patients with KRAS mutant CRC had a nearly 3-fold risk of lung recurrence after liver metastasectomy (HR 2.56; 95% CI 1.54-4.25; \( P < 0.001 \)). The time to surgical failure was defined as the time between between initial liver surgery and diagnosis of metastatic recurrence deemed unresectable.
Time to surgical failure was significantly shorter in KRAS mutant tumors compared to the KRAS wild-type tumors (18.8 versus 39.7 months, \( P = 0.001 \)). Even more striking, the occurrence of unresectable (i.e. multiple, diffuse) lung metastases after liver metastasectomy was significantly more common in patients with KRAS mutant compared to KRAS wild-type CRC (18% versus 41%, \( P = 0.048 \)). This conferred also a marked decrease in the 3-year disease-specific survival of the KRAS mutant group (59.8 versus 83.6%; \( P = 0.016 \)). (Shindoh et al, 2016). The authors draw the conclusion that especially lung recurrence after isolated liver metastasectomy hinders long-term survival in patients with KRAS mutant tumors. Thus, these patients might benefit from adjuvant chemotherapy after liver resection and should be closely monitored by chest CT imaging. Stremitzer et al. described in a cohort of 60 patients receiving liver metastasectomy that the recurrence-free survival as well as the overall survival was decreased in patients with KRAS mutant CRC (Recurrence-free survival: HR 2.48, 95%CI 1.26 – 4.89; \( P = 0.009 \). Overall survival: HR 3.51, 95%CI 1.30 – 9.45; \( P = 0.013 \)) (Stremitzer et al, 2012). Furthermore, Vauthey et al. showed in a study cohort from the U.S. that the time to lung-specific recurrence after liver resection was significantly decreased in patients with RAS (including KRAS and NRAS) mutant tumors (HR 2.0, 95%CI 1.1 – 3.4; \( P=0.01 \)). The time to liver recurrence did not significantly differ between the two groups. Again, the increased likelihood to involve the lungs transduced into inferior 3-year survival of KRAS mutant CRC patients (52.2% versus 81%, \( P=0.002 \)) (Vauthey et al, 2013).

The underlying mechanism for the increased capability of KRAS mutant CRC to metastasize to the lungs remains widely unknown. Pollock et al. demonstrated in vitro using the KRAS mutant HCT116 colon carcinoma cell line that cell adhesion is decreased and cell motility is increased in KRAS mutant cells. The disruption of the cytoskeleton was conferred by uncoupling the Rho-ROCK pathway from the capability to form fibers. Moreover, the increased cell motility was mediated by an increased PI3-kinase activity (Pollock et al, 2005). Schramm et al. demonstrated in SW480 colon cancer cells that KRAS mutant cells exhibit a strongly different phenotype regarding integrin expression at the cell surface (Schramm et al, 2000). This might have implications on the metastatic capability and especially on a potential organ-tropism. Another group demonstrated an increased expression of pro-angiogenic chemokines (CXCL-1 and -8) concomitant to a decreased expression of the anti-angiogenic chemokine CXCL-10 in KRAS mutant cell lines (Khan et al, 2014). Watanabe et al. compared the expression of 30 cancer progression-related genes in 35 KRAS mutant and 78 KRAS wild-type primary CRC. Differentially activated pathways included the Wnt-, NF-Kappa B and TGF beta- signaling pathway (Watanabe et al, 2011). In a recent work, it was demonstrated that the up-regulation of TRAIL-R2 is another possible mechanism of KRAS mutant tumors to rapidly proceed (von Karstedt et al, 2015). These findings shed light on the distinct biological characteristics of KRAS mutant CRC and provide possible explanations for
the different clinical presentation of patients with CRC harboring KRAS mutations. Beyond the routinely tested KRAS mutations in codons 12 and 13, there are several rare and atypical but - despite this - also activating mutations in the KRAS (codons 61 and 146) and NRAS (codons 12, 13 and 61) gene. Interestingly, tumors harboring these atypical mutations show a similar lung specific metastatic pattern like tumors with the traditional mutations in KRAS codons 12 and 13. Furthermore, the signatures of expressed genes were comparable between tissue samples of typical KRAS mutant tumors and atypical KRAS mutant tumors (Morris et al., 2014). Therefore, further studies assessing KRAS and other relevant genes using e.g. next generation sequencing might provide an even more precise prognostic information in patients with metastatic CRC.

In conclusion, we could demonstrate that KRAS mutation is a valuable prognostic marker after pulmonary metastasectomy. There is rising evidence in the literature that KRAS might be one of the most important prognosticators in metastatic CRC so far.

In the second work included in this thesis we assessed the tumor microenvironment of CRC lung metastases and corresponding primary tumors. We could demonstrate that large differences exist between pulmonary metastases regarding accumulation of Hsp27+ fibroblasts in close neighborhood to the tumor cells. Moreover, the density of myofibroblasts in pulmonary metastases significantly exceeded the detected myofibroblasts in liver metastases. Interestingly, patients with CRC lung metastases consisting of highly Hsp27 and alpha-SMA positive stromal cells had a worse clinical outcome in terms of recurrence-free survival and overall survival after pulmonary metastasectomy. Moreover, angiogenesis was increased in tumor samples with highly Hsp27 positive stromal cells compared to samples with low stromal Hsp27 expression. This clinical observation was recently confirmed in an experimental metastasis model using C57BL/6 mice. Injecting activated fibroblasts – by TGF-beta pretreatment – resulted in highly angiogenic and proliferating metastases. Treating animals with a TGF-beta inhibitory peptide altered the angiogenic phenotype resulting in decreased CD31+ vessel counts (Gonzalez-Zubeldia et al., 2015). Thuringer et al. demonstrated a possible mechanism of Hsp27 mediated angiogenesis. Treating endothelial cells with recombinant Hsp27 resulted in autocrine and paracrine VEGFR2 activation and vessel sprouting in vitro. In a rat model of xenograft colon tumors, intraperitoneal treatment with OGX-427 – an antisense oligonucleotide to Hsp27 – significantly reduced microvessel density and tumor growth (Thuringer et al., 2013). This supports our findings of concomitant Hsp27 expression and increased angiogenesis in the tumor-surrounding stroma. Gene expression assays comparing fibroblasts from CRC tumor samples to normal fibroblasts of the colon mucosa revealed the highly distinct characteristics of cancer-associated fibroblasts. More than 2400 genes were significantly (P < 0.05) differentially expressed (Mrazek et al.,
Also in other solid tumors cancer-associated fibroblasts are thought to play a role in metastasis. Her2+ breast cancers are characterized by their capability of rapid progression and thus diminished prognosis. The expression of HSPB1, the gene coding for Hsp27, was demonstrated to be significantly upregulated in cancer-associated fibroblasts of Her2+ tumors compared to triple negative or estrogen receptor positive tumors (Tchou et al., 2012). Park et al. demonstrated in a recent work using a lung fibrosis model and human samples of idiopathic pulmonary fibrosis that pulmonary myofibroblasts are highly positive for alpha-SMA and Hsp27, which are co-localized at the cellular level. Delivery of HSP27 siRNA reversed the bleomycin-induced fibrosis in a mouse model (Park et al., 2016). This finding in benign lung disease supports our findings in lung metastases regarding the Hsp27-alpha-SMA co-expression in tumor-associated myofibroblasts.

To the best of our knowledge, we described for the first time that Hsp27 is strongly expressed by activated fibroblasts in the tumor-surrounding stroma of primary and metastatic CRC. The presence of these Hsp27+ alpha-SMA+ fibroblasts provides prognostic information after pulmonary metastasectomy.

Of course there are several limitations to this work. First of all, patients receiving pulmonary metastasectomy are a highly selected subgroup of patients with metastatic CRC. The findings in this selected cohort of patients can not necessarily be extrapolated to the whole cohort of patients with metastatic CRC. Second, the included patients are a heterogeneous group regarding the previously received treatment. As the patients were referred for metastasectomy from different institutions, various treatment plans were applied for the primary tumors, previous liver metastases etc.. This might have implications on the phenotype of the pulmonary metastases and might affect the outcome. Third, confirmation of our findings in an independent validation cohort is needed. However, despite these limitations, we could investigate and describe a variety of tissue-based prognostic markers in one of the most detailed and stringently followed-up cohorts of patients with CRC lung metastases.

3.9 Conclusion & future prospects

This thesis adds novel and promising prognostic markers in patients with pulmonary metastases of CRC. These markers might help to describe and stratify patients with CRC lung metastases according to the biological characteristics of the tumor rather than to the current clinical presentation of the patient. This might impact the treatment of these patients.
as well as the interpretation of studies assessing the outcome of patients with CRC lung metastases.
CHAPTER FOUR: MATERIALS & METHODS

4.1 Materials

Study population
From April 2009 to October 2012 (study I)/from April 2009 to November 2013 (study II), all patients receiving R0 (histologically complete) pulmonary metastasectomy from primary CRC at the Department of Thoracic Surgery, Medical University of Vienna and appropriate tissue samples were included in this study. Of 33 patients corresponding primary CRC samples were available. Samples from a study cohort of 25 patients receiving liver metastasectomy were used as control group in study II. The diagnostic work-up before surgery included thoracic and abdominal computed tomography (CT). Time from surgery of the primary tumor to pulmonary metastasis was termed lung metastasis free survival (LMFS). Time to recurrence was defined as the period from the first pulmonary metastasectomy to evidence of recurrent disease irrespective of the organ site of recurrence. Time to lung specific recurrence was defined as the period of time between the first pulmonary metastasectomy and pulmonary recurrence verified by CT. Follow-up examinations were carried out in 3 to 6 months intervals. In patients receiving pulmonary metastasectomy before the inclusion period, tissue samples of the first lung metastasis were also assessed and the data of the first metastasectomy was used for outcome calculation.

In 10 patients receiving pulmonary metastasectomy, serum samples before and 3-6 months after metastasectomy were available. Moreover, serum samples from age-, gender- and smoking status- matched healthy volunteers served as control. Patients receiving blood draws gave written informed consent prior to participation. The studies were approved by the ethics committee of the Medical University of Vienna (EK 91/2006, EK1194/2011 and EK1044/2012) and was conducted according to the declaration of Helsinki.

4.2 Methods

KRAS and BRAF mutation analysis
KRAS codons 12 and 13, and BRAF codon 600 mutations were detected using restriction fragment length analysis as described previously. (Szabo et al, 2011) Briefly, macrodissection of FFPE tumor samples was performed. DNA was isolated using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany). AmpliTaqGold PCR Master Mix (Applied Biosystems, Foster...
City, CA) was used for polymerase chain reaction using specific primers to introduce restriction sites in the products generated from the wild-type allele. The primer sequences were as follows: for KRAS codon 12 forward 5'-GAATATAAACTTGTGGTAGTTGGAACCT-3' and reverse 5'-GGTCTCTGCCCAAGTAATG-3', for KRAS codon 13 forward 5'-GAATATAAACTTGTGGTAGTTGGAACCT-3' and reverse 5'-GGTCTCTGCCCAAGTAATATG-3'. BstnI and BglI were used for digestion, respectively. BRAF codon 600 mutation was analysed in a two-step PCR and restriction digestion. The first primers sequences were as follows: forward (Mt-F) 5'-TAAAAATAGGTGATTTTGGTCTAGCTGC-3' and reverse (Wt-R) 5'-CCAAAAATTTAATCAGTGGAAAAATA-3'. The products obtained were then used in a second-stage PCR, which was performed under the same conditions as the first-stage PCR but with Mt-F and Mt-R primer (5'-AAAAATTTAATCAGTGGAAAAATA-3'). BtsI (New England Biolabs, Beverly MA) was used for digestion. The digested products were visualized on 3% agarose gels stained with ethidium bromide.

**Immunohistochemistry and immunofluorescence**

Immunohistochemistry and immunofluorescence conducted following standard protocols. 4-μm thick, formalin-fixed, paraffin-embedded tissue samples were deparaffinized. Heat-mediated antigen retrieval was performed if necessary. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide. Afterwards an appropriate primary antibody was added for 1h at room temperature. For manual immunohistochemistry the polymer- based ImmPRESS kit (Vector Laboratories, Burlingame, California) was used as secondary step. For automated stainings Benchmark Ultra Immunostainer (Ventana, Tucson, AZ, USA) was used with appropriate secondary antibodies according to the manufacturer’s protocol. 3,3'-Diaminobenzidine (DAB) was used as substrate (Vector Laboratories, Burlingame, California). Finally, the sections were counterstained with hematoxylin. Vimentin staining was performed in a Ventana ES Immunostainer System (Ventana Medical Systems Inc., Tucson, AZ, USA). Fluorescent secondary antibodies were used for immunofluorescence. DAPI (Sigma Aldrich, St. Louis, MO, USA) was used as cell nuclei dye. A detailed list of antibodies and dilutions is provided in Table 1.

**Scoring of Hsp27, vimentin and alpha-SMA in stromal cells**

The samples were rated in a semiquantitatively manner as described previously (Henry et al, 2007). The tumor center was screened at low magnification regarding the staining intensity in the stromal cells. The stromal staining was scored from grade 0 (negative, <1% positive stromal cells), grade 1+ (low, 1-10% positive stromal cells), grade 2+ (intermediate, >10-50% positive stromal cells) to grade 3+ (strong, >50% positive stromal cells) by two blinded observers.
Scoring of target protein expression in tumor cells

The immunohistochemistry staining score (IHC score) was used to quantify protein expression in tumor cells according to the literature (Driessen et al, 2006). Staining intensity (0/1+/2+/3+) was multiplied by the percentage of positive tumor cells, resulting in IHC scores ranging from 0 to 300. The median was used as cutoff to dichotomize the IHC score. Staining intensity for EGFR was scored as described by Scartozzi et al. (Scartozzi et al, 2004). Samples showing membranous EGFR staining in ≥1% of the tumor cells were scored as positive. Stainings were assessed by two blinded observers and re-discussed if the ratings differed.

CD3+ microvessel density

Microvessel density (MVD) was determined using the “hotspot” method (Weidner et al, 1992). At low magnification the area with the greatest number of CD31-positive microvessels was identified (“hotspot”). MVD in this hotspot was determined by counting all CD31+ vessels at 200x magnification (corresponding to 0.95 mm²). Mean values from two independently counted densities were calculated.

Enzyme-linked immunosorbent assay (ELISA)

Commercially available ELISA kits (R&D Systems, Minneapolis, USA) were used to assess serum levels of human Hsp27, phospho-Hsp27 and interleukin-8 following the manufacturer's protocol. The samples were assayed in duplicates. The absorbance was determined at a wavelength of 450nm (PerkinElmer, Waltham, USA) and converted to pg/mL.

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**Table 1: Specification of antibodies and dilutions (IF= immunofluorescence; IHC= immunohistochemistry)**

**Statistical analysis**

Data was statistically processed using GraphPad Prism 6 (GraphPad Software Inc., California, USA) and SPSS 19 (SPSS Inc., Chicago, USA). Fisher's exact test and chi-square test were used to compare binominal variables. Mann-Whitney U-test was used to compare medians between two groups. Student’s t-test was used to compare means of two independent groups, paired t-test for dependent groups and expressed as mean±standard deviation (SD). Kaplan-Meier method, log-rank test and Cox regression were used to compare survival functions. All tests were two-sided. P-values < 0.05 were considered statistically significant.
REFERENCES


APPENDIX

Supplementary material “EGFR, BRAF and KRAS status in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma: a prospective follow-up study”

Supplementary Figure 1

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Supplementary material “Stromal expression of heat-shock protein 27 is associated with worse clinical outcome in patients with colorectal cancer lung metastases.”

Supplementary Table 1

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**Supplementary Table 2**

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<th>healthy controls n= 10</th>
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<th>p-value</th>
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<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
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<tr>
<td>male/female</td>
<td>7/3</td>
<td>7/3</td>
<td>ρ= 1.000⁺</td>
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<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>range (median)</td>
<td>45-88 (52.5)</td>
<td>44-76 (62.5)</td>
<td>ρ= 0.430³</td>
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<tr>
<td><strong>Smoking history</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>never smoker/ever smoker</td>
<td>4/6</td>
<td>3/7</td>
<td>ρ= 1.000⁺</td>
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<td><strong>Months after primary tumor resection (range)</strong></td>
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<td>29 (7-91)</td>
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*Chi square test; ³Mann-Whitney test
## Supplementary Table 3

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<td>Total</td>
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<td>N=51</td>
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<tr>
<td><strong>Patients</strong></td>
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<tr>
<td><strong>Sex</strong></td>
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</tr>
<tr>
<td>Male</td>
<td>29 56.9</td>
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<td>Female</td>
<td>22 43.1</td>
<td>9 17.6</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
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<tr>
<td>Median</td>
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<tr>
<td>Range</td>
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<td><strong>Primary tumors</strong></td>
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<td><strong>Location</strong></td>
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<td>Colon</td>
<td>27 52.9</td>
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<tr>
<td>pT2</td>
<td>7 14.6</td>
<td>3 6.2</td>
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<tr>
<td>pT3</td>
<td>34 70.8</td>
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<tr>
<td>pT4</td>
<td>6 12.5</td>
<td>3 6.2</td>
</tr>
<tr>
<td>N/A</td>
<td>3 -</td>
<td>12 25.0</td>
</tr>
<tr>
<td><strong>N stage</strong></td>
<td></td>
<td></td>
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<tr>
<td>pN0</td>
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<td>12 25.0</td>
</tr>
<tr>
<td>pN1</td>
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<tr>
<td>pN2</td>
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<td>7 14.6</td>
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<td>2 3.0</td>
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<tr>
<td>G2</td>
<td>42 82.4</td>
<td>19 37.3</td>
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<td>G3</td>
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<td><strong>Pulmonary metastasis</strong></td>
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<td>Previous liver metastasis</td>
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<td>No</td>
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<tr>
<td>No. of nodules</td>
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<td>33 64.7</td>
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<td>&gt;1</td>
<td>18 35.3</td>
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<tr>
<td><strong>Stromal HSP27</strong></td>
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<td>low</td>
<td>17 33.3</td>
<td>10 19.6</td>
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<tr>
<td>intermediate</td>
<td>17 33.3</td>
<td>8 15.7</td>
</tr>
<tr>
<td>high</td>
<td>17 33.3</td>
<td>7 13.7</td>
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<tr>
<td><strong>Stromal alpha-SMA</strong></td>
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<td>14 27.5</td>
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<td>intermediate</td>
<td>23 45.1</td>
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<tr>
<td>high</td>
<td>14 27.5</td>
<td>5 9.8</td>
</tr>
</tbody>
</table>

*Kruskal-Wallis test
CV
Thomas Sebastian Schweiger

Personal background

Date of birth: May 25th, 1987
Nationality: German

Education

2015/05--/present Residency in Thoracic Surgery, Division of Thoracic Surgery (Head: Prof. Walter Klepetko), Medical University of Vienna, Austria
2014/09--/2015/05 Transplant coordination, Division of Thoracic Surgery (Head: Prof. Walter Klepetko), Medical University of Vienna, Austria
2012/10--/present MDPHd excellence program (Vascular biology), Medical University of Vienna, Austria
2010/04--/present Research Fellow at the Christian Doppler Laboratory for Diagnosis and Regeneration of Cardiac and Thoracic Diseases, Medical University of Vienna, Austria
2008/10--/2014/07 Human medicine (Degree: Dr.med.univ./MD), Medical University of Vienna, Austria
2006/07--/2007/04 Military Service
2006/06 High School Graduation
1997/--2006 Katharinen-Gymnasium Ingolstadt, Germany
1993/--1997 Primary School, Manching, Germany

Clinical Training

2014/04--/2014/05 Clinical Clerkship at the/Division of Thoracic Surgery, Medical University Vienna, Austria
<table>
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<th>Year Range</th>
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<tr>
<td>2012/08 / 2012/09</td>
<td>Clinical Clerkship at the Department of Internal Medicine, Evangelisches Krankenhaus, Vienna, Austria</td>
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<tr>
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<tr>
<td>2011/06 / 2011/07</td>
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<tr>
<td>2010/07 / 2010/08</td>
<td>Clinical Clerkship at the Department of General, Visceral, Vascular/and Thoracic Surgery, Charité Berlin, Germany</td>
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### Scholarships and Honors

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<tr>
<td>2014/12</td>
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<td>Poster Prize of the German Society for Thoracic Surgery</td>
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<td>Research Scholarship of the Medical University of Vienna/</td>
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### Workshops

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<td>Nahtkurs Gefäßchirurgie (OA Dr. C Senekowitsch), Wilhelminenspital, Vienna, Austria</td>
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<td>2011/08</td>
<td>Theodor-Billroth-Academy/Surgical/Summer School 2011/(Prof. Dr. B Brücher, FACS), University of Tuebingen, Germany</td>
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### Congresses and Meetings

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<td>Annual Meeting of the Austrian Society for Pulmonology, Graz, Austria</td>
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<td>2015/09</td>
<td>7th ÖGMBT Annual Meeting, Salzburg, Austria</td>
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<td>2015/05</td>
<td>23rd European Conference on General Thoracic Surgery, Lisbon, Portugal</td>
</tr>
<tr>
<td>2014/10</td>
<td>ACO/ASSO Annual meeting 2014, St. Wolfgang, Austria</td>
</tr>
<tr>
<td>2014/06</td>
<td>Annual Congress of the Austrian Society of Surgery, Vienna, Austria</td>
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<tr>
<td>2014/03</td>
<td>Management of Laryngotracheal Problems, Vienna, Austria</td>
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<tr>
<td>2014/01</td>
<td>EACTS Academic Thoracic Surgery Club, Bern, Switzerland</td>
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Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland

ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria

Annual Congress of the German/Society of Surgery, Munich, Germany

36th/Seminar of the Austrian Society for Surgical Research, Vienna, Austria

Annual Congress of the Austrian Society of Surgery, Vienna, Austria

Annual Congress of the Austrian Society of Surgery, Berlin, Germany

ACO/ASSO Annual meeting/2011, St. Wolfgang, Austria

Annual Congress of the Austrian Society of Surgery, Vienna, Austria

SkillsLab Symposium, Würzburg, Germany

EACTS Meeting on Cardiac and Pulmonary Regeneration, Vienna, Austria

Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Vienna, Austria

SkillsLab Symposium, Münster, Germany

### Original articles (peer-reviewed)

**Schweiger T, Schwarz S, Traxler D, Dodier/P, Aigner C, Lang G, Klepetko W, Hoetzenecker K**  
**Bronchoscopic Indocyanine Green Fluorescence Imaging of the Anastomotic Perfusion After Tracheal Surgery.**  
*IF*: 3.849

**Clinical impact of c-MET expression and mutational status in patients with colorectal cancer lung metastases.**  
*IF*: 3.304

**Increased lymphangiogenesis in lung metastases from colorectal cancer is associated with early lymph node recurrence and decreased overall survival.**  
*IF*: 3.491

**Hoetzenecker K, Schweiger T, Schwarz S, Roesner I, Leonhard M, Denk-Linnert DM, Schneider-Stickler B, Bigenzahn W, Klepetko W.**  
**Summarized institutional experience of paediatric airway surgery.**  
*IF*: 3.304

**Elevated inflammatory parameters and inflammation scores are associated with poor prognosis in patients undergoing pulmonary metastasectomy for colorectal cancer.**  
*IF*: 1.155


Reviews and Case reports


Intubation with flexible bronchoscope and surgical resection of a post-tracheostomy stenosis
HealthMED 2015 March;(9)3:127-130.

Schweiger T, Hoetzenecker K, Bacher A, Aigner C, Klepetko W

Reversible compression of the left lower lobe vein after right pneumonectomy

IF: 3.631

Hoetzenecker K, Schweiger T, Bigenzahn W, Klepetko W

Tracheal resection for post-tracheostomy stenosis

IF: -

Schweiger T, Hoetzenecker K, Taghavi S, Klepetko W

Extended cervico-thoracic metastasectomy for testicular non-seminomatous germ cell tumour masses through an inverse T and combined collar incision

IF: 3.048

Schweiger T, Hoetzencker K, Bacher A, Aigner C, Klepetko W

Reversible compression of the left lower lobe vein after right pneumonectomy

IF: 3.741

Schweiger T, Lang G, Klepetko W, Hoetzenecker K

Prognostic factors in pulmonary metastasectomy: spotlight on molecular and radiological markers

IF: 3.048


Unsuspected finding of a relapsing perichondritis during lung explantation.

IF: 3.741

Published articles (non peer-reviewed)

Hoetzenecker K, Schweiger T, Klepetko W

Neues zur pulmonalen Metastasektomie
Wiener klinische Wochenschrift, ERS Spezial, Dezember 2012

Hoetzenecker K, Schweiger T, Klepetko W

Neues zur pulmonalen Metastasektomie
ÄrzteWoche, Nr. 42, 26. Jahrgang, 2012
Schweiger T.
4. Sommer-Schule der Theodor-Billroth-Akademie in Tübingen - Eine Initiative zur Förderung des internationalen Chirurgennachwuchses
Chirurgie, Nr. 03/11, 2011

Abstracts

Clinical impact of c-MET expression and mutational status in patients with CRC lung metastases
7th ÖGMBT Annual Meeting, Salzburg, Austria

c-MET overexpression and mutational status in patients with pulmonary metastases from primary CRC
56. Österreichischer Chirurgenkongress, Linz, Austria

Summarized institutional experience of pediatric airway surgery
56. Österreichischer Chirurgenkongress, Linz, Austria

Lymphatic invasion in colorectal cancer lung metastases predicts the outcome after pulmonary metastasectomy
56. Österreichischer Chirurgenkongress, Linz, Austria

A Case series of carinal resections with tracheobronchial end-to-end anastomosis and end-to-side reimplantation of the remaining airway into the contralateral bronchus
56. Österreichischer Chirurgenkongress, Linz, Austria

Ghanim B, Schweiger T, Lang G, Klepetko W, Hoetzenecker K
Elevated inflammatory parameters and inflammation scores are associated with poor prognosis in patients undergoing curative pulmonary metastasectomy for colorectal cancer
23rd European Conference on General Thoracic Surgery, Lisbon, Portugal

Summarized institutional experience of pediatric airway surgery
23rd European Conference on General Thoracic Surgery, Lisbon, Portugal

Clinical impact of c-MET expression and mutational status in patients with primary colorectal cancer lung metastases
23rd European Conference on General Thoracic Surgery, Lisbon, Portugal

Hoetzenecker K, Schweiger T, Lang G, Klepetko W
Surgical treatment of diffuse tracheomalacia in adults: posterior stabilization using a polypropylene mesh
Annual Meeting of the Austrian Society for Pneumonology 2014, Salzburg, Austria

Hoetzenecker K


Lymphangiogenesis in patients undergoing pulmonary metastasectomy from metastatic colorectal carcinoma with negative thoracic lymph node staging
23. Jahrestagung der Deutschen Gesellschaft für Thoraxchirurgie 2014, Osnabrück, Germany

Hoetzenecker K, Marta G, Schweiger T, Denk D, Bigenzahn W, Klepetko W
Eine Adaptation der Couraud-Technik ermöglicht laryngotracheale Rekonstruktionen bei Erwachsenen als einzeitige Eingriffe
23. Jahrestagung der Deutschen Gesellschaft für Thoraxchirurgie 2014, Osnabrück, Germany

Korrektur laryngotrachealer Stenosen bei Kindern – eine Fallserie
23. Jahrestagung der Deutschen Gesellschaft für Thoraxchirurgie 2014, Osnabrück, Germany

Hoetzenecker K, Schweiger T, Lang G, Klepetko W
Posterior stabilization using polypropylene mesh in a patient with severe diffuse tracheomalacia
55. Österreichischer Chirurgenkongress 2014, Graz, Austria

Hoetzenecker K, Hochdanninger M, Traxler D, Mitterbauer A, Schweiger T, Hegedüs B, Klepetko W, Ankersmit HJ, Mildner M
High concentrations of antimicrobial peptides in post-operative pleural drainage fluids
55. Österreichischer Chirurgenkongress 2014, Graz, Austria

Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases
55. Österreichischer Chirurgenkongress 2014, Graz, Austria

Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma
55. Österreichischer Chirurgenkongress 2014, Graz, Austria

Hoetzenecker K, Hochdanninger M, Traxler D, Mitterbauer A, Schweiger T, Hegedüs B, Klepetko W, Ankersmit HJ, Mildner M
Antimicrobial peptides are highly abundant and active in post-operative pleural drainage fluids
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery 2013, Basel, Switzerland

Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery 2013, Basel, Switzerland

Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery 2013, Basel, Switzerland

T Schweiger, D Kollmann, C Nikolowsky, Denise Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker
Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery 2013, Basel, Switzerland

Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria


Pulmonary Metastasectomy at the Department of Thoracic Surgery, MUV, 2009-2013 – A computer-based prospective database
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria


Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko

Impact of pulmonary metastasectomy on lung function parameters
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria

T Schweiger, D Kollmann, C Nikolowsky, Denise Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria


Regenerative effects of secreted factors derived from peripheral blood mononuclear cells in cutaneous wound healing after full-thickness skin defects, burn and skin grafting: results of animal studies
54. Österreichischer Chirurgenkongress 2013, Vienna, Austria

D Kollmann, K Hoetzenecker, T Schweiger, HJ Ankersmit, C Aigner, G Lang, S Taghavi, W Klepetko

Unsuspected finding of a relapsing polychondritis during lung explantation
54. Österreichischer Chirurgenkongress 2013, Vienna, Austria


Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation. European Society of Cardiologists Annual Meeting 2012, Munich, Germany


Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction
in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation and vasodilation.
3rd TERMIS World Congress 2012, Vienna, Austria

T Schweiger, D Kollmann, C Nikolowsky, Denise Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker
Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking
130th Annual Congress of the German Society of Surgery, Munich, Germany
Abstract published in: Langebeck’s Archives of Surgery, Volume 398(4), 2013

Regenerative effects of secreted factors derived from peripheral blood mononuclear cells in cutaneous wound healing after full-thickness skin defects, burn and skin grafting: results of animal studies
3rd EACTS Meeting on Cardiac and Pulmonary Regeneration, Berlin, Germany

K Hoetzenecker, M Zimmermann, T Schweiger, D Kollmann, M Mildner, A Mitterbauer, P Birner, M Lichtenaumer, HJ Ankersmit
Secretome from mononuclear cells confers immunosuppression in a murine autoimmune myocarditis model
36th Seminar of the Austrian Society for Surgical Research, Vienna, Austria

T Schweiger, D Kollmann, C Nikolowsky, Denise Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker
Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking
36th Seminar of the Austrian Society for Surgical Research, Vienna, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko
Impact of pulmonary metastasectomy on lung function parameters
European Respiratory Society Annual Meeting 2012, Vienna, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, G Lang, P Birner, HJ Ankersmit, W Klepetko
Hypoxic phenotype in pulmonary metastases of different primary tumors
European Respiratory Society Annual Meeting 2012, Vienna, Austria

K Hoetzenecker, A Assinger, M Lichtenaumer, M Mildner, T Schweiger, A Mitterbauer, P Starlinger, M Ernstbrunner, B Steinlechner, M Gyöngyösi, I Volf, HJ Ankersmit
Secretome of apoptotic peripheral blood cells (APOSEC) attenuates microvascular obstruction in a porcine closed chest reperfused acute myocardial infarction model: role of platelet aggregation in vitro and in vivo
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

K Hoetzenecker, T Schweiger, M Mildner, C Plass, D Traxler, M Lichtenauner, M Gyöngyösi, HJ Ankersmit
Secretome of apoptotic peripheral blood cells (APOSEC) induces coronary vasodilation: Impact on microvascular obstruction during acute myocardial infarction
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria
Secretome from mononuclear cells confers immunosuppression in a murine autoimmune myocarditis model

K Hoetzenecker, M Zimmermann, T Schweiger, D Kollmann, M Mildner, A Mitterbauer, P Birner, M Lichtenauer, HJ Ankersmit


53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

Secretome of apoptotic peripheral blood cells attenuates microvascular obstruction in acute myocardial infarction: role of platelet aggregation

T Schweiger, K Hoetzenecker, A Hoda, A Aliabadi, G Lang, C Aigner, S Taghavi, W Klepetko

Impact of lymphnode downstaging on the prognosis of pancoast tumors

9. PneumoUpdate 2012, Innsbruck, Austria

Impact of pulmonary metastasectomy on lung function parameters

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko

Hypoxic phenotype in pulmonary metastases of different primary tumors

T Schweiger, K Hoetzenecker, L Lehmann, R Wiebringhaus, G Lang, P Birner, HJ Ankersmit, W Klepetko

Chirurgische Therapie pulmonaler Metastasen beim Mammakarzinom

K Hoetzenecker, T Schweiger, L Lehmann, F Gittler, HJ Ankersmit, G Lang, W Klepetko

Clinical impact of c-MET expression and mutational status in patients with CRC lung metastases

P Angleitner*, T Schweiger*, Michael Schmidts und Martin Schindl

P Angelnter, T Schweiger, T Schweiger, Chirurgia Practica – Peer teaching chirurgischer Fertigkeiten

Skills Lab Symposium 2011, Würzburg, Germany

Presentations


Clinical impact of c-MET expression and mutational status in patients with CRC lung metastases

7th ÖGMBT Anuual Meeting, Salzburg, Austria


Clinical impact of c-MET expression and mutational status in patients with primary colorectal cancer lung metastases. (Oral presentation)

23rd European Conference on General Thoracic Surgery, Lisbon, Portugal


Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases. (Oral presentation)

55. Österreichischer Chirurgenkongress 2014, Graz, Austria

**Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma.** *(Oral presentation)*
55. Österreichischer Chirurgenkongress 2014, Graz, Austria

Schweiger T, Hoetzenecker K, Klepetko W

**Posterior stabilization in a patient with severe tracheomalacia.** *(Oral presentation)*
Management of laryngotracheal problems 2014, Vienna, Austria

Hoetzenecker K, Hochdanninger M, Traxler D, Mitterbauer A, Schweiger T, Hegedüs B, Klepetko W, Ankersmit HJ, Mildner M

**Antimicrobial peptides are highly abundant and active in post-operative pleural drainage fluids** *(Oral presentation)*
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland


**Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases** *(Oral presentation)*
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland


**Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma** *(Poster presentation)*
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland

T Schweiger, D Kollmann, C Nikolowsky, Denise Traxler, E Guenova, G Lang, P Birner, W Klepetko, HJ Ankersmit, K Hoetzenecker

**Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking** *(Oral presentation)*
Meeting of the Austrian, German and Swiss Society of Thoracic Surgery, Basel, Switzerland


**Heat-shock proteins 27 and 70 in primary colorectal cancer and corresponding pulmonary metastases** *(Poster presentation)*
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria


**Mutation in KRAS prognosticates early recurrence in patients undergoing pulmonary metastasectomy from primary colorectal carcinoma** *(Poster presentation)*
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko

**Impact of pulmonary metastasectomy on lung function parameters** *(Poster presentation)*
ACO/ASSO Annual meeting 2013, St. Wolfgang, Austria

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Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking (Oral presentation)
130th Annual Congress of the German Society of Surgery, Munich, Germany
Abstract published in: Langebeck’s Archives of Surgery, Volume 398(4), 2013

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Early pulmonary spreading of primary colorectal carcinoma is associated with carbonic anhydrase IX expression and tobacco smoking (Oral presentation)
36th Seminar of the Austrian Society for Surgical Research, Vienna, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko
Impact of pulmonary metastasectomy on lung function parameters (Poster presentation)
European Respiratory Society Annual Meeting 2012, Vienna, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, G Lang, P Birner, HJ Ankersmit, W Klepetko
Hypoxic phenotype in pulmonary metastases of different primary tumors (Poster presentation)
European Respiratory Society Annual Meeting 2012, Vienna, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, HJ Ankersmit, G Lang, W Klepetko
Impact of pulmonary metastasectomy on lung function parameters (Oral presentation)
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

T Schweiger, K Hoetzenecker, C Nikolowsky, L Lehmann, R Wiebringhaus, G Lang, P Birner, HJ Ankersmit, W Klepetko
Hypoxic phenotype in pulmonary metastases of different primary tumors (Oral presentation)
53. Österreichischer Chirurgenkongress 2012, Salzburg, Austria

K Hoetzenecker, T Schweiger, L Lehmann, F Gittler, HJ Ankersmit, G Lang, W Klepetko
Chirurgische Therapie pulmonaler Metastasen beim Mammakarzinom (Poster presentation)
Senologie-ACO 2011, St. Wolfgang, Austria

P Angleitner*, T Schweiger*, Michael Schmidts und Martin Schindl
Chirurgia Practica – Peer teaching chirurgischer Fertigkeiten (Poster presentation)
Skills Lab Symposium 2011, Würzburg, Germany
Review activity

2015-present Transplant International

Clinical Trials

Schweiger T/(Study author), Klepetko W, Hoetzenecker K, Lang G –/EUDRACT: 2013-001725-10 Bronchosscopic indocyanine green fluorescence imaging for the evaluation of tracheal perfusion after surgery – A monocenter, prospective open-label feasibility study

Teaching activity

2016/03 JC Current Topics in Applied Immunology/(summer term 2016; 861.111), Medical University of Vienna
2016/03 TS Applied Immunology and Tissue Regeneration/(summer term 2016; 861.119), Medical University of Vienna
2013/10 Student teaching assistant „Themenspezifische Untersuchungstechniken I/Chirurgische Grundfertigkeiten“ (winter term 2013/14; 805.008), Medical University of Vienna
2012/10 Student teaching assistant „Themenspezifische Untersuchungstechniken I/Chirurgische Grundfertigkeiten“ (winter term 2012/13; 805.008), Medical University of Vienna
2012/03 Hands-on Workshop „Introduction to surgical suturing techniques“, MedSuccess 2012, Medical University of Vienna
2011/10 Student teaching assistant „Themenspezifische Untersuchungstechniken I/Chirurgische Grundfertigkeiten“ (winter term 2011/12; 805.008), Medical University of Vienna
2011/10 Student teaching assistant „Themenspezifische Untersuchungstechniken I“ (/ winter term 2011/12; 805.008), Medical University of Vienna
2011/03 Hands-on Workshop „Introduction to surgical suturing techniques“, MedSuccess 2011, Medical University of Vienna
2010/10-/ongoing Co-Founder of „Chirurgia Practica“ (peer-teaching of surgical techniques) together with Dr. Philipp Angleitner (Supervisor: Ao. Univ.-Prof. Dr. Martin Schindl), Medical University of Vienna

Memberships

2015-/present Membership at Austrian Society of Cardio-Thoracic Surgery (ÖGHTC)
2015-/present Membership at European Society of Thoracic Surgeons (ESTS)
2014-/present Membership at Austrian Society of Surgical Oncology (ACO-ASSO)
2011-/present Membership at Berufsverband Österreichischer Chirurgen (BÖC)