# Prognostic and predictive role of EGFR protein expression in colorectal cancer

**PhD thesis**


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**Abbreviations**

| | |
|---|---|
| APC | adenomatous polyposis coli gene |
| CIMP | CpG island methylator phenotype |
| CIN | chromosomal instability |
| CMS | consensus molecular subtype |
| CNV | copy number variation |
| CRC | colorectal cancer |
| DNA | deoxyribonucleic acid |
| EGFR | epidermal growth factor receptor |
| FFPE | formalin-fixed, paraffin-embedded |
| 5-FU | fluorouracil |
| G | grade |
| GCN | gene copy number |
| HNPCC | hereditary nonpolyposis colorectal cancer |
| IHC | immunohistochemistry |
| KRAS | Kirsten rat sarcoma viral oncogene homolog |
| LSCRC | left-sided colorectal cancer |
| MAB | monoclonal antibody |
| MMR | mismatch repair |
| MSI | microsatellite instability |
| MSS | microsatellite stable |

| | |
|---|---|
| NRAS | neuroblastoma RAS viral oncogene homolog |
| NSCLC | non-small-cell lung carcinoma |
| OS | overall survival |
| PD1 | programmed cell death protein 1 |
| PFS | progression free survival |
| PI3KCA | phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha |
| RR | relative risk |
| RSCRC | right-sided colorectal cancer |
| RTK | receptor tyrosine kinase |
| SCNA | somatic copy number alteration |
| SSC | saline sodium citrate |
| TCGA | The Cancer Genome Atlas |
| WT | wild type |

# 1 Introduction

## 1.1 Epidemiology

Cancer-related mortality cases account for 25% of all deaths in Hungary (**1**). Among the countries of the European Union, the standardised mortality rate of colorectal cancer (CRC) is the highest in Hungary, at 56 reported deaths per 100,000 inhabitants (**2)**. The incidence of colorectal cancer increased from 9,401 to 10,684 cases per year between 2007 and 2014. However, the mortality rate did not increase significantly (4,979 to 5,017 cases per year between 2004 and 2013) in the same period of time (**3).** Approximately 35% of all colorectal cancer patients have metastatic disease at the time of diagnosis. In those patients who were succesfully operated on in stage II-III, metastases occured in 20-50% of all cases during the course of the disease (**4)**. The most common location of the metastasis is the liver. Liver metastases are unresectable in 85% of cases at the time of diagnosis (**5).**

## 1.2 Carcinogenesis and mutation landscape in colorectal cancer

Genetic and epigenetic alterations are equally responsible for the development of CRC. Three different genomic instability pathways are distinquished in colorectal carcinogenesis:

1. Activation of the WNT pathway, due to mutation of the APC (adenomatous polyposis coli) or other supressor genes, characterized by chromosomal instability (CIN) phenotype. CIN is considered to be the most frequent patomechanism in the development of CRC, accounting for 85% of all cases.

2. Hypermethylation of the genome leads to the inactivation of the tumor suppressor gene through an epigenetic pathway. This is considered to be the second major patomechanism observed in sporadic CRC; the phenotype is called CpG island methylator phenotype (CIMP). CIMP also includes the sporadic form of MSI (microsatellite instability) high colorectal carcinomas.

3. Germline mutations in the mismatch repair (MMR) genes could also occur in the microsatellite instability (MSI) phenotype, known as Lynch syndrome or hereditary nonpolyposis colorectal cancer (HNPCC). In these cases MLH1, MSH2, MSH6 and PMSS gene mutations should be evaluated (**6,7**).

Microsatellite instability could be verified in 15% of all colorectal cancer cases; 3% are associated with germline mutation of MMR genes, while hypermethylation of the prometer region of MLH1 is responsible for the other 12% of cases (**8**). The mutation landscape of CRC was evaluated in several large whole-genome sequencing studies; the results are available in the Cancer Genome Atlas (TCGA) database (**9**). The genomic basis of microsatellite stable (MSS) CRC heterogeneity has been examined in a cohort of 1,027 patients. The most frequently mutated genes were APC (79%) and TP53 (78%); both are oncosuppressors. The most frequently mutated oncogenes were KRAS (44%), PI3KCA (18%), BRAF (8.5%), and NRAS (3.9%) (**Table 1)** (**10**).

**Table 1 Most frequently mutated genes in CRC**

| **Gene** | **Gene Type** | **Frequency (%)** |
|---|---|---|
| APC | oncosuppressor | 79 |
| TP53 | oncosuppressor | 78 |
| KRAS | oncogene | 44 |
| PI3KCA | oncogene | 18 |
| BRAF | oncogene | 8.5 |
| NRAS | oncogene | 3.9 |

### 1.3 Consensus molecular subtype classification system of colorectal cancer

Recently, based on a new classification system, four consensus molecular subtypes (CMS) of colorectal cancers were distinguished**.** Approximately 14% of all colorectal cancers are considered to be CMS1. CMS1 or MSI Immune tumors are hypermutated, microsatellite unstable, CIMP high, and frequently BRAF mutated, but the prevalence of somatic copy number alterations (SCNA) is low in these tumors. CMS2, also called Canonical type, is responsible for 37% of CRC. CMS2 tumors are chromosomally unstable, CIN high, and the activation of WNT and MYC signaling pathway is frequent. CMS3, the Metabolic type represents 13% of CRC cases. CMS3 has a different genomic and epigenomic profile that other CIN tumors: CMS3 tumors are CIMP low, SCNA low, their MSI status is mixed, KRAS mutations are frequent, and metabolic dysregulation is detectable. CMS4**,** referred to as the Mesenchymal type, represents 23% of cases. This

group of CRC is characterized by stromal infiltration, TGFb activation, and high levels of SCNA (**Table 2**) (**11**)**.**

**Table 2 Consensus molecular subtypes (CMS) of colorectal cancers**

| CMS type | Frequency (%) | Characteristics |
|---|---|---|
| CMS1: MSI Immune | 14 | hypermutated, microsatellite unstable, CIMP high, frequently BRAF mutated, low somatic copy number alterations (SCNA) |
| CMS2: Canonical | 37 | chromosomally unstable, CIN high, WNT and MYC signaling pathway activation |
| CMS3: Metabolic | 13 | CIMP low, SCNA low, mixed MSI status, frequently KRAS mutated, metabolic dysregulation |
| CMS4: Mesenchymal | 23 | stromal infiltration, TGFb activation, high level of SCNA |

### 1.4 The role of epidermal growth factor receptor (EGFR) in colorectal carcinogenesis

EGFR is a tyrosine kinase transmembrane receptor, expressed in all epithelial tissues along the human gastrointestinal (GI) tract. EGFR activation is important for embryogenesis and organogenesis. EGFR activation is induced by ligand binding (EGF, TGF-α, hbEGF, amphyregulin, BTC), and different EGFR ligands may differentially regulate EGFR signaling (**12**). EGFR is one of the key factors in gene transcription and cell proliferation, leading to the progression of the cancer. EGFR is overexpressed on the surfaces of several types of cancer cells. EGFR belongs to the human epidermal growth factor receptor (HER) family, and is also called HER1/ErbB1. The first step in EGFR activation is the ligand (eg. EGF, TGF-α, amphiregulin) binding to the extracellular domain of the receptor**.** The second step is conformational change and dimerisation (homo- or heterodimerisation) of the receptor. The dimerisation leads to the

autophosphorylation of the tyrosine kinase, which activates the signal transduction pathways. RAS-MAPK, PI3K-Akt and STAT signaling pathways are the main triggers involved (**Figure 1**) (**13**).

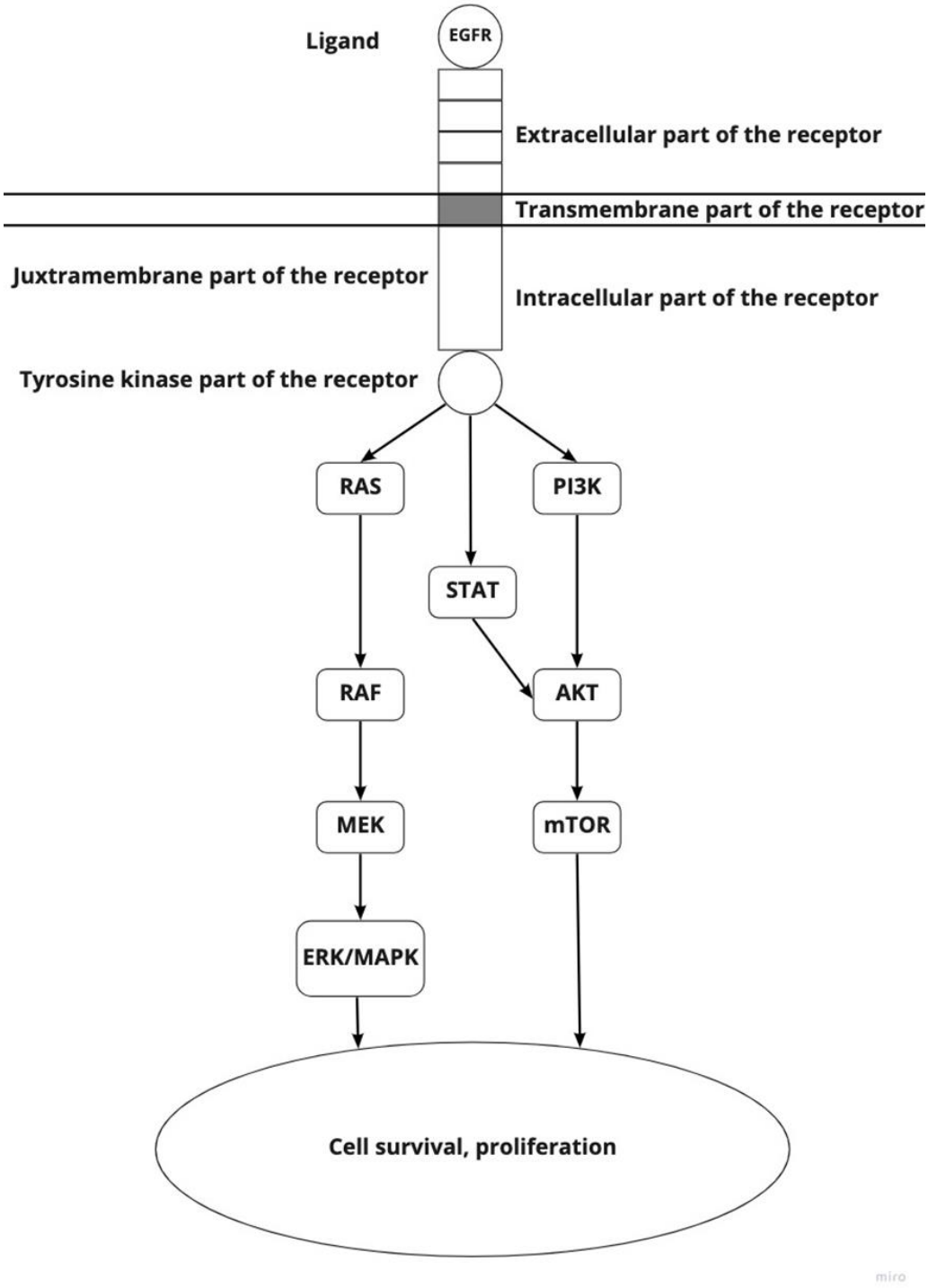



**Figure 1 EGFR receptor and signaling pathways**

(self modification, based on Ref.12,13,14)

Unlike in glioblastomas and lung cancers, EGFR mutations affecting the extracellular domain or the tyrosin kinase part of the receptor are rare in colorectal cancers (**15**). The EGFR mutation rate is found to be lower than 1% in human colorectal cancer (**13**), while in other HER family receptors the incidence of gene mutations are as follows: ERBB4 3.5%, HER2/ERBB2 3.1%, and HER3 /ERBB3 3.0%. Receptor tyrosine kinase (RTK) amplifications in MSS CRC are also rare; the most common of these are ERBB2 amplifications, which were found in 4% of cases (**10).**

### 1.5 Systemic therapy for metastatic colorectal cancer

In cases of unresectable metastatic CRC, systemic therapy is the preferred therapeutic option. Fluorouracil (5-FU), leucovorin, capecitabine, irinotecan, and oxaliplatin should be considered as a part of combination chemotherapy. The chemotherapy backbone should also be combined with vascular endothelial growth factor- (VEGF), or EGFR-targeted agents, such as bevacizumab, cetuximab or panitumumab, according to currently available guidelines (**16**). Recently, the anti-PD1 (programmed cell death protein 1) antibody pembrolizumab was accepted for the treatment of MSI high metastatic disease in later lines (**17**). The choice of targeted therapy for inoperable metastatic CRC depends on the goal of the therapy. Preoperative, conversion systemic therapy should be considered in selected cases to downsize metastases and convert an inoperable case into a resectable one. In cases of unresectable disease, only palliative systemic therapy is feasible. In both cases, targeted agents have an important role. Bevacizumab /Avastin is a vascular endothelial growth factor (VEGF) inhibitor. For bevacizumab treatment, to date we do not have an effective and reliable predictive biomarker of its potential efficacy. There is no biomarker for patient selection in routine clinical practice either (**18).** Cetuximab/Erbitux and panitumumab/Vectibix are anti-EGFR monoclonal antibodies. Cetuximab is a chimeric monoclonal IgG1 antibody used in combination with chemotherapy, or as a single agent in some cases (**19**). Panitumumab is a fully human monoclonal IgG2 antibody which also can be used in combination with chemotherapy or as a single agent in the treatment of metastatatic colorectal cancer (**20).**

### 1.6 Predictive molecular markers of EGFR targeted therapy

Not all patients respond to anti-EGFR therapy, therefore it would be beneficial to use positive and/or negative predictive markers reflecting the efficacy of anti-EGFR therapy during patient selection. Unfortunately, to date only proven/first-line evidenced negative predictive molecular pathological factors are known, such as RAS and BRAF mutations. Anti-epidermal growth factor receptor (EGFR) monoclonal antibodies cetuximab and panitumumab should only be considered as a part of treatment in RAS and BRAF wild-type metastatic colorectal cancer patients. KRAS exon2 and other rare KRAS, NRAS, and BRAF mutations are proven negative predictors of the efficacy of anti-EGFR antibody therapies (**21-23**). The KRAS exon2 mutation rate is 35-40% in colorectal cancers (**24**), but other rare mutations occur in 18-26 % of all cases (**25,22**). In Hungary, the overall prevalence of RAS mutations was found to be 46.7% (**26**). Therefore, approximately 50% of all colorectal cancer cases could be excluded from anti-EGFR therapy. The importance of these negative predictors was highlighted by the results of early clinical trials. In a cohort of RAS non-selected colorectal cancer patients, the response rate for cetuximab-chemotherapy combination was found to be 46.9% compared to 38.7 % for the control, chemotherapy-only group (**27**). Another study enrolled patients with expanded RAS wild status. In this case, the response rate in the combination (chemotherapy-cetuximab) group was 58% compared to the 29% of the chemotherapy-only group (**22**). Based on these results, despite the use of negative predictors in daily practice approximately 40% of patients are not expected to respond to anti-EGFR antibody therapy. Therefore, it would be useful to have positive predictive markers as well.

### 1.7 The localisation of the primary tumor as a potential prognostic and/or predictive marker

The localisation of the primary tumor as a potential prognostic and/or predictive marker has recently received more attention due to the different characteristics of left- and right-sided colorectal cancers. The right part of the large intestine develops from the midgut, while the left side develops from the hindgut. Right-sided colorectal cancer (RSCRC) occurs in older subjects and it is more frequent in females than left-sided colorectal cancer (LSCRC). RSCRC tends to disseminate on the peritoneum, while LSCRC metastasizes

to the liver and the lung. Pathological and molecular analyses have confirmed that RSCRC is characterized by mucinous histology, contains a strong lymphocytic infiltration, is frequently MSI-high (mismatch deficient) and immunogenic, therefore it responds well to immunotherapy. LSCRC is usually polyploid, has a tubular or villous morphology, is characterized by chromosomal imbalances (CIN-high), and is poorly immunogenic, but it responds well to chemotherapies **(28-31)**.

Data on the prognostic value of sidedness are controversial. In a recently published large meta-analysis, it has been confirmed that right-sided tumors have a worse prognosis compared to left-sided CRCs. The difference remains consistent also in early stage or metastatic colon cancers. Right-sided colorectal cancers (RSCRC) were determined to be tumors up to splenic flexure, and left-sided colorectal cancers (LSCRC) were defined as tumors of the descending colon, sigmoid and/or rectosigmoid regions (**32**). Interestingly, in another, earlier paper based on the published data of stage I-III colorectal cancers, the paradigm of worse survival in right-sided colon cancer was disputed; the authors found better survival in right-sided versus left-sided colon cancer patients (**33**). The importance of sidedness as a predictive factor for anti-EGFR antibody therapy has been evaluated based on the results of CRYSTAL and FIRE-3 trials. It was confirmed that the expected progression free survival (PFS) and overall survival (OS) are far worse in cases of right-sided tumors than in those of left-sided ones (**34-36)**. Based on re-evaluation of the NCIC CO.17 clinical trial, in a cohort of chemotherapy refractory, cetuximab-treated metastatic colorectal patients the prognostic value of sidedness has not been proven; it was considered to be a predictive factor for cetuximab therapy **(37**).

### 1.8 Prognostic and predictive role of EGFR expression

The potential prognostic role of EGFR expression has been evaluated in several studies. EGFR overexpression is a proven negative prognostic marker of disease-free status and overall survival in head-and-neck, ovarian, cervical, urinary bladder and oesophageal cancer. The results are similar in gastric, endometrial, and colorectal cancers, but the correlation is not as pronounced as in the cancers mentioned above **(38).** In colorectal cancer, EGFR protein expression of the tumor cells was found to be a powerful predictor of prognosis. Tumors with high EGFR expression are characterised by a more advanced stage, lymphovascular invasion, increased metastatic potential, and poor prognosis **(13).**

The predictive value of the epidermal growth factor receptor (EGFR) protein expression for EGFR antibody treatment is still questionable. Only patients with EGFR-positive CRC were enrolled in the first clinical trials with cetuximab **(27)**, so the protocol of cetuximab/Erbitux still states that the drug can only be used in the treatment of EGFR-positive colorectal cancers **(19)**. In the case of panitumumab/Vectibix, the Summary of Product Characteristics does not contain any information about EGFR status requirements **(20)**. Even though the hypothesis is logical that a targeted treatment will not work in cases where the tumor does not have the target, the efficacy of anti-EGFR antibody therapies for advanced colorectal cancer seems to be independent of the EGFR expression level of the tumor cells. Retrospective analyses of large clinical trials confirm this observation **(39,40)**. Interestingly, there are even reports of the effectiveness of anti-EGFR agents in EGFR-negative cases **(41)**.

EGFR expression could be evaluated with immunohistochemistry (IHC) using commercially available kits. The reason for the above mentioned controversies could be explained by inconsistencies in testing and evaluation. Tissue samples of low or negative EGFR protein expression became positive in 4 of 7 cases after changing the protocol of the antigen retrieval or incubation time. The use of various different anti-EGFR antibodies could be another explanation. The use of different monoclonal antibodies yielded controversial results in the same cohort of patients (**42,43**). The diagnostic problems of EGFR expression and the observation that EGFR-negative colorectal cancer patients responded to EGFR-targeted antibody therapy (**41**) diminshed the use of EGFR immunohistochemistry (IHC) in colorectal cancer.

Recently, activated EGFR was found to be a potential predictive protein biomarker of the efficacy of anti-EGFR therapy. Phospho-EGFR (activated) was determined by reverse phase protein arrays. Higher-than-median expression of phospho-EGFR was associated with significantly better survival compared to the lower-than-median cases in an anti-EGFR antibody treated cohort of mCRC pateints (**44**).

There have been attempts to analyze the connection between EGFR copy number variation (CNV) and protein expression. EGFR overexpression correlated with increased gene copy number per cell in non-small-cell lung carcinoma (NSCLC), but high gene copy number cases showed only a trend toward poor prognosis (**45**). EGFR protein expression and copy number were found to be closely related in cases of sporadic CRC,

and EGFR expression as shown by IHC was found to be an independent prognostic factor of overall survival (**46**). Data on the correlation between EGFR-CNV and the response to an anti-EGFR antibody therapy are still controversional. Amplification of EGFR was a positive predictor of efficacy (**47**), even though earlier reports did not confirm this correlation. In this report, neither EGFR gene copy number (GCN) nor HER2 GCN were found to have any predictive value for response to treatment with cetuximab (**48**), meanwhile extracellular EGF receptor mutations at codon 492 or 465 could be a possible explanation of cetuximab resistance (**49,50**).

### 1.9 Localisation of the primary tumor and EGFR expression

There are available data on the correlation between sidedness and EGFR expression of colorectal tumors. Comparative molecular analyses have been performed, and EGFR protein expression of right- and left-sided colon cancers and rectal tumors has been evaluated in a large cohort (N=1424) of primaries. EGFR expression was evaluated by immunohistochemistry. According to the results (using simple positive/negative thresholding), EGFR expression is more frequent in right-sided tumors compared to left-sided CRCs. Data also demonstrated that EGFR protein expression was significantly higher in right-sided tumors than in left-sided and rectal tumors, independently of their MMR and RAS mutation status (**31**). The difference between the EGFR status of the primaries and their paired metastases was reported (**51**). This result was not confirmed by another report; authors found 93.8% of samples had concordant EGFR status between the paired primary tumor and distant metastatic lesions, however the low cutoff set by the authors did not exclude differential expressions (**52**).

## 2 Objectives

2.1 Evaluate the difference between the EGFR expression of left- and right-sided tumors in a KRAS exon2 wild-type metastatic colorectal cancer cohort of patients treated with anti-EGFR therapies, where survival data (PFS, OS) were available.

2.2 In a small proportion of these patients, we have compared the EGFR copy numbers (CN) in tumor cells with their corresponding EGFR protein scores, and investigated the potential correlation.

2.3 Evaluate the predictive role of EGFR protein expression in the efficacy of anti-EGFR antibody (cetuximab) therapy, and test the predictive value of the EGFR H-score of primary tumors and metastases in a multivariate analysis.

## 3 Methods

The approval number of the local Institutional Review Board (IRB) of this study is 19/1043.

### 3.1 Patient characteristics

We collected data on 99 patients diagnosed with metastatic colorectal cancer and treated with anti-EGFR antibody therapy at the Hungarian Defence Forces Medical Center between 2008 and 2014. In total, 97 primary tumors and 33 corresponding metastatic tissues of the 99 patients were available for further evaluation. In 31 cases, we had samples from both the primaries and their corresponding metastases for comparison. In 2 cases, only biopsies were taken from a metastases due to the advanced stage of CRC. The end of the data collection was defined as the 16th of August, 2017. At that time, only 5 patients had survived. The characteristics of the 67 male and 32 female patients, and the data on localisation of the primary tumor, TNM stage and grade (G) are summerized in **Table 3**. In rectal tumor cases, the majority (N=15) originated from the middle third, 6 from the lower third, and 7 from the upper third of the rectum. The TNM stage was evaluated based on histopathological reports in those cases where the resection of the primary was performed. In 15 patients, the resection of the primary was not possible. In this group, the staging radiology reporting on T and N stage was available in only one case; in the other 14 cases it was not applicable (NA). All patients were diagnosed with multiple, unresectable metastases. The majority of the patients (67/99) had sycnchronous metastatic disease, where the metastases were confirmed less than three months after the diagnosis of CRC. In 32 cases (32/99), patients had metachronous metastatic disease**,** which is defined as metastases that were confirmed later than three months after the diagnosis of the CRC. We have also evaluated the number of metastatically involved organs by collecting data on the first staging computer tomography (CT) performed at the diagnosis of metastatic disease. Seventy-one (71) patients had only one involved organ. The following organs were involved: liver in 49 cases, lungs in 9, peritoneum in 8, distant lymphnode in 3, soft tissue in 1, and stomach in 1 case. Twenty-eight (28) patients were diagnosed with multiple metastatic organ involvement. According to the available protocols used at the time of our patient's treatment, the majority of the patients (90/99) were treated with a cetuximab-FOLFIRI combination, and one with a cetuximab-De Gramont protocol. Only 8 (8/99) patients received panitumumab in monotherapy. In 64

(64/99) cases, the anti-EGFR therapy was administered in a second-line setting. In 8 (8/99) cases, it was given as a first-line therapy. All other patients (27/99) were treated with anti-EGFR therapy in later lines. Before the initiation of anti-EGFR therapy, all patients were tested for RAS mutations as required. KRAS exon2 (N=84), then later expanded RAS testing (N=15) were performed. Thirty-three (33) metastatic tissue samples were available for testing. We performed EGFR IHC in cases of 18 liver, 2 lung, 2 lymphnode, 1 cerebellum, 1 skin, 1 ovarian, 6 peritoneal, 1 soft tissue, and 1 mesocolon metastases.

**Table 3 Patient's characteristics- all patients (N= 99)**

| **Sex** | | [ N] | [ % ] |
|---|---|---|---|
| | Male | 67 | 67.7 |
| | Female | 32 | 32.3 |
| **Age (years)** | | [ N] | [ % ] |
| | Median | 64 | |
| | Range | 24-79 | |
| **Primary tumor location** | | [ N] | [ % ] |
| | Rectum | 28 | 28.3 |
| | Rectosigmoid | 6 | 6.1 |
| | Sigma | 33 | 32.3 |
| | Descending colon | 7 | 8.1 |
| | Lienal flexure | 2 | 3.0 |
| | Transverse colon | 4 | 4.0 |
| | Hepatic flexure | 1 | 1.0 |
| | Ascending colon | 10 | 9.1 |
| | Coecum | 8 | 8.1 |
| **Primary tumor T** | | [ N] | [ % ] |
| | NA | 14 | 14.1 |
| | pT1 | 0 | 0.0 |
| | pT2 | 7 | 7.1 |
| | pT3 | 58 | 58.6 |
| | cT3 | 1 | 1 |
| | pT4 | 19 | 19.2 |
| **Primary tumor N** | | [ N] | [ % ] |
| | NA | 14 | 14.1 |
| | pN0 | 22 | 22.3 |
| | cN0 | 1 | 1 |
| | pN1 | 30 | 30.3 |
| | pN2 | 31 | 31.3 |
| | pN3 | 1 | 1.0 |

| **Primary tumor grade** | | [ N] | [ % ] |
|---|---|---|---|
| | NA | 10 | 10.1 |
| | 1 | 4 | 4.0 |
| | 2 | 66 | 66.7 |
| | 3 | 19 | 19.2 |
| **Resection of primary** | | [ N] | [ % ] |
| | Yes | 84 | 84.8 |
| | No | 15 | 32.3 |
| **Time of the diagnosis of metastatic disease** | | [ N] | [ % ] |
| | synchron | 67 | 67.7 |
| | metachron | 32 | 32.3 |
| **Organs involved, diagnosis by CT** | | [ N] | [ % ] |
| | Liver | 49 | 49.5 |
| | Lung | 9 | 9.1 |
| | Peritoneum | 8 | 8.1 |
| | Lymph node | 3 | 3.0 |
| | Soft tissue | 1 | 1.0 |
| | Stomach | 1 | 1.0 |
| | Multiorgan | 28 | 28.3 |
| **Site of metastases evaluated by IHC (N = 33)** | | [ N] | [ % ] |
| | Liver | 18 | 54.6 |
| | Lung | 2 | 6.1 |
| | Lymphnode | 2 | 6.1 |
| | Cerebellum | 1 | 3.0 |
| | Skin | 1 | 3.0 |
| | Ovarium | 1 | 3.0 |
| | Peritoneum | 6 | 18.2 |
| | Soft tissue | 1 | 3.0 |
| | Mesocolon | 1 | 3.0 |
| **RAS testing** | | [ N] | [ %] |
| | KRAS exon2 | 84 | 83.3 |
| | Extended RAS | 15 | 16.7 |

c: clinical, p: pathological

### 3.2 Definition of sidedness

We have defined sidedness according to the definition used in the Crystal and Fire-3 clinical trials (**34**). Tumors originating from the appendix, cecum, ascending colon, hepatic flexure, or transverse colon were considered to be right-sided CRCs (RSCRC). Primary tumors localised in the splenic flexure, descending colon, sigmoid colon, or rectum were classified as left-sided CRCs (LSCRC). We have evaluated 22 RSCRC and 75 LSCRC samples. The ratio of left- and right-sided tumors of our patient cohort was comparable to the results of the large clinical trials (**34**).

### 3.3 EGFR protein expression

The EGFR protein expression of colorectal cancer tumor cells was determined by immunohistochemistry, using a Benchmark Ultra automatic stainer (Ventana, Tucson, AZ). A ready-to-use mouse monoclonal anti-EGFR antibody 3C6 (Confirm anti-EGFR antibody, Ventana) was used, and the antibody bound was revealed by an Ultraview Universal DAB detection kit (Ventana). This antibody is directed against the internal domain of the human Epidermal Growth Factor Receptor (**53**). Slides were counterstained with hematoxylin. The membranous EGFR protein expression levels of tumors were determined by light microscopy using a 40× lens. The evaluation was carried out by applying the H-score (0–300) semiquantitative methodology **(54**). Where multiple metastatic samples were available, only one was utilized for evaluation. In each tumor sample, three representative areas were assessed for measurement of the percentage and intensity of EGFR-positive tumor cells. The intensity of the membrane labeling was defined in a three-tier system (1 = weak, 2 = moderate, 3 = strong). In each intensity category, the percentage of the tumor cells was defined and multiplied with the respective intensity range (1–3). Finally, the H-score was calculated as the sum of the results of each intensity category. A completely negative result was defined as HS = 0, and a maximal HS was defined as 300 (100% of tumor cells with 3+ intensity) **(Figure 2)**.

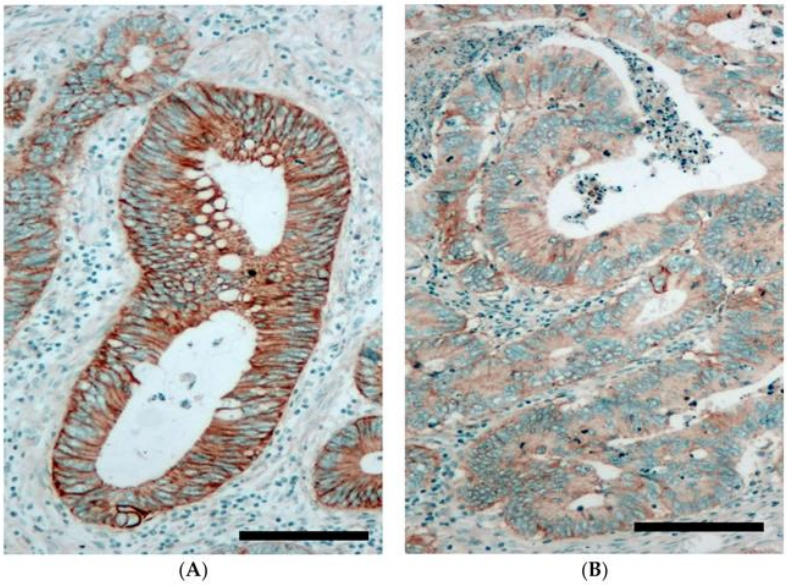



**Figure 2 Epidermal growth factor receptor (EGFR) protein expression of primary colorectal cancer tissue by immunohistochemistry** (brown membrane signal in basolateral position) (A) H- score =248, (B) H-score = 31. Cell nuclei are stained by hematoxillin (blue). Bar = 200 μm.

### 3.4 RAS testing

RAS mutation testing was a prerequisite for anti-EGFR antibody therapy, according to the available protocol at the time of the patient's treatment (**13,22**). RAS testing was performed at the 1st and 2nd Department of Pathology, Semmelweis University, and data were obtained from reports. Initially, KRAS exon2 mutation analysis was performed. DNA isolation was performed from formalin-fixed, paraffin-embedded (FFPE) tissue, blocks of primary tumors, or metastases. KRAS exon2 mutations were identified by microcapillary-based restriction fragment length analysis. Deoxyribonucleic acid (DNA) was extracted using the MasterPure™ DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). The microfluid-based restriction fragment detection system was characterized by 5% mutant tumor cell content sensitivity. The density ratio of the mutated band to the wild-type one was calculated. Mutation positivity was defined as samples containing >5% of the non- wild type (WT) band due to the sensitivity threshold. The base-pair substitutions in the mutant samples were verified and determined by sequencing on the ABI 3130 Genetic Analyzer System (Life Technologies, Carlsbad, CA,

USA) with the BigDye® Terminator v1.1 Kit (**55,56**). After protocol changes, extended RAS mutation analysis was used in a smaller fraction of our cohort; extended RAS mutation analysis was done using Idylla KRAS- and NRAS-BRAF-EGFR Mutation Assays (Biocartis) (**57**).

### 3.5 Evaluation of EGFR gene copy number using interphase fluorescence in-situ hybridization (iFISH)

The EGFR gene copy number status was evaluated by iFISH analysis; 5µm-thick FFPE tissue sections were mounted onto Superfrost Plus positively charged slides, deparaffinized, and rehydrated in distilled water. For antigen retrieval, sections were incubated in citric acid-based antigen unmasking solution (Vector Laboratories, Inc. Burlingame, CA, USA) at 95 °C for 20 min. In the prehybridizational steps, sections were incubated in Triton X-100 (AppliChem GmbH, Ottoweg 4, 64,291 Darmstadt, Germany)–SSC solution at 65 °C for 30 min to lyse cells, followed by digestion in pepsin solution for 12 min at 37 °C, and washing twice in SSC for 5–5 min with the ZytoLight® FISH-Tissue Implementation Kit (ZytoVision GmbH, Bremerhaven, Germany). Sections were air-dried prior to denaturation at 73 °C for 10 min. Hybridization was performed in an automated hybridization chamber (ZYTOMED Systems GmbH Berlin, Germany) using 7µl of ZytoLight SPEC EGFR/CEN 7 Dual Color Probe (ZytoVision Gmbh, Bremerhaven, Germany) per slide at 37 °C overnight. Slides were then washed in buffer SSC for 30 min at 45 °C to remove unbound probes, rinsed in water for 10 min, and air-dried. Cell nuclei were counterstained with DAPI in antifade solution (Vector Laboratories, Inc., Burlingame, CA, 94010, USA). A Leica DM RXA fluorescent microscope equipped with a Leica DFC 365FX high-performance CCD camera (Leica Microsystems GmbH, Wetzlar, Germany) and appropriate filters was used to evaluate the hybridization results. Areas with well-separated cell nuclei and overall good hybridization signals were selected for analysis. At least two FISH images per case were digitally captured at 63x magnification. For each case, green (EGFR) and red (CEN7 centromeric region) fluorescent signals were counted separately in at least 50 non-overlapping interphase nuclei. Finally, average EGFR copy number/cell, average CEN7 copy number/cell, EGFR/CEN7 ratio, average EGFR copy number/cell in amplified cell population, and percentages of polysomic or amplified cells were calculated (**58**).

### 3.6 Statistical analysis

The investigated patient cohort was divided into low- and high-expression groups based on their EGFR H-scores. We used different EGFR-HS threshold ranges (0, 50, 100, 200) to define low/high groups. The H-score of EGFR was analyzed by a Mann-Whitney test. Overall and progression-free survival analyses were carried out using the Kaplan–Meier method. Progression-free and overall survival intervals were determined as the time elapsed from the start of anti-EGFR therapy till the establishment of progression or death, respectively. The comparison between survival data of different strata was assessed by log-rank statistics. A multivariate analysis was performed by the Cox proportional hazard model. In cases of numeric variables (age and EGFR H-score), risks were calculated by individual values. In cases of categorical variables (sex, sidedness, metastasis), subgroups were applied. Statistical significance was confirmed in those cases where $p$ values were $<0.05$. Statistical analysis was performed using Statistica 13.0 software (StatSoft, Tulsa, OK).

# 4 Results

## 4.1 Aim 1

### Difference between the EGFR expression of left- and right-sided tumors

We evaluated and compared the EGFR-H-scores of left- (LSCRC) and right-sided colorectal cancers (RSCRC) in our KRAS wild type, anti-EGFR antibody treated, metastatic colorectal patient cohort. Ninety-seven (97) primary tumors were available for evaluation. From the whole patient population (N=99), the two excluded patients were those who did not have samples from their primaries (1 RSCRC and 1 LSCRC patient). The majority of the primaries consisted of LSCRC (N=75), while RSCRC cases were in the minority (N=22). Of the 33 available metastases, we evalutated 21 left-sided and 10 right-sided samples for EGFR expression. In 31 cases, samples from both the primaries and the corresponding metastases were available for evaluation. EGFR H-scores showed large variability in KRAS-wt primary as well as metastatic tumors from 0 to 300. In primary tumor cases, we found that the EGFR H-scores of the LSCRC were significantly lower than in the RSCRC: 89.9 ± 66.7 versus 141 ± 72.2 ($p$ = 0.04) **(Figure 3**). In metastases cases, a similar comparison of the right-sided and the left-sided samples was executed. According to our results, EGFR scores of the left-sided CRC metastases were significantly lower than those of the right-sided samples: 86.6 ± 65.2 versus 142.5 ± 87.8 ($p$=0.018) (**Figure 3).**

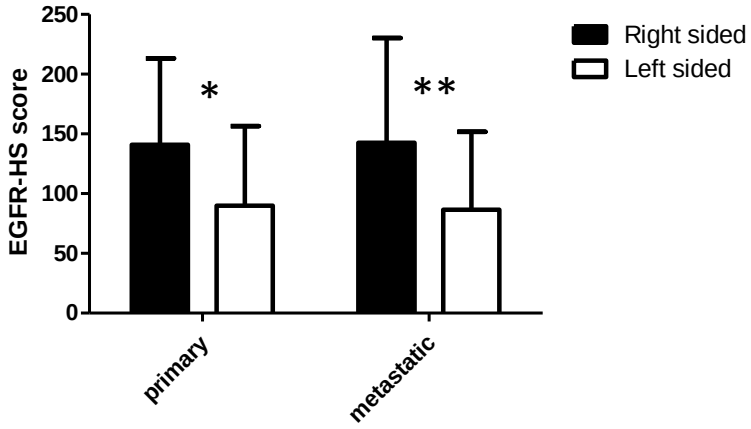
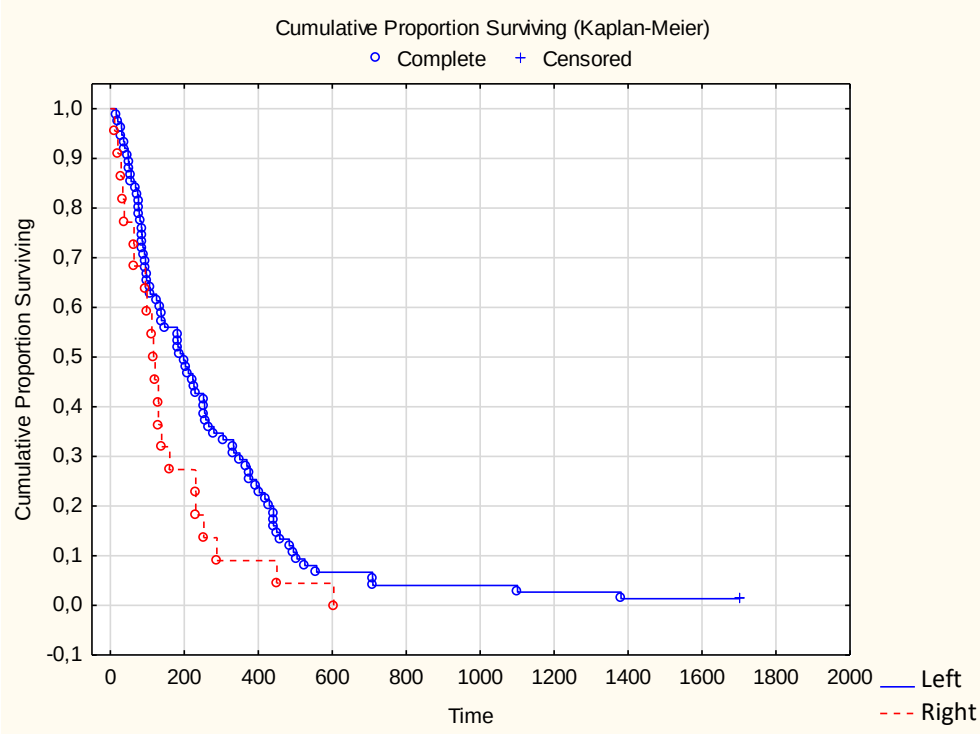



**Figure 3 Higher EGFR protein expression levels of right-sided primary and metastatic colorectal cancers compared to left-sided tumors.** Measured by H-scoring. Primary tumors (N=97), metastases (N= 31). Data are expressed as mean ± SD. *$p$ = 0.04, ** $p$ = 0.018.

In addition, progression-free survival (PFS) and overall survival (OS) data of anti-EGFR antibody treated RSCRC and LSCRC patients (N=22 v.75) were investigated using Kaplan-Meier analysis **(Figure 4**). In cases of PFS, RSCRC patients showed numerically poorer survival compared to the LSCRC cohort (**Figure 4A**, $p$ = 0.064). In cases of OS, the difference was significantly worse in cases of RSCRC patients compared to those with LSCRC (**Figure 4B**, $p$ = 0.047). The median PFS was 189 days for LSCRC and 117 days for RSCRC patients. The median OS for LSCRC was 423 days and 265 days for RSCRC (**59**).

A



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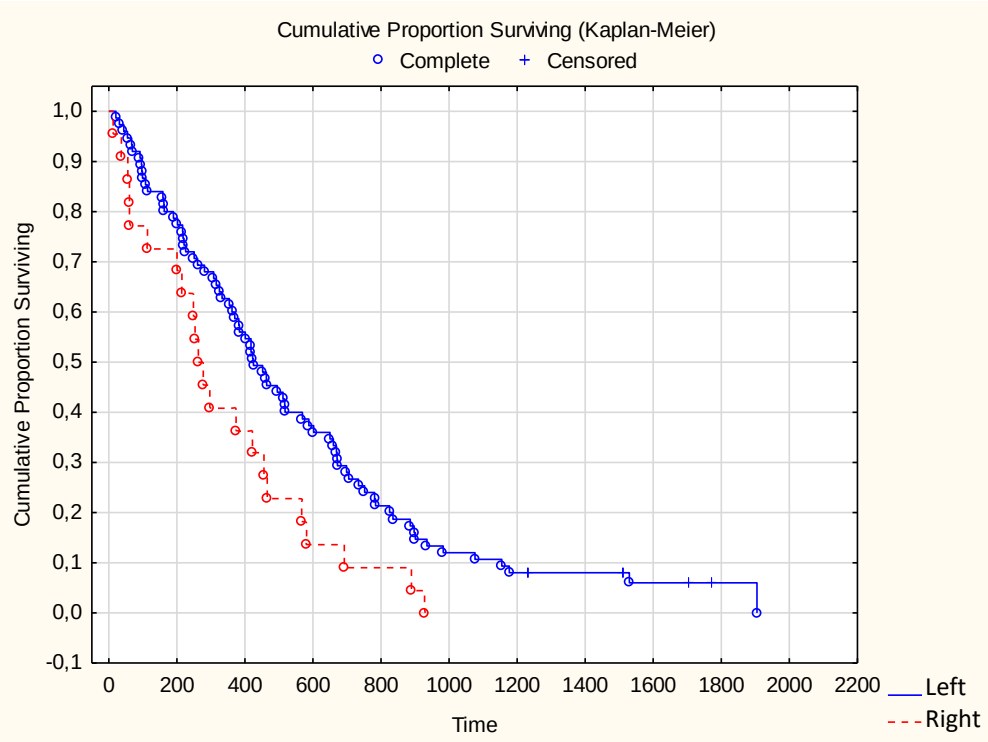



**Figure 4 Favourable survival of LSCRC compared to RSCRC patients treated with anti-EGFR antibody therapies** (Kaplan-Meier analysis).

(**A**) Presentation of progression free survival. $p$ = 0.064. (**B**) Presentation of overall survival. $p$ = 0.047. Y axis: Cumulative proportion surviving, X axis = days.

### 4.2 Aim 2

**Correlation between EGFR copy number (CN) and corresponding EGFR protein score.**

EGFR protein expression and copy number were found to be closely related in cases of sporadic CRC (**46**). We therefore compared the EGFR copy numbers (CN) in tumor cells to the corresponding EGFR protein scores in 7 cases (**Table 4**). We found that in these selected cases, CN/cell varied between 1.9 (diploid) and 5.04 (amplified), and the EGFR H-scores varied between 5 and 250. However, there was no association between the CN and EGFR protein expression in these cases. Moreover, extremely low protein scores were associated with amplified tumors, and high scores with near-diploid statuses (**59**).

**Table 4 Lack of association between the EGFR copy number and EGFR protein H-score in KRASwt colorectal cancer cases**

| case No | H-score | EGFR CN/ tumor cell | % of tumor cells with amplified EGFR |
|---|---|---|---|
| 1 | 5 | 4.44 | 22.81 |
| 2 | 25 | 1.9 | 0 |
| 3 | 30 | 4.77 | 13.33 |
| 4 | 70 | 4.08 | 5.77 |
| 5 | 70 | 4.26 | 40.0 |
| 6 | 200 | 2.73 | 16.13 |
| 7 | 250 | 5.04 | 7.69 |

CN= copy number, EGFR amplification= EGFR/cen7 ratio >2

### 4.3 Aim 3

**Predictive role of EGFR protein expression in the efficacy of anti-EGFR antibody (cetuximab) therapy**

We analysed the correlation between EGFR-HS and the progression-free survival (PFS) and overall survival (OS) populations (N=90). At the time of the diagnosis of advanced disease, all patients had multiple metastases. Sixty-seven (67) patients had only one organ involvement, while 23 patients had multiple organ involvement. The majority of the cases (61/90) was characterized by synchronous metastatic disease, while in the remaining cases (29/90) metachronous metastases developed. In this cohort, the primary tumor was resected in 77 cases (77/90). In the case of 11 (11/90) patients, only biopsies were carried out from the primaries. Overall, 88 patients had tissue samples from their primaries; 19 of these were considered to be right-sided tumors, and 69 were left-sided. Tumor sample pairs – primaries and their corresponding metastases – were available for comparison in 27 cases. In 2 (2/90) cases, only biopsies from the corresponding metastases were available, and there were no samples from their primaries. Altogether, 29 metastatic tissues were evaluated; 11 of these were right-sided, and 18 were left-sided samples. Eleven (11) of the 29 metastatic samples were considered to be metachronous, and 18 were synchronous. All sampling was carried out before any anti-EGFR therapies. KRAS exon2 (75/90) and later extended KRAS/NRAS exon2,4 (15/90) mutation analyses were performed according to the appropriate guidelines. Patient characteristics are summerized in **Table 5**.

**Table 5 Patient characteristics - cetuximab treated population (N= 90)**

| **Sex** | | [ N ] | [ % ] |
|---|---|---|---|
| | Male | 63 | 70.0 |
| | Female | 27 | 30.0 |
| **Age (years)** | | [ N ] | [ % ] |
| | Median | 64 | |
| | Range | 24-79 | |
| **Primary tumor location** | | [ N ] | [ % ] |
| | Rectum | 26 | 28.9 |
| | Rectosigmoid | 6 | 6.7 |
| | Sigma | 28 | 31.1 |
| | Descending colon | 8 | 8.9 |
| | Lienal flexure | 3 | 3.3 |
| | Transverse colon | 4 | 4.4 |
| | Hepatic flexure | 1 | 1.1 |
| | Ascending colon | 8 | 8.9 |
| | Coecum | 6 | 6.7 |
| **Primary tumor pT** | | [ N ] | [ % ] |
| | NA | 13 | 14.4 |
| | 1 | 0 | 0.0 |
| | 2 | 6 | 6.7 |
| | 3 | 54 | 60.0 |
| | 4 | 17 | 18.9 |
| **Primary tumor pN** | | [ N ] | [ % ] |
| | NA | 13 | 14.4 |
| | 0 | 21 | 23.3 |
| | 1 | 26 | 28.9 |
| | 2 | 29 | 32.2 |
| | 3 | 1 | 1.1 |
| **Resection of primary** | | [ N ] | [ % ] |
| | Yes | 77 | 85.6 |
| | No | 13 | 14.4 |
| **Number of metastates evaluated by IHC (n =29)** | | [ N ] | [ % ] |
| | Liver | 17 | 58.6 |
| | Lung | 2 | 6.9 |
| | Lymphnode | 2 | 6.9 |
| | Cerebellum | 1 | 3.4 |
| | Skin | 1 | 3.4 |
| | Ovarium | 1 | 3.4 |
| | Peritoneum | 3 | 10.3 |
| | Soft tissue | 1 | 3.4 |
| | Mesocolon | 1 | 3.4 |

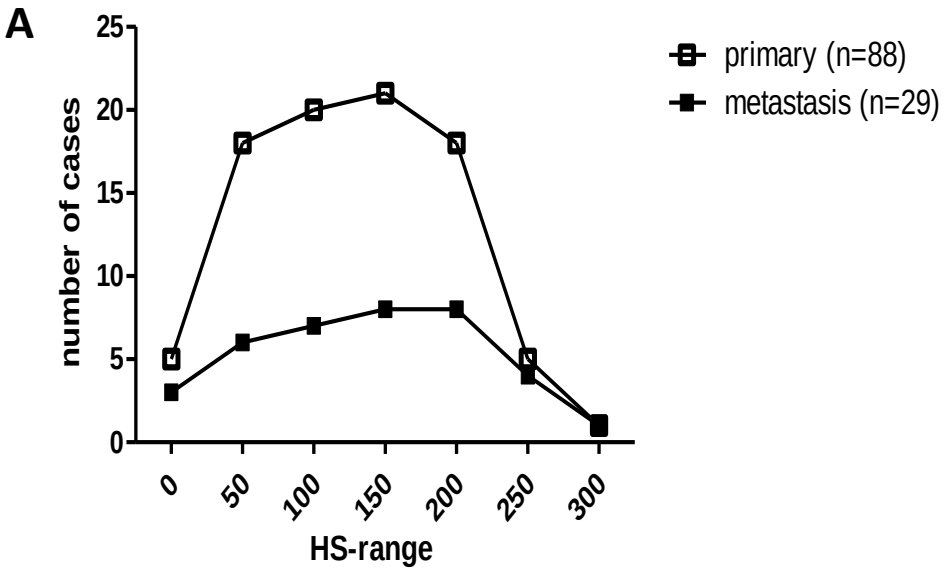
| **RAS testing** | [ N ] | [ % ] |
|---|---|---|
| KRAS exon2 | 75 | 83.3 |
| Extended RAS | 15 | 16.7 |

Patients received the cetuximab and FOLFIRI therapy as second-line therapy in the majority of cases (63/90). Nine (9/90) patients were treated with this combination in first line, the remainder of the patients (18/90) in later lines. Three patients survived at the end of the study from this cohort. In those cases where multiple metastatic samples were available, only one sample/case was used for EGFR score calculation.

We first evaluated the EGFR protein expression of the primaries and their metastases. The median EGFR-HS was similar in both the primary and the metastatic tumor tissues (100 ± 66 versus 110 ± 75, respectively). Distribution of the EGFR-H-scores (by 50 increments) were very similar in the primary and the metastatic colorectal tumors (**Figure 5A**).

Comparison of the HS of 27 metastases to their corresponding primaries was also carried out. The resulting individual alterations (decrease or increase) were plotted in **Figure 5B**. We found significant differences and extreme alterations in both directions (higher or lower) in the majority of cases. The metastases maintained the EGFR-HS range of the primary tumor only in a minority of cases (no difference: 3/27, 11.1 % ; ± 10% difference: 8/27, 29.6%) (**Figure 5B**).

EGFR H-scores of the primary tumors with different metastatic potentials (single versus multiple metastatic diseases) were also compared. EGFR protein expression was significantly higher in primary tumors with multiple metastases ($p = 0.007$), (**Figure 5C**). We evaluated the possible correlation between the metastatic potential and the EGFR expression of the tumors. Our data revealed that in both primary tumors and their metastases, the tissue samples of multiple metastatic cases expressed significantly higher EGFR-HS compared to the EGFR expression of samples of single metastatic cases ($p = 0.007$ and $p = 0.004$ respectively) (**Figure 5C**) (**60**).


A
25
20
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number of cases
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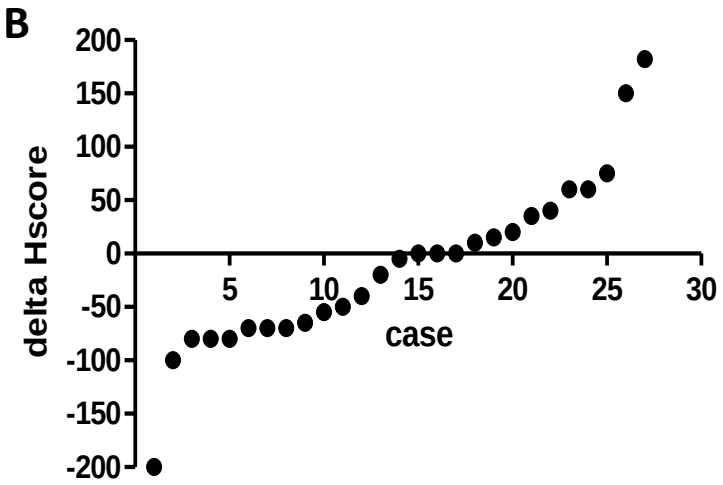
HS-range
primary (n=88)
metastasis (n=29)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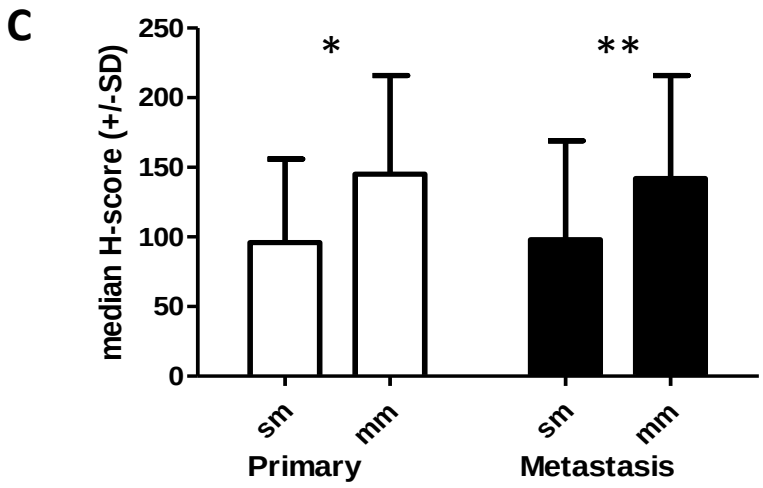


**Figure 5 Comparison of the EGFR protein expression in primary and metastatic colorectal cancer tissues.** (**A**) Distribution of EGFR expression levels in primary versus metastatic tumor tissues as represented with various H-score ranges. (**B**) Variations of EGFR-HS (H-score) in colorectal cancer metastases compared to the corresponding primary tumor (N = 27). Data are expressed as H-score differences of metastatic minus primary tumor at individual case level. (0 = no change; negative value = decrease; + value = increase). (**C**) Higher EGFR H-score of primary tumors with multiple-metastases compared to tumors with single metastases (single metastasis, sm, N = 22) versus multiple-metastasis, (mm N = 66), * $p$ = 0.007. Comparison of metastatic tumors with single metastastasis (sm) versus multiple metastases (mm), ** $p$ = 0.04. Data are expressed as median ± SD, Mann–Whitney test.

In the same cohort of our patients, we also analyzed the correlation between EGFR-HS and the progression-free survival (PFS) and overall survival (OS). We used different EGFR-HS threshold ranges (0, 50, 100, 200) to define low/high groups, and evaluated the PFS and OS using Kaplan-Meier statistics.

Our data indicated that in primary tumors with values below the threshold, EGFR protein expression was associated with favourable PFS and OS. The differences were statistically significant in OS at the 200 threshold exclusively ($p$ <0.05) (**Figure 6 A,B**). In metastatic tissues, our data indicated that values below the applied threshold of EGFR-HS were associated with longer PFS. The differences were significant at the 50 and 200 thresholds in cases of PFS, and at all thresholds in cases of OS. In particular, the difference was greatest at the lowest thresholds, gradually decreasing with increasing EGFR-HS thresholds (**Figure 6 C, D)** (**Table 6**) (**60**).

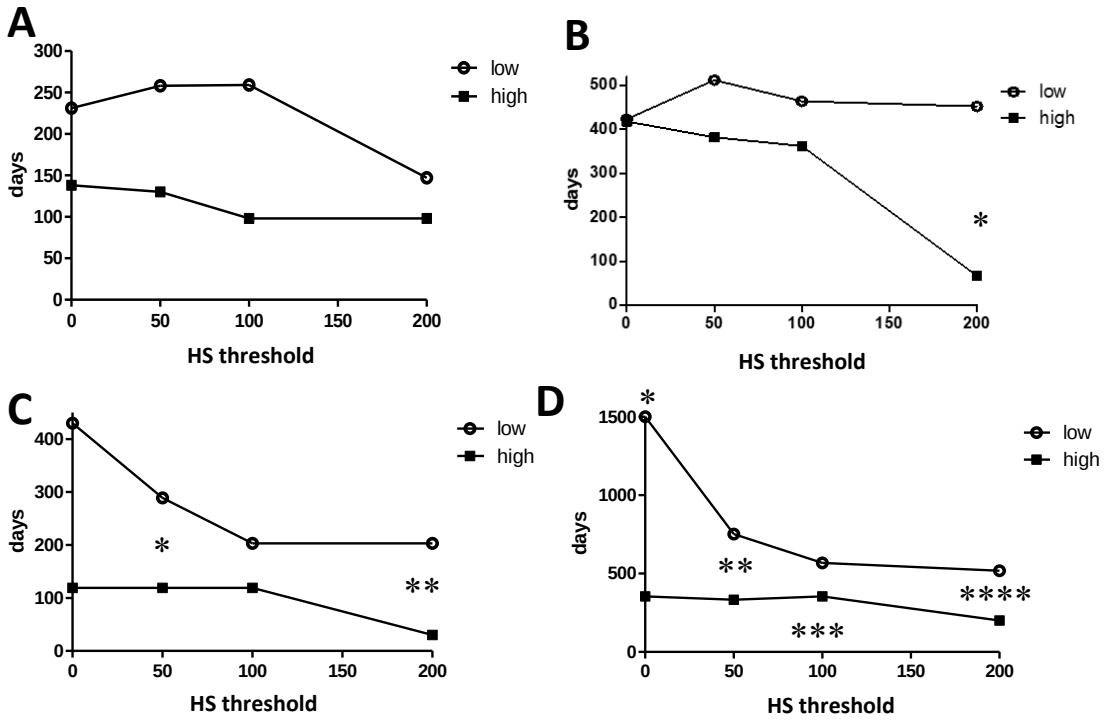


**Figure 6 Progression-free and overall survival of cetuximab-treated colorectal cancer patients** (expressed in days) in relation to the level of EGFR protein expression defined as H-score. Kaplan–Meier statistics. (**A**, **B**): Primary tumors (N = 88). (**C**, **D**): Metastatic tissue (N = 29). (**A**, **C**): Progression-free survival, (**B**, **D**): Overall survival in days. Low=below-, high=above HS threshold. (**B**): * $p$ = 0.042, (**C**): * $p$ = 0.024, ** $p$ = 0.046, (**D**): * $p$ = 0.008, ** $p$ = 0.05, *** $p$ < 0.018, **** $p$ = 0.053

**Table 6 PFS and OS results by EGFR expression in primary and metastatic tumors**

| | Primary tumor (N=88) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of patients (N) | | **PFS (day)** | | P value | **OS (day)** | | P value |
| **HS-Threshold** | **high** | **low** | **high** | **low** | | **high** | **low** | |
| 0 | 83 | 5 | 138 | 231 | 0.453 | 418 | 423 | 0.492 |
| 50 | 65 | 23 | 130 | 258 | 0.268 | 382 | 512 | 0.201 |
| 100 | 40 | 48 | 98 | 258 | 0.131 | 362 | 464 | 0.355 |
| 200 | 6 | 82 | 97 | 147 | 0.112 | 67 | 452 | 0.042 |
| | Metastases (N=29) | | | | | | | |
| | No. of patients (N) | | **PFS (day)** | | P value | **OS (day)** | | P value |
| **HS-Threshold** | **high** | **low** | **high** | **low** | | **high** | **low** | |
| 0 | 26 | 3 | 115 | 430 | 0.067 | 354 | NR | 0.008 |
| 50 | 20 | 9 | 119 | 289 | 0.024 | 333 | 752 | 0.005 |
| 100 | 13 | 16 | 118 | 203 | 0.143 | 333 | 579 | 0.018 |
| 200 | 5 | 24 | 30 | 203 | 0.046 | 201 | 518 | 0.053 |

NR= not reached

The predictive power of the EGFR H-score of primary tumors and their metastases was also investigated. We applied the Cox proportional hazard model and tested the EGFR-H score in multivariate analysis, with other factors such as sidedness, number of involved metastatic organs (single versus multiple), age and sex. The analysis confirmed that in our cetuximab-treated cohort, EGFR H-score was a very weak independent predictor of OS; it approached the border of significance only in the case of metastatic tissue. In the same analysis, sidedness was found to be a strong, significant predictor either in the group of primary tumors or in cases of metastases (**Table 7,8**). It should be noted that there was a significant difference between the EGFR expression of left- and right-sided CRCs (**31,60**). Age was also found to be an independent predictor as well, with a questionable clinincal significance, since the corresponding relative risk (RR) levels were minimal. Our results did not confirm the significant predictive roles of sex, or the number of metastases (**60**).

**Table 7 EGFR H-score of primary tumors is a weak independent predictor of OS**

Multivariant analysis of various prognostic/predictive factors of cetuximab efficacy using Cox proportional hazard model of survival: EGFR protein expression of the primary tumors (N = 88).

| Variables | P | RR (95% CI) |
|---|---|---|
| age | 0.001 | 0.967 (0.943-0.993) |
| sex | 0.715 | 1.099 (0.661-1.826) |
| EGFR-HS | 0.53 | 1.001 (1.193-1.006) |
| sidedness | 0.009 | 2.028 (1.193-3.448) |
| metastases (S vs M) | 0.1 | 0.643 (0.38-1.089) |

M= multiple metastatic disease, RR= relative risk, S= single metastatic disease, sidedness= left or right

**Table 8 EGFR H-score of metastases is a weak independent predictor of OS**

Multivariant analysis of various prognostic/predictive factors of cetuximab efficacy using the Cox proportional hazard model for overall survival: EGFR protein expression of the metastases (N = 29).

| Variables | P | RR (95% CI) |
|---|---|---|
| age | 0.001 | 0.916 (0.871-0.964) |
| sex | 0.427 | 1.468 (0.570-3.784) |
| EGFR-HS | 0.083 | 1.007 (0.999-1.016) |
| sidedness | 0.006 | 5.694(1.641-19.757) |
| metastases (S vs M) | 0.19 | 0.469 (0.151-1.455) |

M= multiple metastatic disease, RR= relative risk, S= single metastatic disease, sidedness= metastasis derived from left or right sided primary

Finally, we conducted a subgroup analysis of survival data of the left- and right-sided cetuximab-treated cases based on EGFR-HS. We performed the analysis using Kaplan–Meier statistics; EGFR-HS low- versus high status was determined by the median of the analysed subgroup. In both left- and right-sided primary tumors, there was no statistical difference observed in OS between EGFR-low and EGFR-high tumor cases (**Figure 7 A,B**). In cases of metastases, Kaplan–Meier analysis demonstrated that low EGFR-HS patients are characterized by a nominally better median OS at both sides (left side low:

766.5 days versus high: 368 days, right side low: 283.5 days versus high: 55 days). This result was significant only in the case of left-sided tumors (N = 18, $p = 0.016$), which can be explained by the low number of right-sided metastatic cases (N = 11) (**Figure 7 C, D**) (**60**).

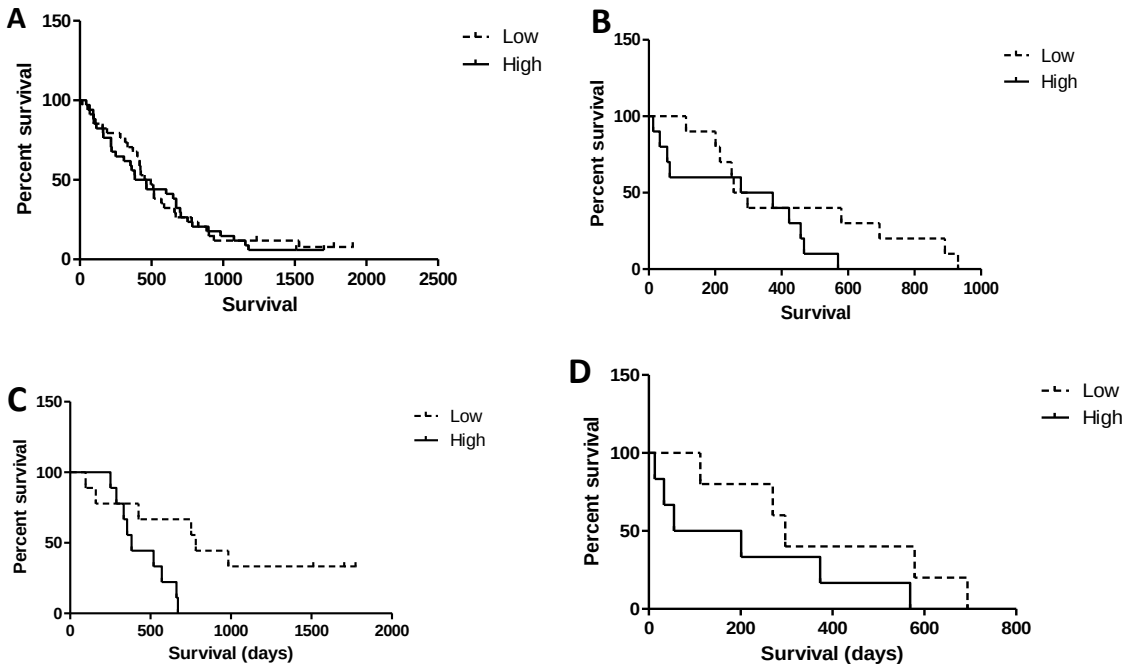


**Figure 7 Overall survival analysis of left- and right-sided colorectal cancer patients treated with cetuximab stratified by the median EGFR-HS scores as low versus high of the primary tumors** (**N = 88**) **or metastastatic tissues** (**N = 29**). (**A**) Left-sided primary tumors (N = 69), EGFR HS median = 95. (**B**) Right-sided primary tumors (N= 19), EGFR HS median = 125. (**C**) Left-sided tumor metastases (N = 18), median = 80. (**D**) Right-sided tumor metastases (N = 11), median = 150. Kaplan–Meier statistics.

## 5 Discussion

Our results support the previously reported findings that metastatic KRAS-wt LSCRC responds significantly better to anti-EGFR antibody therapy than RSCRC (**34-37, 59**).

In cases of metastatic colorectal cancer, the significance of sidedness in terms of response to targeted therapies was already evaluated by several clinical trials. In cases of anti-VEGF antibody therapy, no difference was found between the responses of left- and right-sided tumors (**61**). Moreover, in cases of bevacizumab, the combination of anti- VEGF-antibody therapy and chemotherapy reduced the mortality of either metastatic LSCRC or RSCRC cases compared to the chemotherapy-only group. In cases of anti-EGFR therapy, the use of the cetuximab-chemotherapy combination was useful only for patients with left-sided, wild-type KRAS mCRC compared to the chemotherapy-only group, and it was associated with significantly higher mortality among patients with right-sided mCRC (**62**)**.** The patomechanism of this difference is still controversial, and it is supposed to be selective EGFR-signaling related, and it is independent of RAS mutation.

Previous reports analysed the molecular profiles and also compared the EGFR expression of LSCRC to RSCRC using a simple ± thresholding. According to their results, EGFR protein expression was higher in RSCRC than in LSCRC tissues. In this study, the possible relationship of EGFR expression and KRAS mutation status was not evaluated **(31**). Therefore we investigated and compared the EGFR protein expression of KRAS exon2-wt LSCRC and RSCRC. In our EGFR antibody-treated, KRAS exon2-wt metastatic colorectal cancer patient population, we evaluated the EGFR protein expression of the primary tumors and also their corresponding metastases using a semi-quantitative measurement of H-scoring, and found similar results. Our data confirmed that RSCRC has a significantly higher EGFR protein expression level than LSCRC even in cases of KRAS-wt setting. We investigated this phenomenon in cases of metastatic tissues as well. Our analysis indicated that even in cases of visceral tumor metastases, RSCRC maintains a higher EGFR protein expression level as compared to LSCRC (**Figure 3**).

In our preliminary research we have already attempted to analyse the predictive role of EGFR protein expression in this KRAS exon2 wild-type metastatic colorectal cancer cohort (N=99) treated with anti-EGFR (cetuximab or panitumumab) therapies. Ninety-seven (97) primary colorectal carcinoma and 33 corresponding metastatic FFPE tissues

were used. Our results suggested that lower-than-chosen-threshold EGFR protein levels are associated with longer progression-free survival (PFS) or overall survival (OS) **(63**). Based on these findings, we evaluated the correlation between EGFR protein expression and survival data in a homogeneous, cetuximab-treated group of KRAS exon2 wild-type metastatic colorectal cancer patients. In this cohort, low EGFR protein expression levels of tumor tissue were associated with significantly better survival. This result suggests that high EGFR protein experession could be another negative predictor of the response to anti-EGFR antibody therapy. The major differences in survival were found at the lowest EGFR-HS thresholds: 0 and 50; 9 and 27% of the cetuximab-treated KRAS exon2 wild-type patient population belongs to these subgroups respectively. We found that the predictive value of EGFR-HS is more favourable in the cohort of metastases. This observation can be explained with the significant difference we found between the EGFR protein expression of the primaries and their corresponding metastases (**Figure 5B**).

The survival results of our patient cohort are consistent with the previously discussed data in the anti-EGFR antibody treated patient population (**34,35**); PFS and OS were better/ favourable for the LSCRC cohort compared to the RSCRC subgroup **(Figure 4)**. It should be noted that EGFR expression of the RSCRC group was significantly higher compared to the LSCRC group both in the primary tumors and in their corresponding metastases (**Figure 3).**

Considering the action mechanism of anti-EGFR antibodies, our observations are relatively unexpected. Previous data on the value of EGFR protein expression as a predictive marker for anti-EGFR therapies in CRC are still conflicting. Early clinical trials with cetuximab confirmed response in colorectal cancer patients with tumors that do not express the epidermal growth factor receptor by immunohistochemistry (**41).** In cases of panitumumab monotherapy, the lack of correlation between epidermal growth factor receptor status and the treatment response was also reported in metastatic colorectal cancer cases (**39**). There is even a report which found that EGFR protein expression is a negative predictor of the anti-EGFR therapies in CRC (**40**). Recent publications suggested that EGFR IHC should be combined with SISH to select patients who will respond to cetuximab therapy. The authors recommended to evaluate several factors together, such as quantitative image analysis of aberrant EGFR expression, ligand expression, gene copy number variation and mutational status for patient selection (**64**). Other authors analysed

the expression and activation of proteins involved in cell signaling in a cohort of RAS WT mCRC treated with anti-EGFR therapy. With the method of reverse phase protein arrays, they found that activated EGFR and HER3 are predictive biomarkers for better overall survival. Interestingly, left-sided mCRC displays active ErbB2/3 and Wnt pathways, and was found to respond better to anti-EGFR therapy compared to right-sided mCRC. Active EGFR signaling and downstream PI3K were found as positive predictors of response to anti-EGFR treatment (**44**).

Correlations of EGFR protein levels with copy number status, mRNA levels, and miRNA regulation were also recently investigated. The authors stated that EGFR protein expression and copy number are closely related (**46**). In earlier reports, a positive correlation between EGFR copy number and EGFR protein level had already been reported (**47**). Data also suggested that in general, increased GCN was associated with a better objective response in cases of anti-EGFR monoclonal antibodies (mAbs) treatment, however in these cases the EGFR protein was not analysed in parallel (**65**). The clinical utility of EGFR GCN is limited because of inappropriate sensitivity and specificity of the scoring system (**66**). Our result however did not support a correlation between EGFR copy number and EGFR protein levels (**Table 2**) (**59**).

We also tested the predictive value of EGFR expression with multivariate analysis, using other factors such as age, sex, sidedness, and number of metastases. The analysis indicated that EGFR protein expression of both primary and metastatic tissue is not an independent predictor of cetuximab efficacy **(Table 5,6)**. Sidedness is an already-confirmed, well known predictor for anti-EGFR antibody therapy (**34,35)**. Based on earlier reports and our results, sidedness and EGFR expression are closely related; the poorly responding right-sided tumors express EGFR at significantly higher levels than left-sideded CRCs (**30,59**). These observations suggest that EGFR protein expression could also be a negative predictive factor for anti-EGFR antibody therapy. EGFR protein expression above the median value on both sides is a negative predictor of the efficacy of anti-EGFR therapy in metastases. Based on earlier reports, high EGFR protein expression of the KRAS exon2 wild-type primary colorectal cancer is a negative prognostic factor (**31**). EGFR expression was related to advanced stage lymph node and liver metastases at the diagnosis of CRC (**46**). Our data also suggest that high EGFR expression is a negative

prognostic factor, since EGFR expression levels were higher in colorectal cancers with multiple metastases compared to those with single metastases (**59,60**).

There are some limitations regarding the evaluation of these results. Cetuximab recognises the ligand-binding extracellular domain of EGFR, while the Ventana antibody used here for immunohistochemistry recognizes the juxtamembrane extracellular domain (**54**). Moreover, the method is unable to detect extracellular domain mutations or splice variations (vIII), which negatively affect the efficacy of cetuximab in colorectal cancer (**49,50**). Further studies are required to explore this issue.

The mechanism of action of anti EGFR therapy also needs further evaluation due to the major differences in cetuximab efficacy observed in low EGFR protein-expressing tumors; in addition, there are also reports on responses in case of EGFR negative tumors (**41**). Other, unknown mechanisms should be involved in the mode of action of anti-EGFR therapy.

Despite using negative predictors of efficacy, not all patients will respond to anti-EGFR antibody therapy. The resistance mechanism to cetuximab therapy of advanced colorectal cancer is under intensive investigation. Several mechanisms are supposed to contribute to primary resistance for anti-EGFR antibody treatment, such as KRAS, NRAS and BRAF mutations (**34**). MAP2K1, PIK3CA, ERBB2, MET, and FGFR1 gene mutations were also recently investigated as potential resistance mechanisms, but have not been validated (**67**). There are other potential predictors: EPHA2 overexpression proved to be a strong negative predictor for cetuximab efficacy (**68**), and the Prospect-C Cetuximab trial revealed miR-31-3p to be a potential negative predictor of efficacy **(69)**. Recently, CCR7 protein overexpressions in the tumors were found to correlate with insensitivity to cetuximab **(70).** In cases of an acquired resistance to cetuximab therapy, systematic analysis of pre- and post-treatment tumors revealed a switch from consensus molecular signature-2 to consensus molecular signature-4 (a stromal expression signature), an increase in immune cell infiltrate and in the upregulation of checkpoint regulators. These results suggest a possible immune mechanism of resistance to anti-EGFR antibody therapies (**67**).

## 6 Conclusions

EGFR protein expression of CRC is an already well known, strong negative prognostic factor. Our “real life” study confirmed that EGFR expression of right-sided tumors is significantly higher compared to left-sided ones. Our results strongly support the notion that EGFR protein expression in colorectal cancer is not only a negative prognostic factor, but also a negative predictive factor of anti-EGFR antibody therapies. Furthermore, low- but not high-level EGFR protein expression on KRAS wild-type metastatic colorectal cancer cells may be a prerequisite for successful anti-EGFR antibody therapy. These paradoxical findings deserve further rigorous investigation.

The new observations provided by the present study are the following:

1. Our results support the findings that metastatic KRAS-wt LSCRC responds significantly better to anti-EGFR antibody therapy than RSCRC.
2. Our data also confirmed that RSCRC has a significantly higher EGFR protein expression level than LSCRC, even in KRAS-wt settings.
3. Based on our results, there is no correlation between EGFR CNV and protein expression in CRC.
4. Our data confirmed that in a cetuximab-treated group of KRAS exon2 wild-type metastatic colorectal cancer patients, low EGFR protein expression levels of tumor tissue are associated with significantly better survival.
5. The multivariate analysis indicated that EGFR protein expression of both the primary and the metastatic tissues is not an independent predictor of cetuximab efficacy.
6. Based on our results, sidedness and EGFR expression are closely related: the poorly responding right-sided tumors express EGFR at significantly higher levels compared to left-sided CRCs.

## 7 Summary

Despite the recent progress in treatment selection for patients with metastatic colorectal cancer, approximately 40% of cases will not respond to anti-EGFR antibody therapy. In daily practice we use negative predictors, such as RAS and BRAF mutations for patient selection, but we do not have any positive predictive factor for the efficacy of anti-EGFR antibody therapy.

Therefore in the present study we revisited the issue of EGFR protein expression as a potential predictive factor of anti-EGFR antibody treatment. The level of EGFR protein expression was determined by immunohistochemistry and evaluated by H-score (HS) methodology. We examined progression-free survival (PFS) and overall survival (OS) at different EGFR expression thresholds in primary and metastatic tissues. We also compared the EGFR expression and survival data of left- and right sided tumors. The prognostic role of EGFR expression was investigated as well in the same cohort of our anti-EGFR treated, metastatic colorectal cancer patients.

In all cases, lower than chosen threshold EGFR expression was associated with numerically favourable PFS and OS. In the cohort of the primary tumors, the difference in OS was significant only at threshold HS 200, but in metastatic tissues, all levels lower than the EGFR-HS thresholds were associated with significantly longer OS.

In terms of sidedness, the EGFR-HS of RSCRC was significantly higher compared to LSCRC, both in the primary tumors and their metastases. These data demonstrated for the first time that the EGFR protein expression is significantly higher in KRAS wild type RSCRC as compared to LSCRC. Kaplan-Meier survival analysis demonstrated that anti-EGFR antibody therapies were more effective in LSCRC compared to RSCRC. However, in a multivariate analysis, sidedness remained a strong independent predictive factor of survival.

The negative prognostic role of high EGFR expression was also confirmed, since high EGFR expression levels in either primary tumors or metastatic tissues were associated with multiple metastatic disease.

In summary, our results support the opinion that EGFR protein expression in colorectal cancer is not only a negative prognostic factor, but also a negative predictive factor of anti-EGFR antibody therapies.

## Összefoglalás

A metasztatikus colorectalis tumorok terápiaválasztásában bekövetkezett javulás ellenére az esetek közel 40 %-a nem fog reagálni az anti -EGFR kezelésre. Betegszelekció céljából a mindennapi gyakorlatunkban negatív prediktorokat használunk, mint pl. a RAS valamint BRAF mutációk, azonban az EGFR terápia hatékonysága szempontjából pozitív prediktív faktorral nem rendelkezünk.

Jelen vizsgálatunkban ezért az EGFR protein expressziót, mint az anti-EGFR antitest kezelés lehetséges pozitív prediktorát értékeltük újra. Az EGFR expressziót immunhisztokémiai módszerrel vizsgáltuk, és a H-score (HS) módszerével értékeltük. A progressziómentes (PFS), és teljes (OS) túlélést különböző EGFR küszöbértékek mellett vizsgáltuk, mind a primer mind a metasztatikus daganatok csoportjában. Összehasonlítottuk a jobb -, és bal- colonfél EGFR expressziójának mértékét, valamint a túlélési adatokat is. Anti- EGFR kezelésben részesült metasztatikus colorectalis tumor miatt kezelt betegeink ugyanezen csoportjában az EGFR expresszió prognosztikus szerepét is vizsgáltuk.

A választott küszöbérték alatti EGFR expresszió minden esetben numerikusan kedvezőbb progressziómentes, illetve teljes túléléssel járt együtt. A teljes túlélés különbsége a primer tumorok esetében a 200-as küszöbértéknél bizonyult csak szignifikánsnak, azonban a metasztázisok csoportjában minden küszöbérték alatti csoportban szignifikánsan jobb volt.

Az oldaliság szempontjából a jobb oldali colorectalis tumorok (RSCRC) EGFR expressziója szignifikánsan magasabbnak bizonyult a baloldali ( LSCRC ) tumorokénál, mind a primer tumorokban mind a metasztázisokban. Eredményeink első alkalommal igazolják, hogy a KRAS vad típusú jobboldali colon tumorok EGFR expressziója szignifikánsan magasabb, mint a baloldali tumoroké. A Kaplan – Meier túlélési adat-elemzés alapján az anti -EGFR kezelés hatékonyabbnak bizonyult a baloldali tumorokban, mint a jobboldali daganatokban. Azonban multivariáns analízis alapján az oldaliság továbbra is a túlélés erős, független prediktív faktora maradt.

A magas EGFR expresszió negatív prognosztikus szerepe szintén megerősítésére került, ugyanis a magas EGFR expresszió mind a primer tumorok, mind a metasztázisok esetében a többszörös/ többszervi metasztázis képződéssel mutatott összefüggést.

Összefoglalva, eredményeink azt a véleményt támasztják alá, hogy az EGFR expresszió colorectalis daganatok esetében nem csak negatív prognosztikus faktor, hanem az anti-EGFR terápia szempontjából negatív prediktív szereppel is bír.

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## 9 Bibliography of candidate's publications

### Publications related to the thesis

**Uhlyarik A**, Piurko V, Vizkeleti L, Pápai Z, Rásó E, Lahm E, Kiss E, Sikter M, Vachaja J, Kenessey I, Tímár J.
EGFR Protein Expression of KRAS Wild-Type Colorectal Cancer: Predictive Value of the Sidedness for Efficacy of Anti-EGFR Therapy.
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**Uhlyarik A**, Piurko V, Papai Z, Raso E, Lahm E, Kiss E, Sikter M, Vachaja J, Kenessey I, Timar J.
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EGFR protein is a negative prognostic and predictive factor in wild-type RAS colorectal cancer.
*Magy Onkol. 2021 65:121–12*

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