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Clinical and translational studies in metastatic colorectal cancer

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Abstract

In metastatic colorectal cancer (mCRC) chemotherapy +/- targeted therapy with palliative intent aims at prolonging survival with sustained quality of life. Maintenance of tumour response by a period of less intense treatment may delay progression and accumulation of unacceptable toxicity. We studied the combination of targeted treatment with the angiogenesis inhibiting antibody bevacizumab (bev) and the epidermal growth factor tyrosine kinase inhibitor erlotinib (erlo) as maintenance treatment in two clinical trials.

The Nordic ACT trial (paper I) included 249 mCRC patients. Following first line induction doublet chemotherapy plus bev, responding patients were randomised to maintenance treatment with bev or bev+erlo. We found no significant difference in survival outcomes between the arms. We then hypothesized that KRAS mutation of the tumour would have a negative impact on the erlo effect.

In the Nordic ACT2 trial (paper II, N=233), the KRAS wildtype (wt) patients were randomised in the same manner as in Nordic ACT. The KRAS mutated (mut) patients recieved bev alone or metronomic low dose capecitabine and arms were compared without significant difference in effect or safety. The KRASwt patient cohorts from the both Nordic ACT trials were pooled in an analysis of survival outcomes (N=126) with no statistically significant gain from the addition of erlo to bev as maintenance.

There are no validated biomarkers of anti-angiogenic therapy. Treatment induced hypertension has been associated with better response to angiogenesis inhibition. The vasoactive peptides (VPs) atrial natriuretic peptide, adrenomedullin, and copeptin are linked to regulation of blood pressure and angiogenesis. In paper III, the stable pro-peptides of each VP were analysed in plasma from ACT2 study patients with documented progressive disease (N=97). IncreasingVP levels during the first six weeks of induction chemotherapy + bev were significantly associated with better clinical outcome.

In paper IV, we collected serum samples at start of induction, start of maintenance and at progression from ACT2 patients (N=22). Analyses of 55 circulating, angiogenesis-related proteins were performed at each time point by antibody array membrane technology. Levels of some, mostly pro-angiogenic, proteins decreased significantly during response and/or increased at progression.

In summary, these studies demonstrate that mCRC patients may not benefit from bev+erlo as maintenance therapy in terms of efficacy, and that the clinical benefit can be further questioned due to tocixity concerns. KRAS status is not likely a predictive biomarker for erlo in mCRC. Microarray methodology for simultaneous detection of multiple proteins in serum is convenient for exploration of signalling patterns related to the response and resistance to angiogenesis inhibition. Our translational results support the evidence of an interaction between host-related vascular effects and response to chemotherapy plus bev. Both VPs and other counterbalancing pro-angiogenic factors are promising biomarkers that warrant further studies in this setting.

Key words: colorectal cancer, maintenance therapy, bevacizumab, erlotinib, angiogenesis inhibition, biomarkers, vasoactive peptides, multiplex protein array

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To my patients

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Thesis at a glance

	Clinical trials		Translational trials	
Paper	I	II	III	IV
Study	ACT	ACT^2	Vasoactive Peptides	Angiogenesis Protein Array
N =	249	233	97	22
Patients	All RAS	KRAS wildtype/ KRAS mutant	Evaluable pts ACT^2 Progressive disease	Beva arms ACT^2 Progressive disease
Method	RCT prospective	RCT prospective	Biomarker retrospective	Biomarker retrospective
Design	Induction + maintenance: Bevacizumab vs. Beva + erlotinib	Induction + KRAS- guided maintenance: Wt: Beva +/- erlotinib Mut: Beva vs. metronomic Capecitabine	Plasma Vasooactive peptides Hypertension; Baseline (A) 6 weeks (B)	Serum Proteinarray; Baseline Start of Maint. End of Treatmt.
Endpoints	PFS OS, Safety	PFS rate 3 months Pooled data KRAS wt	Correlation, peptides HT, TTP, ORR	Feasibility, correlation Proteomics, TTP

Timeline: 2007 2009 2010 2012 2013 2015

Patient recruitment

Analysis of circulating biomarkers

Papers included in the thesis

I. Johnsson A, Hagman H, Frödin J-E, Berglund Å, Keldsen, N, Fernebro E, Sundberg J, DePont Christensen R, Garm Spindler K-L, Bergström D, Jakobsen A. **A randomized phase III trial on maintenance treatment with bevacizumab alone or in combination with erlotinib after chemotherapy and bevacizumab in metastatic colorectal cancer – the Nordic ACT trial**. *Ann Oncol 2013 Sep;24(9):2335-2341*

II. Hagman H, Frödin J-E, Berglund Å, Sundberg J, Vestermark L, Albertsson M, Fernebro E, Johnsson A. **A randomized study of KRAS-guided maintenance therapy with bevacizumab, erlotinib or metronomic capecitabine after first line induction treatment of metastatic colorectal cancer: the Nordic ACT2 trial**. *Ann Oncol 2016 Jan;27(1)140-147*

III. Hagman H, Bendahl PO, Melander O, Sundberg J, Johnsson A, Belting M. **Vasoactive peptides associate with treatment outcome of bevacizumab-containing therapy in metastatic colorectal cancer**. *(Acta Oncologica, accepted)*

IV. Hagman H, Bendahl PO, Lidfeldt J, Belting M, Johnsson A. **A Feasibility Study of Protein Array Profiling of Angiogenesis-Related Factors During Bevacizumab Containing Treatment in Metastatic Colorectal Cancer**. *(Manuscript)*

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Abbreviations

5-FU	5-Fluorouracil
ACT	"<u>A</u>vastin and <u>C</u>hemotherapy followed by Avastin +/- <u>T</u>arceva"
ADH	Antidiuretic hormone, Vasopressin
ADM	Adrenomedullin
AE	Adverse event
AKT	Protein kinase B
ANP	Atrial natriuretic peptide
ASCO	American Society of Clinical Oncology
ATP	Adenosine triphosphate
BL	Baseline
BNP	B-type (brain) natriuretic peptide
BRAF	B-Raf proto-oncogene (gene)
BSC	best supportive care
CAPEOX	capecitabine + oxaliplatin
CAPIRI	capecitabine + irinotecan
CI	confidence interval
CIN	chromosomal instability
CR	Complete response according to RECIST
CRC	colorectal cancer
CRF	Case report form
CRO	Contract research organization
CT	computed tomography scan
CTCAE	Common terminology criteria for adverse events
dMMR	deficient mismatch repair
DNA	Deoxyribonucleic acid
ECCO	European Cancer Organisation
ECOG	Eastern Cooperative Oncology Group performance status scale
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EOT	end of treatment
ERK/MAPK	Extracellular signal-regulated kinase/Mitogen activated protein kinase
ESMO	European Society of Medical Oncology
FAS	full analysis set

FGFb	Fibroblast growth factor basic, FGF-2
FLV	5-FU+leucovorin single chemotherapy schedule
FOLFIRI	5-FU/leucovorin + irinotecan
FOLFOX	5-FU/leucovorin + oxaliplatin
FOLFOXIRI	5-FU/leucovorin+oxaliplatin+irinotecan
FTD	trifluridine
GCP	Good clinical practise
HB-EGF	Heparin binding epidermal growth factor
HGF	Hepatocyte growth factor
HR	Hazard Ratio
ICH	International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use
IFL	5-FU/leucovorin+irinotecan
IGF	Insulin growth factor
IGFBP	Insulin growth factor binding protein
IL	Interleukin
ITT	intention to treat
JAK	Janus kinase
KRAS	Kirsten rat sarcoma viral oncogene (gene or protein)
mCRC	metastatic colorectal cancer
MDT	multidisciplinary team
MedDRA	Medical Dictionary for Regulatory Activities
MEK	Mitogen- activated protein kinase kinase
mOS	median overall survival
MR	mid regional (portion of peptide)
MRI	magnetic resonance imaging
MSI	microsatellite instability
MSS	microsatellite stability
mTOR	mechanistic/mammalian target of rapamycin
mut	mutated
N=	number of patients
NO	Nitric oxide
NRAS	neuroblastoma RAS viral oncogene homolog (gene)
NSCLC	non-small cellular lung cancer
OS	overall survival
PD	progressive disease according to RECIST

PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol-3-kinase (protein)
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (gene)
PlGF	Placental Growth factor
PP	per protocol
PR	partial response according to RECIST
PTX-3	Pentraxin-related protein 3
QoL	Quality of Life
RAS	KRAS + NRAS
RCT	randomised controlled trial
RECIST	Response Evaluation Criteria in Solid Tumours
RNA	Ribonucleic acid
SAE	serious adverse event
SAP	statistic analysis plan
SD	stable disease according to RECIST
SNP	single nucleotide polymorphism
SOM	start of maintenance treatment
STAT	Signal transducer and activator of transcription protein
TF	Tissue factor, Coagulation factor III
TGF-α	Transforming growth factor alpha
TKI	tyrosine kinase inhibitor
TNM	Tumour Node Metastasis stage
TPI	Tipiracil hydrochloride
TRACE	time-resolved amplified cryptate emission
TTP	time to progression
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
wt	wild type (non-mutated)
XELIRI	capecitabine + irinotecan
XELOX	capecitabine + oxaliplatin

Preface

"Omnia mirari etiam tritissima" – Everything is worth our attention, even the insignificant

- Carl von Linné

In March 2009 I first met Lars, a 52 year-old married man, father of two teenagers and skilled carpenter. He lived a fully active life despite his diagnosis that had brought him to my practice at the outpatient oncology ward at Ryhov County Hospital in Jönköping. Lars had been diagnosed the previous year with rectal cancer that was spread to the liver. Following rectal surgery, the colorectal surgeon had informed him that the disease was incurable. It was by all accounts, a straightforward verdict. Shortly thereafter, Lars chose to participate in the clinical randomised ACT trial described in this thesis.

After an initial period of treatment, including chemotherapy, Lars experienced a good partial tumour response; and so the liver surgeons were consulted. Unfortunately they determined that liver surgery would not be successful since it could not give a chance for cure. Lars was then randomised to the experimental arm of maintenance treatment (without chemotherapy), as part of the ACT trial. This involved treatment with the targeted drugs bevacizumab and erlotinib in combination. Three years later, Lars' tumour scans would reveal an 80% reduction in size of the liver metastases against the baseline; and the results would revise the same liver surgeon's opinion on the likely success of surgery. Lars went from a bad surgical prognosis, to a positive one.

At the time of our second appointment I shared his latest CT scan that showed his liver metastases were still in good remission after one year on maintenance therapy. This was of some relief, as Lars was hoping to spend the summer sailing and golfing, and particularly as he was planning for a trip abroad with his family and friends a couple of months later. From a therapy perspective, I was planning for his upcoming journey along lines of therapy, with the prospect of a future beyond christmas and potentially through another summer. I was partly grateful and bursting with joy at the successes, but was silently preoccupied with our mutual challenge of how to approach his disease at tumour progression in the future. Why and how does this experimental combination therapy work? Is there a magic swarm of bullets? If so, how far will they reach? It was challenging not to be able to rely on any familiar prognostic signs or experience. It was obvious that I needed more tools besides my eyes and ears to empower me to maintain quality of life and prolong extension of life for each patient. When I saw my next patients with advanced colorectal cancer, the questions continued to pile up. What signs and patterns do we need to distinguish? How do we best discern the threats and the

targets? When is it possible to predict tumour response or progression? What if we understood how to tailor, not only the combination of drugs, but also the strategic sequencing of treatment options for each patient more efficiently?

Clearly, many aspects of patient-focused and personalised medecine, as we know it, are to a great extent still hidden to us. It´s like an iceberg with the majority of its mass hidden under water. We just see the tip of the iceberg as we navigate our patients through the deepest waters, knowing that a lethal hit is inevitable. Our aim is to provide a safe and durable journey for each individual as we try and avoid hitting the iceberg too soon in the course of his or her disease. And of course, with each patient having varied experiences and situations, the sea we are navigating in the land of cancer is vast.

I have learned, that every day and every detail matter in this continuum of cancer care. My personal aim with this thesis is to continue to care, not solely for my own patients, but for the questions still calling for answers.

<div align="right">

Helga Hagman

January 2017

</div>

Background

"Tumor angiogenesis exploits the same pathways that are used when blood vessels are created to heal wounds. Nothing is invented; nothing is extraneous. Cancer´s life is a recapitulation of the body´s life, its existence a pathological mirror of our own. Susan Sontag warned against overburdening an illness with metaphors. But this is not a metaphor. Down to their innate molecular core, cancer cells are hyperactive, survival-endowed, scrappy, fecund, inventive copies of ourselves."

- Siddharta Mukherjee in "The emperor of all maladies"

In this introduction chapter I will begin with an outline of the epidemiology of metastatic colorectal cancer (mCRC). I then sum up the treatment strategies of this setting, including a description of the current targeted therapy agents used in mCRC, introducing the concept of maintenance treatment. Finally, I present a brief summary of the current knowledge on predictive biomarkers with a focus on anti-angiogenic treatment of cancer.

Epidemiology

Incidence

There is no internationally recognised coloured ribbon to be proudly worn on the sleeve to raise awareness of colorectal cancer. Many people hesitate to talk about their changing toilet habits or intermittent rectal bleedings. Our large bowel, the colon, with its distal 15 cm ending called the rectum, seems to be an organ somehow concealed to the world in the stigma formed by its contents.

Still, it is estimated that cancer of the colon and rectum is the third most common cancer worldwide in males and the second most commonly diagnosed cancer in females after breast cancer. In total almost 1.4 million men and women were diagnosed with colorectal cancer in 2012, compared with 1.7 million cases of female breast cancer and 1.1 million cases of prostate cancer which is estimated to be the second most common cancer diagnosis in men after lung cancer[1]. More than half of the patients with colorectal cancer are above 65 years of age at diagnosis[2, 3].

The incidence for colorectal cancer (CRC) is increasing. In 2015 around 2000 cases of rectal cancer and 4300 new cases of colon cancer were diagnosed in Sweden.[3]

Staging

At the time of diagnosis, histopathological confirmation of invasive adenocarcinoma and radiological staging with computed tomography (CT) and/or magnetic resonance imaging (MRI) of the thorax and abdomen are of essential importance. A multidisciplinary approach to the diagnosis, treatment recommendation and information to the patient is the golden standard of care[4]. The primary focus of the multidisciplinary team (MDT) meetings, apart from the patient´s symptoms, is to map the loco-regional extent of the disease and rule out distant metastasis by clinical staging of the tumour burden. For CRC TNM (Tumour Node Metastasis) stage I-III, curative surgery of the tumour is the primary treatment option. Many patients with rectal cancer are recommended neo-adjuvant radiotherapy or chemo-radiotherapy preoperatively, or for a few cases as a definite substitute for surgery. Adjuvant chemotherapy can be discussed postoperatively based on the pathological tumour stage.

In approximately 20% of the cases, distant metastases are present at diagnosis (TNM stage IV), with liver being the most commonly affected organ, followed by the lungs [4, 5]. After initial curative treatment of TNM stage I-III cancer, half of the patients are diagnosed with relapse of the disease. The scope of this thesis covers the palliative treatment of patients diagnosed with advanced, i.e. metastatic CRC (mCRC).

Survival and treatment options

The 5-year age-standardised relative survival for colorectal cancer (all stages) in Sweden was 64% for men and 67% for women for the period of diagnosis 2010-2014. This can be compared with 36% and 39% 5-year age-standardised relative survival rates for patients diagnosed in 1970-1974[6]. Survival continues to improve due to better diagnostics, endoscopic and surgical techniques, chemotherapy and more advanced treatment options, even for the more advanced stages[7]. According to European cancer registry studies, the survival rates of CRC stage IV is increasing, and outcomes are likely to improve above the reported median survival of up to a year[5, 8]. In a report from the Nordic countries, patients diagnosed with stage IV mCRC during 2006-2008 had a 3-year survival rate of 21%, compared to around 11% and 7% for patients diagnosed in the periods 1996-2000 and 1980-1985 respectively[8].

Surgery of metastatic disease is possible not only in cases with liver-only metastases, but has shown to be effective also for limited metastatic spread to *e.g.* the lungs and the peritoneal cavity[5]. The indication for liver surgery has continued to widen our perception of the multidisciplinary goals. An increasing number of patients are re-evaluated for surgery after the initial months of systemic anti-tumoral therapy, referred to as first-line therapy, including chemotherapy with or without targeted agents. In many cases the decision of the MDT will be to recommend oncologic evaluation for conversion therapy, *i.e.* anti-tumoral treatment with intent to shrink and reduce tumour burden to enable surgical procedures[9].

The median survival time after liver resection in mCRC is around four years, and for patients undergoing surgery of metastases limited to the liver the 5-year survival rate is around 40%[10, 11]. Supplementary techniques include chemo-ablation, radioisotope ablation and radiofrequency ablation, which are locally directed procedures aiming to reduce metastatic burden in the liver, primarily with palliative intent. This encouraging progress in treatment options and results has forced the oncologist profession to be on guard, ready to change treatment plan at every point of evaluation of the disease. According to European guidelines the current goal should be to offer each patient intervention aiming at "no evidence of disease" whenever possible[9].

For patients like Lars who I mentioned in my preface, this will imply hope for a longer life and sometimes, even cure. At the same time thorough risk evaluation concerning postoperative mortality and morbidity, toxic effects and resistance to treatment, must be made. Many mCRC patients describe their situation as a rollercoaster ride between hope and despair. A focus on quality of life must always be adopted in order to at least stabilize the pace of the ride in informed decision making.

Prognosis in advanced disease

A common purpose of anti-tumoral treatment strategies is to achieve a qualitative and quantitative prolongation of the patient´s life. In this context there is sometimes a thin line between clinically meaningful and harmful treatment effects. Scientific evidence needs to be incorporated into clinical praxis in such a way that this line of best practise is clarified.

The oncological treatment options in mCRC have ameliorated in parallel to surgical achievements. Advances in radiotherapy are seen along with the steady introduction of new drugs and combination regimens for systemic anti-tumoral treatment. As a result, the median overall survival (mOS) for mCRC has increased with approximately six months every decade (Figure 1), and median survival times

have now in some clinical trials reached 30 months[9, 12, 13]. Nevertheless, these promising results from clinical trials are not paralleled in the general patient population or registry data, although improvements are seen as mentioned above[7]. It is believed that the discrepancies can be explained by disparities in baseline characteristics such as age, performance status, co-morbidity and socioeconomic factors, limiting the options and outcome of a pro-active therapy approach in many patients not enrolled in clinical protocols[8, 14].
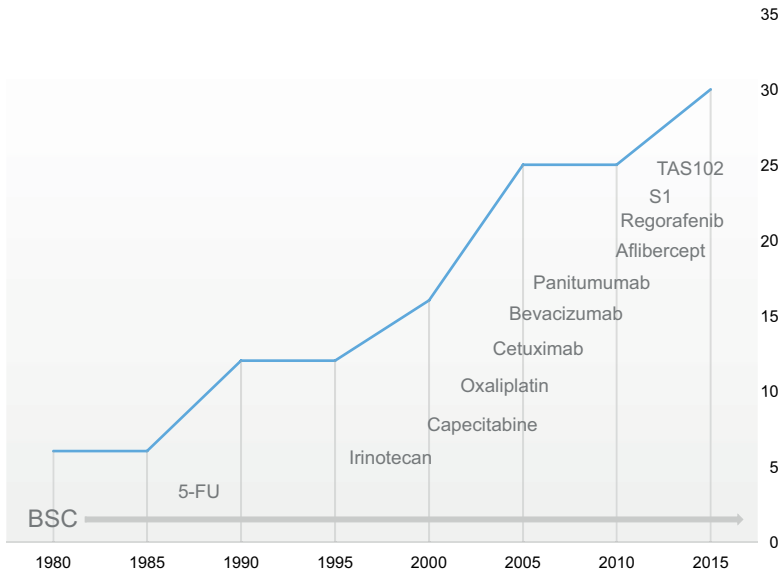

Figure 1.
Median overall **survival (months)in clinical trial reports** is improving with the introduction of new anti-tumoral compounds used in metastatic colorectal cancer. BSC, Best supportive care should always be applied to optimise palliative care in combination with anti-tumoral treatment; 5-FU, 5-Fluorouracil. (Author´s own figure)

Chemotherapy in mCRC

Intravenous chemotherapy agents

Fluorouracil

5-Fluorouracil (5-FU) is the mainstay chemotherapeutic drug for colorectal cancer. It has established effects on survival and quality of life when used in combination with different biochemical modulating agents as demonstrated by the early trials in 1989[15, 16]. 5-FU is used in continuous intravenous infusion regimens, and/or as bolus dose [17, 18]. It is an antimetabolite agent that causes death of rapidly growing cells through deprivation of the DNA nucleoside compound thymidine. To supplement inhibition of DNA synthesis, biochemical modulation with folinic acid/leucovorin/calcium folinate is used to increase the effect of 5-FU as shown by the clinical trials performed in the early 1990´s[19].

5-FU is also used in adjuvant treatment settings of stage II-III CRC[20]. The most common side effects of 5-FU are myelosuppression, mucositis, fatigue, diarrhoea, and hand-foot syndrome (a sometimes painful, oedematous inflammation and skin-scaling of palmar and plantar areas). Cardiac related symptoms and ischemia are rare but potentially serious toxic effects of 5-FU[21].

Irinotecan

Irinotecan (CPT-11, Campto®) inhibits the enzyme topoisomerase-I in the cell nucleus, which leads to DNA breaks that eventually cause cell death[22]. Since the late 1990´s irinotecan has been established both as a useful alternative and as a partner to 5-FU when used in first line and second line mCRC[22, 23]. It is also effective in combinations with oxaliplatin and/or targeted antibody agents[12, 13, 24]. Toxic side effects include diarrhoea, nausea, myelosuppression, fatigue, cholinergic reaction, alopecia and mucositis.

Oxaliplatin

Like other platinum compounds oxaliplatin (Eloxatin®) cause DNA damage by reacting with the DNA molecule, blocking DNA replication and transcription. The combination with 5-FU was evaluated during the 1990´s, and proved superiority to single use of 5-FU in mCRC[25]. The French GERCOR group (Groupe Coopérateur Multidisciplinaire en Oncologie) established the bi-monthly FOLFOX regimens in second and first line treatment of mCRC[26]. Later trials demonstrated improved survival with oxaliplatin plus 5-FU versus 5-FU alone for postoperative adjuvant treatment of stage III and high-risk stadium II colorectal cancers[20]. The toxic effects of oxaliplatin are often dose limiting due to accumulated damage on nerve structures causing acute and chronic peripheral neuropathy. Other toxicities

include nausea, laryngopharyngeal dysesthesia, allergic reactions, myelo-suppression and fatigue.

Oral chemotherapy agents

Capecitabine

The oral compound capecitabine (Xeloda®) is itself a non-toxic 5-FU precursor that is selectively activated by stepwise metabolisation in the liver. In the final step the tumour associated thymidine phosphorylase converts the precursor 5´-DFUR (5´-deoxy-5-fluorouridine) into 5-FU, thus optimising the exposure of the active cytotoxic agent in tumour tissue.

With a randomised phase II trial by van Cutsem *et al.*[27] the intermittent dosing schedule of bi-daily intake for 14 days followed by 7 days pause was proposed for phase III evaluation in CRC, and established as standard. Trials in the early 2000´s demonstrated at least equivalent efficacy compared to 5-FU infusional regimens as both palliative and adjuvant treatment in CRC[28, 29]. The doublet combination schedules with Oxaliplatin (XELOX/CAPEOX) or Irinotecan (XELIRI/CAPIRI) are comparable in terms of efficacy to the corresponding doublet regimens with intravenous 5-FU-leucovorin replacing Capecitabine (FOLFOX/FOLFIRI)[30, 31]. Capecitabine has some advantages to infusional 5-FU by the convenient oral administration and the toxic profiles are similar. Capecitabine more commonly gives rise to hand-foot syndrome whereas stomatitis is seen less often than with intravenous 5-FU regimens[28].

S1 and TAS-102

The development of oral agents has given some impetus to the exploration of varying fluoropyrimidine-based therapeutic strategies in mCRC. UFT (Tegafur-Uracil) and the combined agent S-1 belongs to the latest generation of fluoropyrimidines initially explored in an Asian setting of gastrointestinal cancers[32].

S-1 (Teysuno®) is an orally administered combination of three compounds: Tegafur, gimeracil and oteracil[32]. Tegafur is a prodrug of 5-FU whereas gimeracil acts as an effect modulator by inhibition of the degradation enzyme resulting in prolonged half-life of the active substance. Oteracil reduces the action of 5-FU in the gut. S-1 is associated with less skin toxicity than capecitabine[33].

TAS-102 (Lonsurf®) is an oral compound including the active agent FTD (trifluridine), which is a nucleoside analogue that incorporates into DNA causing DNA dysfunction leading to cell death. The adjuvant compound TPI (tipiracil hydrochloride) improves bioavailability of FTD by suppression of FTD

degradation. TAS-102 is shown to improve survival as single agent compared to placebo in mCRC patients with tumours refractory to standard therapies including 5-FU[34]. Toxic effects include myelosuppression, mild nausea and diarrhoea.

Targeted therapy in mCRC

Targeting vascular endothelial growth factor and angiogenesis

In order to grow and disseminate malignant tumours require certain acquired capabilities, which are shared by all neoplastic disease, and frequently referred to as the hallmarks of cancer[35]. One of these is the ability of sustained angiogenesis in the tumour. Angiogenesis is the sprouting of new blood vessels from the existing vasculature, which is essential to supply normal tissue or a tumour with oxygen and nutrients for sustained cell function and survival. Vasculogenesis is the formation of new blood vessels from circulating endothelial progenitor cells that derive from the bone marrow and differentiate into endothelial cells to supply the angiogenesis process[36]. The relative contribution of angiogenesis and vasculogenesis in tumour neovascularisation is currently ill defined. Angiogenesis is regulated by a complex balance between pro-angiogenic signalling factors that stimulate this process and the counter-balancing anti-angiogenic inhibitory signalling molecules. In the adult, this balance is transiently altered under physiological conditions such as the female menstruation cycle and during wound healing[37].

However, in the multistep pathogenesis of cancer growth, it has been proposed that the up regulation of pro-angiogenic factors released from the cancer cells and stroma initiates an angiogenic phenotype, *i.e.* a vascular-dependent state of the tumour cell mass, in a process which is denoted "the angiogenic switch"[38](Figure 2). This continuous process is regulated by a complex interaction between the cancer cells and the stromal cells *e.g.* fibroblasts, pericytes and immune cells contained in the extracellular matrix that support the tumour and its vasculature[37, 39]. The activity that causes normally quiescent vasculature to form new capillaries, is triggered by genetic changes (*e.g.* expression of oncogenes in the cancer cell), and by external stress factors such as hypoxia[37]. It has been shown that this switch can occur early in the development of cancer, including CRC[40].
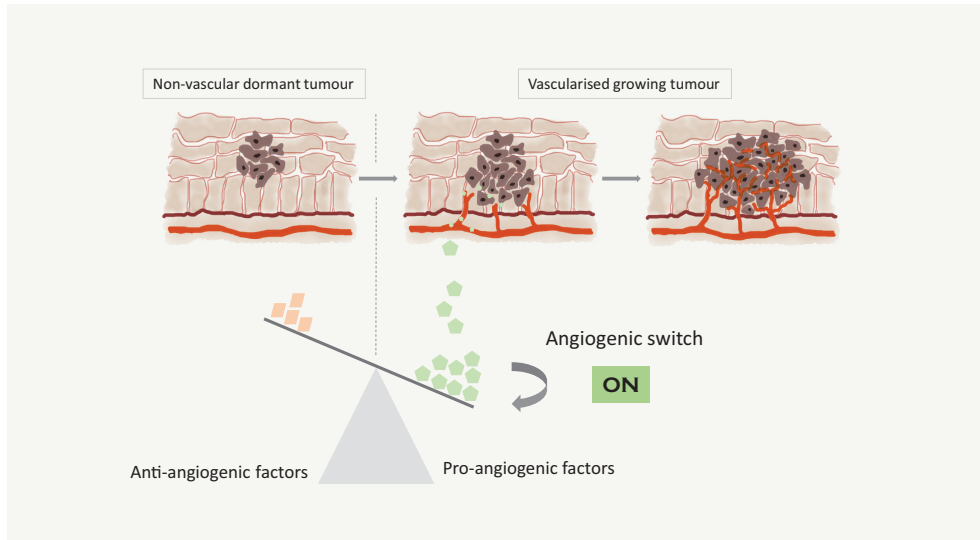
Figure 2.
The angiogenic switch hypothesis. In the tumour, the balance of angiogenesis is switched to a pro-angiogenic state to promote vascularization, survival, growth and metastasis. Pro angiogenic factors include *e.g.* growth factors (VEGF, EGF, TGF-α and β, TNF-α, FGF, IGF, PDGF) and cytokines (interleukins 1α, 6 etc). Examples of anti-angiogenic factors are angiostatin, endostatin, thrombospondin-1, interferon-α and angiopoetin-2. (Author´s own figure, modified from ref[38])

Tumour vasculature differs from the normal capillary bed in that tumour vessels are winding, and chaotic in their organization, and leaky and highly permeable in their structure (Figure 3). This induces intra-tumoral hyperosmosis, deficient perfusion, and hypoxia and causes diffusion of growth factors and cancer cells, which further stimulate tumour progression in a vicious cycle [41]. Judah Folkman coined the term "anti-angiogenesis" in the early 1970´s[42]. He proposed that treatment of cancer could be effected by targeted inhibition of the angiogenesis pathways. A decade later this led to the discovery of the pro-angiogenic Fibroblast Growth Factors (FGF acid and FGF basic) and the human Vascular Endothelial Growth Factors (VEGF-A, -B, -C, -D)[36]. VEGF-A (here denoted VEGF) is the most important mediator of physiological and tumour angiogenesis, promoting endothelial cell growth, proliferation and migration leading to the formation and invasion of new blood vessels[36, 43]. VEGF also enhances the permeability of vessels. Conversely, VEGF-C and VEGF-D are involved in lymphangiogenesis. These molecules are ligands to the tyrosine kinase VEGF-receptors (VEGFR-1, VEGFR-2 and VEGFR-3)[43](Figure 4). VEGF-A binds with a higher affinity to VEGFR-1 than to VEGFR-2, but the resulting signalling transduced by the VEGF-2 receptor is stronger and more important to the VEGF mediated effects on tumour angiogenesis[36]. VEGF-B binds only to VEGFR-1 and VEGF- C and D bind to VEGFR-2 and VEGFR-3. VEGF receptors are mainly located on the surface of

endothelial cells as well as on cells of hematopoietic origin, but can also be found on tumour cells[43, 44].

Apart from physiological angiogenesis, VEGF is produced mainly by tumour cells and has been found highly expressed in many cancers, including colorectal cancer[36, 44]. The stromal cells are also a source of VEGF and other angiogenic factors[37]. Binding of VEGF to VEGFR-2 results in the receptors to bind together in pairs, so called dimerization, followed by a tyrosine kinase phosphorylation process that activates the downstream intracellular signalling pathways. Consequently, angiogenic activation of the cell occurs, as well as up-regulation and secretion of proteins that degrade the extracellular matrix allowing for cell migration[37, 43].

It was initially demonstrated that inhibition of angiogenesis causes a diminished number of microvessels, so called capillary rarefaction in both tumour and normal tissue[45]. It was suggested that this effect would literally starve the tumour to death, although it was later demonstrated by the work of Jain and colleagues that there is also an increased blood flow in the tumour as a consequence of the remodelling of dysfunctional vessels of the tumour bed[46]. Vascular normalisation is believed to be of crucial importance to the effect of anti-angiogenic treatment (Figure 3). The net result is a more normal yet sparse vascular support of the tumour tissue. As a consequence, there is reduced shedding of tumour cells into the circulation, and enhanced supply of oxygen as well as cytotoxic compounds to the tumour.

A

B

C

Normal vasculature

Abnormal tumour vessels

Normalised tumour vasculature
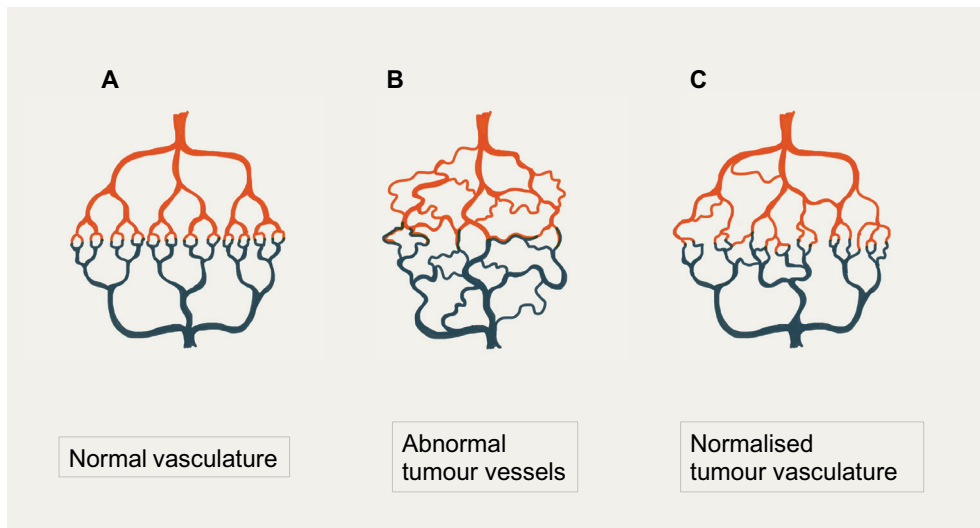
Figure 3.
The vascular normalisation process. In the normal vasculature there is a balance between pro and anti-angiogenic factors resulting in an organized network of vessels (A). In tumours, excessive amounts of pro-angiogenic factors cause a dysfunctional network of microvessels (B). During VEGF inhibition, the balance is restored and a more normal arrangement of vessels is seen (C). (Author´s own figure, adapted from ref.[41])

Bevacizumab

Along with the increased understanding of the main regulatory pathways of tumour angiogenesis, the discovery and development of angiogenesis targeted medical agents began. Bevacizumab (Avastin®) was the first angiogenesis targeted agent to be approved by the United States Food and Drug Administration for treatment of solid tumour disease. It is a recombinant humanised monoclonal antibody that binds to VEGF-A and prevents the ligand from interaction with the VEGFR-1 and VEGFR-2 receptors (Figure 4)[47].

Approval of the drug for first line treatment of mCRC in 2004 was based on the results from the phase III placebo controlled randomised clinical trial by Hurwitz *et al.* that resulted in an mOS increase from 15.6 months to 20.3 months (HR 0.66, p<0.001) in favour of the addition of bevacizumab to bolus 5-FU and irinotecan[24]. At the time of the initiation of the trial, the study backbone regimen (IFL) had been introduced as the standard first line doublet in the United States[23], but IFL was later substituted to the 5-FU-infusional doublet chemotherapy regimen (FOLFIRI)[48]. The survival gain of bevacizumab addition from the pivotal trial was confirmed in a Chinese trial[49] also using IFL as backbone but has not been replicated in later randomised trials exploring the effect of bevacizumab addition to doublet first line chemotherapy in the treatment of mCRC[50-52].

Addition of Bevacizumab to oxaliplatin-5FU/capecitabine combination regimens resulted in a small progression-free survival (PFS) gain but did not increase OS significantly in first line[51]. In second line[53], and in studies investigating bevacizumab continuation beyond progression after first line bevacizumab plus chemotherapy[54, 55], only a modest 1-2 months of mOS gain has been demonstrated with bevacizumab addition. The additional effect of bevacizumab to triplet chemotherapy (FOLFOXIRI) has not been evaluated in randomised trials, although the combination is feasible and effective as first line option in selected patients with mCRC[13]. Nonetheless, bevacizumab in combination with 5-FU or capecitabine prolongs PFS but not OS compared to single chemotherapy. This is an option to patients who are not expected to tolerate doublet chemotherapy up front, as demonstrated in trials of older patients with mCRC[56, 57]. Many reports demonstrate increased tumour response rates by approximately 10% with the addition of bevacizumab to chemotherapy. Maintenance treatment and treatment with bevacizumab as single drug, aiming to maintain tumour response in mCRC, will be covered in the next chapter and in the discussion of this thesis.

Bevacizumab is effective in addition to chemotherapy also in other cancer forms, and is currently approved for palliative treatment in ovarian cancer, fallopian tube or primary peritoneal cancer and cervical cancer, in renal cell carcinoma, glioblastoma, and in non-small cell lung cancer[58]. The most common side effects of bevacizumab are hypertension and proteinuria, which are rarely perceivable to

the patient unless the hypertension needs medical treatment. Patients also frequently report a mild epistaxis (nosebleed). Other more serious but rare adverse events include gastrointestinal perforation, venous and arterial thromboembolism, fistulation, mucosal bleeding and wound healing complications[58].

Aflibercept and Ramucirumab

In second line treatment of mCRC, there is a lack of evidence for a survival gain of bevacizumab introduction to irinotecan-based combinations. However, two other anti-angiogenic targeted drugs are proven effective in this setting: Aflibercept and Ramucirumab. Ziv-aflibercept (Zaltrap ®) is a recombinant protein fusion between part of the human immunoglobulin-G1 molecule and the extracellular VEGF-binding domains of the VEGFR 1 and 2. This protein forms a VEGF-trap preventing binding of the pro-angiogenic ligands VEGF-A, VEGF-B and placental growth factor (PlGF) to their receptors. Ramucirumab (Cyramza®) is a VEGFR-2 targeted human monoclonal antibody that binds to the VEGFR-2 extracellular domain and inhibits the activation of the receptor pathways by its ligands[59].

In second line treatment of mCRC aflibercept has been shown to prolong OS with 1.4 months (Hazard Ratio, HR 0.82) compared to placebo in combination with FOLFIRI[60]. This is approximately the same magnitude of benefit as reported with switch of antibody to ramucirumab after progression to an oxaliplatin doublet regimen plus bevacizumab (1.6 months, HR 0.84)[59], or as seen with continuation of bevacizumab compared to no anti-angiogenic agent continuation in the second line TML trial (1.4 months, HR 0.81)[54]. Ramucirumab is also effective in second line treatment in advanced gastric cancer, but in conclusion, there is at present no obvious superior challenger to bevacizumab in the second line anti-angiogenic treatment options of mCRC[61].

Regorafenib

Multi-tyrosine-kinase inhibitors (TKIs) are small molecule drugs obstructing multiple of the intracellular signalling pathways implicated in tumour angiogenesis and oncogenesis. TKIs are oral treatment options proven effective in many solid cancer forms, *e.g.* urothelial cancer and gastrointestinal stroma cell tumour (GIST), although none of the available agents have yet come to play a significant role in the advancements of medical treatment of mCRC. The approval of the TKI regorafenib (Stivarga®) for mCRC in 2012 was based on a placebo controlled trial, which showed an mOS gain of 1.4 months (HR 0.77) in a heavily pre-treated international patient population with refractory disease, also exposed to bevacizumab in former lines of treatment[62]. The results were confirmed with a to some degree better result in an Asian population also refractory to former treatment lines but less frequently exposed to bevacizumab[62].

As a result of the subjective toxicity of regorafenib, the drug is for many practising oncologists and their patients not an evident treatment option in a very late line setting. Instead, in the late continuum of care, optimisation of symptoms and quality of life, often have higher priority than a potential minor gain in survival.

Targeting the epidermal growth factor receptor

In parallel with the genuine anti-angiogenic agents, other targeted agents were developed that aim at inhibiting the pathways modulating tumour cell growth and apoptosis, differentiation and proliferation, as well as the angiogenic and metastatic potential of cancer. One key target molecule is the epidermal growth factor receptor (EGFR, ErbB-1), which belongs to a family of four related receptors (ErbB-1 to 4), including the Her-2/neu receptor (target for the inhibiting antibody trastuzumab)[63].

EGFR is overexpressed and has aberrant activity in many cancer forms including CRC[64]. The ErbB receptors dimerises upon ligand binding resulting in phosphorylation of the tyrosine kinase intracellular domain and activation of the intracellular signalling cascade of protein kinases including the RAS-RAF-MEK-ERK/MAPK, JAK-STAT and PI3K-AKT-mTOR pathways (Figure 4)[63]. In malignant cells, mutations are frequent in the genes coding for these proteins and deregulation of the signals can endow tumours with the hallmarks of cancer, including sustained proliferation, evasion of growth suppression and apoptosis, as well as activation of adhesive and invasive functions promoting metastasis[35, 65]. Known ligands of the EGFR receptor are *e.g.* EGF, transforming growth factor alpha (TGF-α), amphiregulin, epiregulin and heparin binding EGF-like growth factor (HB-EGF)[63]. EGFR also plays a role in tumour angiogenesis[64].
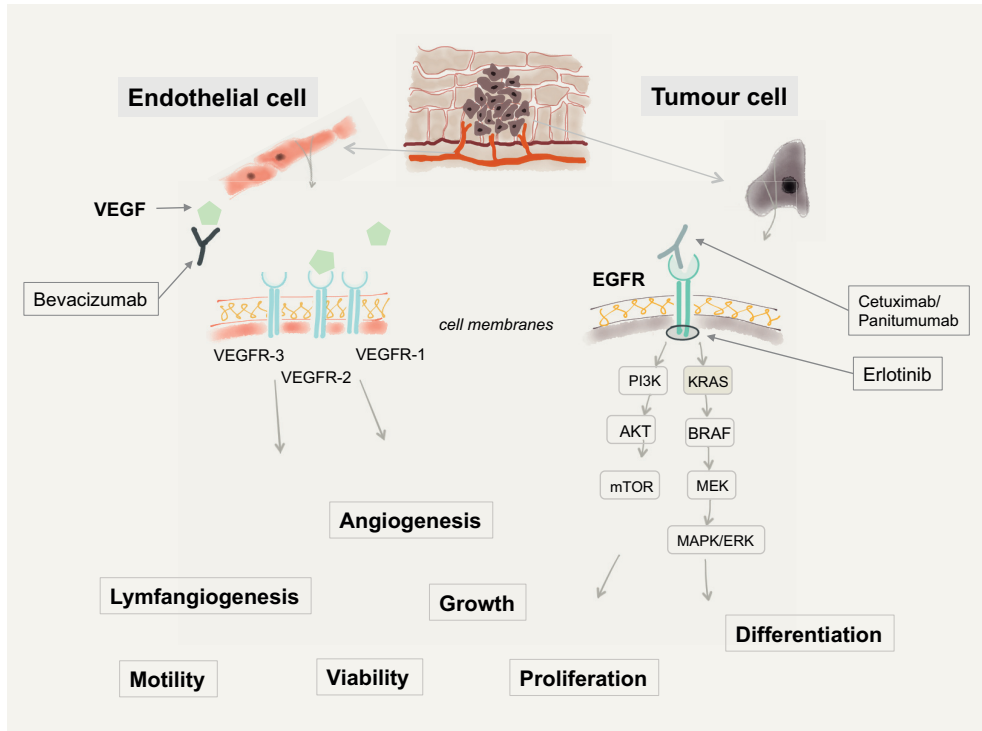
Figure 4.
Target molecules and intracellular pathways involved in cancer growth. The targeted agents (bevacizumab, cetuximab/panitumumab and erlotinib) inhibit different parts of the signalling pathways. VEGF(R), vascular endothelial growth factor (receptor); EGFR, epithelial growth factor receptor. (Author´s own figure)

Cetuximab and Panitumumab

The first EGFR inhibitor to be introduced for treatment of mCRC was cetuximab (Erbitux®), a chimeric monoclonal antibody that inhibits the activating dimerization of the EGFR by binding to its surface (Figure 4). The agent was initially approved in 2004 for treatment of EGFR positive CRC tumours after progression on irinotecan-based therapy. This was based on the BOND trial demonstrating an improved median (m)PFS with cetuximab plus irinotecan versus cetuximab as single agent[66]. A significant gain in mOS with preserved quality of life was later reported in favour of single cetuximab (6.1 months) compared to best supportive care (4.6 months) from the CO.17 trial in the third line setting of mCRC. The survival gain for cetuximab was almost doubled in the patients with tumours that harboured no mutation in the gene coding for the Kirsten-rat sarcoma virus oncogene protein (KRAS)[67]. First line treatment with cetuximab plus FOLFIRI in the CRYSTAL trial confirmed the value of KRAS mutation status as a predictive biomarker of the EGFR-inhibiting antibody effect[68]. For cetuximab in

second line the EPIC trial reported a better PFS and response, but with exception of the CO.17 trial, no studies have demonstrated any gain in OS with addition of anti-EGFR antibody in a KRAS unselected population[69].

The combination of cetuximab to oxliplatin-based regimens has been debated due to the negative results from the first line OPUS, MRC COIN and Nordic VII studies, however this could also in the latter two trials be attributed to suggested elusive negative interaction mechanisms of cetuximab with capecitabine or bolus 5-FU[69, 70]. In recent years, partly given the preliminary results of the CALGB/SWOG 80405 study[71] that demonstrated equivalent outcomes in terms of survival between FOLFOX/FOLFIRI in combination with either bevacizumab or cetuximab in chemotherapy-naïve mCRC patients, the acceptance for FOLFOX plus an EGFR-inhibiting antibody as first line treatment has widened[63].

The humanised monoclonal anti-EGFR antibody panitumumab (Vectibix®) was approved in 2006, initially as single agent treatment in third line[72]. Panitumumab was later investigated in first line combination with FOLFOX in the PRIME study and in second line with FOLFIRI. Effects were seen in terms of PFS and response rates but no significant OS gains were demonstrated in the non-RAS selected mCRC population [69].

In randomised clinical trials investigating cetuximab or panitumumab as single agents or in combination chemotherapy in mCRC, the antibody activity in terms of absolute gain in response rate (complete or partial tumour response) is around 10% in the RAS unselected populations[69]. Following the collected results from clinical trials, the two antibodies are considered equal in terms of effect in mCRC. An advantage with panitumumab is the lower risk of allergic toxic reactions to the drug, owing to its fully humanized molecular structure. This also accounts for possible differences in the mechanism of action, as cetuximab is observed to elicit tumour cell apoptosis also by antibody-dependent immune-cell-mediated cytotoxicity[63]. Nowadays the use of the anti-EGFR antibodies is restricted only for patients with tumours not harbouring any of the specific mutations in the RAS genes, owing to the predictive biomarker studies which will be discussed further below.

Cetuximab is also an approved agent for advanced head neck cancer[73]. Common side effects for EGFR inhibiting agents are dry skin, and nail changes such as paronychia and acneiform skin rash unfolding mainly in the central face- scalp and thoracic areas. Both antibodies can also cause hypomagnesaemia and sometimes associated disturbances of electrolyte balance as well as diarrhoea.

Erlotinib

Erlotinib hydrochloride (Tarceva®) is an oral small molecule TKI drug targeting the intracellular domain of the EGFR (Figure 4). It competes with the ATP

binding site of the kinase preventing the crucial ATP-dependent phosphorylation step of the EGFR dimer that precedes activation of the downstream signalling cascade. Erlotinib has never been approved for use outside of clinical trials in CRC. An early phase II trial evaluated erlotinib in 38 mCRC patients formerly exposed to a maximum of two standard chemotherapeutic agents for advanced disease, and showed that 39% of the evaluable patients had stable disease for in median four months[74]. Although none had objective (*i.e.* partial or complete) response the effect and safety profile of erlotinib was considered enough promising to perform phase II trials combining erlotinib with capecitabine or cetuximab[75, 76]. However, no phase III studies have been completed, due to the toxic effects of combining erlotinib with chemotherapy, as well as disappointing results from conference reports and parallel trials combining the EGFR-TKI gefitinib (Iressa®) plus chemotherapy[63, 77].

The main toxic events include the typical EGFR-inhibitor associated skin rash, diarrhoea and fatigue. Erlotinib is approved for non-small cellular lung cancer (NSCLC) harbouring EGFR mutations (which will be discussed in a following chapter), and was shown to produce a median of less than two weeks gain in survival in combination with gemcitabine for pancreatic cancer[77]. Based on these results, erlotinib was approved for pancreatic cancer, but is generally not recommended to use as a result of the lack of a clinically relevant benefit.

Combined VEGF/EGFR inhibition

The pharmacological blocking of specific targets in the cellular signalling pathways can be bypassed through primary or acquired resistance to the biological agent. To overcome these escape mechanisms, a combination of targeted drugs appears to be an attractive approach. There is a strong background rationale in the literature to support simultaneous inhibition of VEGF and EGFR. This combination of targeted biological agents has been investigated in several preclinical and clinical trials in many solid tumours. Others have extensively reviewed the early encouraging data in two publications from 2007-2008[77, 78].

Results from clinical trials with single targeted agents as described above, have proven added value to some but not all patients. Theoretically, as understood in the early era of chemotherapy trials, combination in different regimen protocols might lead to better success also for biological drugs. Furthermore, there are links between targetable signalling pathways, *e.g.* parts of the EGFR signalling pathway are shared by VEGFRs[78]. VEGF expression in tumour and associated endothelial cells is stimulated upon EGFR activation. Also, inhibition of EGFR only partly blocks the angiogenic process, and it is suggested that VEGF overexpression can

promote resistance to EGFR inhibitors[79]. Thus, acquired resistance of EGFR inhibition could presumably be overcome by dual inhibition of VEGF.

The phase II BOND2 trial investigated cetuximab plus bevacizumab with or without irinotecan in late line mCRC and showed a response rate of 20% in the non-chemotherapy group. Furthermore, the addition of bevacizumab to the irinotecan-cetuximab combination seemed superior in cross-trial comparison with the first BOND study[78]. Despite this, results from the ensuing trials combining a VEGF-antibody with EGFR-antibody have been disappointing.

In the phase III PACCE trial doublet chemotherapy plus bevacizumab with or without panitumumab was compared in first line treatment of mCRC[80]. The results from the planned interim analysis showed a lack of additional benefit and increased toxicity in the experimental panitumumab-arm, why the study was discontinued with the conclusion not to recommend this combination strategy. The results from the CAIRO2 trial confirmed the inferior outcomes of the antibody combination and could also demonstrate inferior quality of life in the patient group randomised to first line cetuximab in combination with oxaliplatin, capecitabine and bevacizumab[81]. Finally, a third study comparing bevacizumab-5-FU with addition of oxaliplatin (FOLFOX) or cetuximab in first line, established evidence that the antibody combination should be avoided in mCRC[82]. Accordingly, the aforementioned CALGB/SWOG 80405 trial had in its initial design in 2004 a third arm combining doublet chemotherapy with bevacizumab and cetuximab, which was later deleted in a first amendment[71].

Why do the antibodies bevacizumab and cetuximab or panitumumab not have synergistic effects in combination with chemotherapy in the clinical setting? One explanation could be pharmacodynamical interactions between the antibodies, but the mechanisms are not fully understood[80]. More frequent dose reductions due to higher grades of adverse events in the combination arms are not likely to be the cause[83].

On the other hand, the evidence in preclinical studies for an additive effect of bevacizumab and the oral EGFR-inhibiting TKI agent erlotinib was more solid than combination of antibodies targeting VEGF and EGFR[77, 83]. Accordingly, this fuelled the clinical research to combine these agents in phase II trials of renal cancer, squamous cell carcinoma of the head and neck, breast cancer, hepatocellular cancer, carcinomas of unknown origin and in lung cancer[77, 78, 84]. The results were promising along with manageable toxicity. A small phase II trial was performed to investigate the combination of bevacizumab and erlotinib in combination with chemotherapy (FOLFOX) as first line treatment in mCRC [85]. Seventy-seven percent of patients withdrew from study due to side effects and no patient was followed until documented progression, why PFS was not reported.

The grade 3/4 toxicity rate was 86% and the partial response rate of 34% was not better than expected with chemotherapy alone.

In parallel with the non-randomised trials mentioned above, our Nordic ACT study was launched with design and results as presented and discussed in the first paper of this thesis.

Palliative systemic treatment strategies in mCRC

Fluorouracil compounds remains the backbone of medical palliative treatment for mCRC, and is often prescribed in a so-called doublet regimen in combination with oxaliplatin or irinotecan as described above. Addition of targeted antibody agents is optional from first line through second to third line for selected patients. Triplet combination with FOLFOXIRI can be offered to selected patients in good performance status when maximum tumour regression is the primary goal. Conversely single treatment with fluoropyrimidine +/- bevacizumab is a relevant option in frail or older patients, and in cases with limited tumour burden where less aggressive treatment is preferred[9].

Although questions remain to be answered about the best combination of drugs, currently the focus and debate in this research area is also about the optimal intensity and sequencing of these anti-tumoral regimens. Another important concern is to find associated biomarkers to established treatments, which will be covered in the following chapters.

Chemotherapy pause

During a course of chemotherapy treatment for malignant disease, there is often accumulation of toxic effects, which in clinical trials are described under the term adverse events. Consequently, the more effective therapies, the longer the patient must withstand the negative impact of treatment toxicity. Under these circumstances, it is evident that many patients need to pause treatment at some time point. Chemotherapy free intervals are sometimes essential to avoid deterioration of quality of life or even harmful, chronic and potentially lethal effects of a still effective cancer therapy.

Is continuous chemotherapy better than intermittent treatment pause?
The effect of a planned full treatment pause was initially investigated in the extended MRC CR06 trial, presented in 2003 (N=354) [86]. This study showed that after three months of induction with single 5-FU or ralitrexed there was no clear

benefit of continuous treatment compared to a treatment stop until disease progression.

The GERCOR group presented in the first OPTIMOX trials the so-called "stop and go concept" in treatment of mCRC. First the OPTIMOX-1 trial (N=620) demonstrated that de-escalation, *i.e.* intermittent omission of oxaliplatin from the FOLFOX treatment schedule, was safe compared to continuous doublet chemotherapy[87]. The OPTIMOX-2[88] and MRC COIN[89] trials further explored the effect on full treatment breaks, both after three months of first line induction doublet chemotherapy. The OPTIMOX-2 trial (N=202) used a modified FOLFOX7 as induction, and compared de-escalation maintenance 5-FU (as in OPTIMOX-1) with complete treatment pause. At evidence of progressive disease FOLFOX7 was reintroduced for three more months. The larger MRC COIN trial (N=1630) used oxaliplatin plus 5-FU or capecitabine as induction and randomised between continuation of doublet induction regimen or complete treatment pause, until progression. Given the designs and endpoints of these studies, the investigators could not exclude a detrimental effect on survival by introducing early treatment pause followed by re-introduction of the first line regimen at evidence of progressive disease.

Finally, the GISCAD trial (N=337) compared continuous FOLFIRI with 2 months on and 2 months off treatment intermittently, *i.e.* stop-and-go of all drugs, until progression (Figure 5)[90]. No statistically significant drop in efficacy was seen in the intermittent arm compared to continued treatment with the induction regimen.

In conclusion, taking into consideration cumulative treatment-related toxicity, continuous doublet chemotherapy is not convincingly better than intermittent treatment in mCRC. Thus, a treatment pause can be recommended after response to therapy in selected patients who are exposed to oxaliplatin in the first line setting, as an option to non-stop continuous treatment until progressive disease or unacceptable toxicity[9].

Maintenance treatment

Oxaliplatin and irinotecan have equal potential to provide treatment gain in first and second line by doublet combination with 5-FU[91]. Regardless of the choice of first line regimen, one of the most important issues to address in treatment decisions with mCRC patients is how to fit in as many of the drugs as possible throughout the continuum of care[92]. Accumulation of toxicity during chemotherapy is an essential drawback of many regimens, best managed with a pro-active approach. Here, the strategic concept of so-called maintenance treatment is a suggested option to consider as an alternative to treatment break.

The purpose of maintenance therapy is to stabilize disease burden by maintaining the anti-tumoral response achieved by the initial treatment. This strategy also aims to reduce toxicity and optimise symptoms that could induce deterioration of quality of life. Preferably, the change to maintenance phase should precede the occurrence of unacceptable toxicity. By definition, in most clinical trials the maintenance treatment is thus a pre-planned change in treatment schedule after a definite period of first line treatment, which is referred to as the induction phase. A maintenance therapy regimen comprises either only a de-escalation of induction treatment intensity, or introduction of a new therapeutic compound, also referred to as "switch maintenance" (Figure 5).
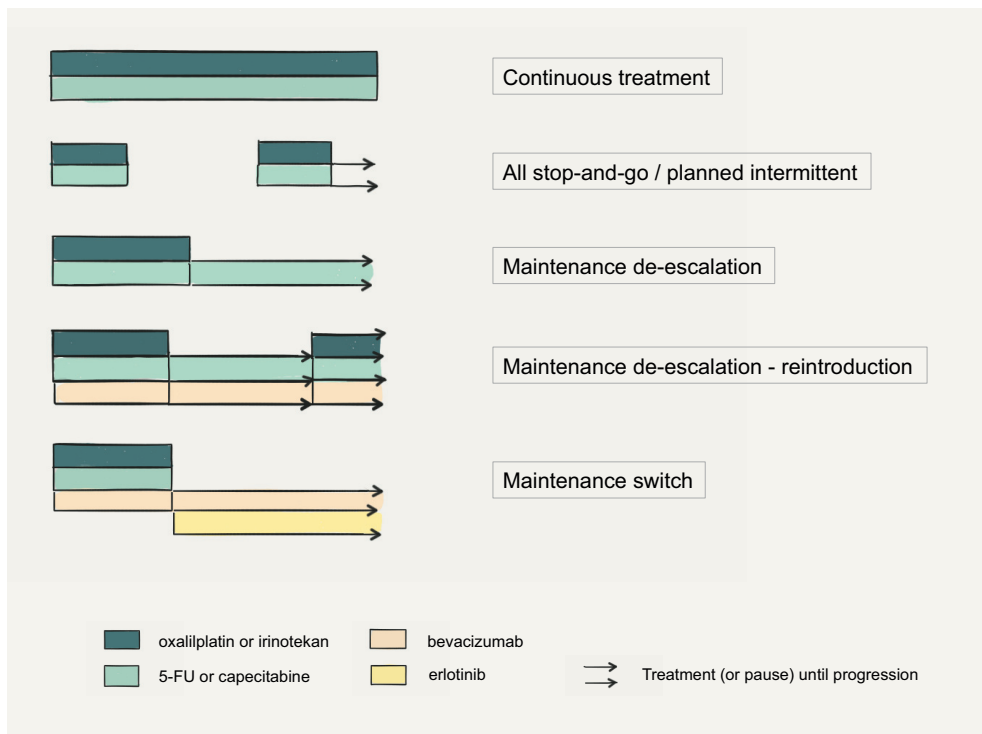
Figure 5.
Treatment concepts in advanced colorectal cancer. The stop-and-go concept is here illustrated as omission of all drugs, with defined shorter length of treatment and pause. Induction - maintenance – reintroduction of the same regimen as used in induction is another first-line strategy, here illustrated with addition of the targeted agent bevacizumab. Switch maintenance treatment is here illustrated by the combined targeted drugs bevacizumab and erlotinib, as presented in this thesis (paper I and II). (Author´s own figure)

Is continuous doublet chemotherapy better than intermittent de-escalation/maintenance?

In the OPTIMOX-1 trial the impact of varying oxaliplatin dose intensity was studied by use of 5-FU as maintenance treatment for a maximum of six months[87]. The continuous FOLFOX4 as standard arm was compared with the more oxaliplatin intense FOLFOX7 schedule for 3 months followed by de-escalation to 5-FU for six months or until progression, followed by reintroduction of FOLFOX7 for three months (Figure 5). From the results of this study the conclusion was drawn that oxaliplatin can safely be omitted during a defined period of time in first line treatment of mCRC. The benefit is decreased toxic effects of oxaliplatin, particularly with concerns of the dose-dependent peripheral neurotoxicity.

With the introduction of bevacizumab in first line regimens, the efficacy of bevacizumab based maintenance de-escalation has been explored. Two randomised trials investigating maintenance strategies have used 5-FU or capecitabine with continuous bevacizumab as intermittent de-escalation/maintenance strategy after induction with bevacizumab plus either FOLFOX or XELOX[93, 94]. A shared conclusion from these trials was that intermittent maintenance with bevacizumab and a fluoropyrimidine was superior to continuation with bevacizumab plus doublet chemotherapy including oxaliplatin until progression.

Maintenance treatment with bevacizumab alone until progression or unacceptable toxicity has been compared to continuous doublet chemotherapy (XELOX) plus bevacizumab as standard arm in the randomised non-inferiority phase III MACRO TTD trial[95]. This trial found no significant differences in survival endpoints between the arms, even if non-inferiority of bevacizumab was not statistically proven.

Thus, according to results from the early maintenance trials in mCRC, patients do not benefit more from continuous doublet chemotherapy than from de-escalation to maintenance treatment with 5-FU or capecitabine with or without bevacizumab. In the final chapter of this thesis I will discuss the findings from more recent trials comparing different maintenance strategies to treatment pause.

Is there a role for switch maintenance?

Previously mentioned clinical trials explored the combination of two targeted antibody agents with chemotherapy, which was obviously very toxic[71, 80-82]. Other studies were designed to investigate the sequencing of chemotherapy and targeted drugs in a maintenance protocol. Similarly, our Nordic ACT trials (paper I and II) investigated the combination of bevacizumab and erlotinib in a first-line maintenance setting. In one phase II trial, XELOBER, the investigators switched to erlotinib plus bevacizumab combination as maintenance treatment in 52 patients

after response to 18 weeks of first line treatment with bevacizumab-XELOX[96]. Munoz *et al.* reported the preliminary results at the 2010 American Society of Clinical Oncology (ASCO) annual meeting (abstract # 3539), *i.e.* after the launch of our first ACT study. Final results showed a median PFS of 11.1 [95% CI: 9.0-15.7] months, and a median OS of 29.5 [95% CI: 23.7-36.7] months, with an acceptable toxicity profile.

The first phase III trial to show prolonged PFS with the addition of erlotinib versus bevacizumab alone as maintenance was performed in advanced NSCLC after first line induction with chemotherapy plus bevacizumab. Preliminary results from the ATLAS trial[97] were presented at the 2009 European Cancer Organisation (ECCO) congress and suggested KRAS mutation as a biomarker for the erlotinib effect (see also the following chapter regarding predictive biomarkers). This formed the background for the design of our ACT2 trial, which is presented in the second paper of this thesis.

Metronomic chemotherapy

At the time of initiation of the Nordic ACT and Nordic ACT2 studies (paper I and II) in 2006 and 2009 respectively, the effect of bevacizumab alone as maintenance in mCRC was not known. Another maximal de-escalation therapy studied in paper II, is so called metronomic low dose capecitabine. By standard, chemotherapy is prescribed in the maximum tolerated dose as pulse-treatment, in cycles. A common example is the cyclic dosing of intravenously administered drugs for two consecutive days which is repeated every second week, *e.g.* as in the FLV or FOLFOX/FOLFIRI schedules. The treatment is toxic to the cancer cells, but also for rapidly proliferating normal cells. This causes the general toxicity of chemotherapy such as hair loss, oral and intestinal mucositis and bone marrow suppression. The days of treatment free interval in each cycle allows for recovery of these symptoms.

The term metronomic chemotherapy, introduced by Hanahan *et al.* in 2000[98], denotes a treatment schedule of daily continuous dosing without planned break between cycles. Instead of using the maximum tolerated dose aiming at a maximised anti-tumoral cytotoxic treatment effect on each treatment cycle, the metronomic treatment is comprised of lower chemotherapy doses in a continuous, daily, more rhythmic frequency of administration. Figure 6 shows the suggested basic principles for these mechanisms of action. Early studies from *in vitro* and *in vivo* cancer models have shown that many chemotherapeutic compounds can induce anti-angiogenic effects in low metronomic doses[99, 100]. Notably, the tumour associated vascular endothelial cells have a more stable genetic phenotype than cancer cells, and are more sensitive to low doses of cytotoxic agents. Metronomic

dosing of chemotherapy was also shown to inhibit tumour growth and indirectly vasculogenesis by decreased mobilisation of endothelial progenitor cells [101]. Secondly, metronomic chemotherapy can cause up regulation of anti-angiogenic factors such as Thrombospondin-1[102], as well as reduction of pro-angiogenic VEGF and Platelet derived growth factor (PDGF)-B in humans[103]. Based on these results, there are reasons to believe that metronomic treatment may shut off the angiogenic switch (see Figure 2), and prevent it from being turned on in the dormant cancerous cell mass.

It is believed that the metronomic treatment effect does not only include angiogenesis inhibition, but can also involve a direct cytotoxic action on the cancer cells. Moreover, studies on mice and humans have shown that the immune system can be triggered by metronomic low doses of chemotherapy in such a way that the tumour cells are more easily recognised and destroyed by the cytotoxic T-cells and natural killer cells of the immune response[104]. Together, these biologic properties of metronomic therapies are thought to result in a dormancy state of the malignant cell mass. Intriguingly, metronomic cancer therapy has also been shown to induce new responsiveness by re-introduction /re- challenge of a drug to cancer that was formerly shown to be resistant to the specific agent. Thus, the daily dose of the chemotherapeutic compound can be kept very low and still have a cytostatic effect.
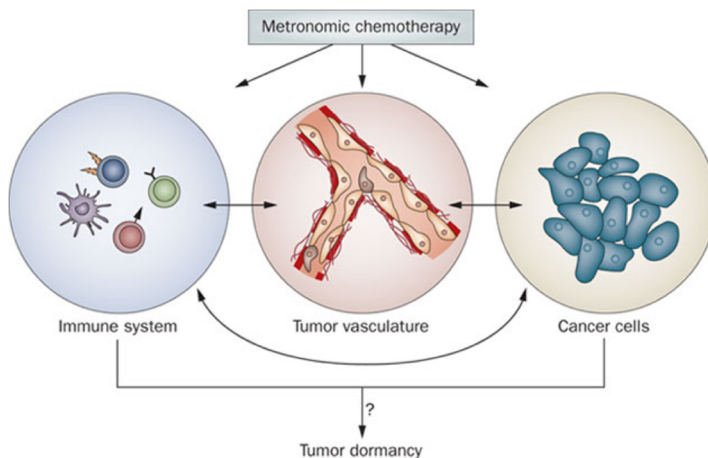
Figure 6.
Metronomic chemotherapy as multi-targeted therapy. By inhibition of angiogenesis and stimulation of anticancer immune response metronomic chemotherapy affects the complex interactions of the cancer and its microenvironement, which may lead to additional anticancer effects and potential (re)induction of tumour dormancy[104]. Adapted by permission from Macmillan Publishers Ltd: Nature Reviews Clinical Oncology. Pasquier, E *et al.* Metronomic chemotherapy: new rationale for new directions, ©2010 Nature Publishing Group.

The standard dosing for capecitabine is to administer the maximum tolerated dose twice daily for 14 consecutive days, followed by seven days pause, based on the above mentioned phase II study[27]. In that trial, a metronomic arm was not convincingly inferior to the later preferred standard arm. Therefore, this metronomic dosing of capecitabine was later re-used in the CAIRO3 trial as maintenance in combination with bevacizumab for mCRC[105]. Lower doses of metronomic capecitabine have been investigated in smaller cohorts and case reports of gastrointestinal cancers, sometimes also in combination with bevacizumab[106-110]. Given the heterogeneity of the studied cancers, patients and settings, as well as the different schedules used, there is no standardised model to determine the most optimal dose level or combination of metronomic oral anti-cancer compounds. Safety concerns are important in the administration of a chronic dose of a cytotoxic compound in the clinical setting. A fixed capecitabine schedule using oral intake of 500 mg twice or three times daily (regardless of body surface area) is practical and feasible.

In a review on the future of metronomic chemotherapy from 2001 Gasparini proposed a change in the therapeutic anti-cancer paradigm, from aiming at cancer shrinkage and eradication to cancer cytostatic control[100]. He suggested metronomic scheduling to be investigated as maintenance treatment to optimise patient survival. In line with this, we decided to explore two "maximum de-escalation" maintenance strategies in a subgroup of the ACT2 trial, by comparing bevacizumab single to very low dose metronomic capecitabine as an alternative, potentially anti-angiogenic approach.

Biomarkers of predictive importance in mCRC

Treatment decisions in oncology are based on clinical and pathological data, including biomarkers. The collected information serves as important tools in the practise of so called personalised medicine. The term biomarker can be described as a sign that is objectively measured and evaluated as an indicator of either a normal or pathological biological process, or of responses to a therapeutic intervention[111]. The biomarker measurement should also be reproducible.

In medicine, diagnostic biomarkers are used to confirm the manifestation of a disease, and spans from basic signs such as fever or abnormal blood pressure to more complex laboratory tests. Prognostic biomarkers carry information about the outcome of a diagnosis, exemplified by *e.g.* cancer stage, a blood test or a specific molecular analysis of pathology specimens. A predictive biomarker can be helpful to predict the effect of a specific intervention to treat or cure the disease. The

informed biomarker guided selection to which patients are most likely to gain from treatment is increasingly important.

For many targeted therapies, such as bevacizumab, there are still no validated molecular predictive biomarkers despite the well-defined target of the drug. A core focus of the research project covered in this thesis was to explore potential predictive biomarkers of interest to targeted therapy in mCRC.

Biomarkers of EGFR inhibition

KRAS/NRAS

The most important component for selection of which biologic targeted drug to use in mCRC is analysis of the RAS mutational status of the tumour. RAS genes are frequently mutated in CRC and the most common mutations (~40% of cases) occur in the KRAS gene[112]. RAS proteins effectuates the intracellular signals downstream the EGFR. Consequently, specific somatic mutations in the RAS genes result in modified proteins leading to constant activation of the important pathways regulating tumour cell growth and proliferation, such as the MAPK pathway (Figure 4). It is now well established that the mutation driven downstream pathway signalling is not blocked by antibody-inhibition at the extracellular domain of the EGFR.

Initially, retrospective analyses of several randomised clinical studies investigating the effect of cetuximab and panitumumab in mCRC, demonstrated that KRAS mutations in codons 12 and 13 of exon 2 of the gene were associated with lacking efficacy of these antibodies. This led to the implementation in 2008 of KRAS status as the first predictive molecular biomarker in personalised treatment of mCRC[67]. However, not all tumours that lack the specific exon 2 KRAS mutations respond to anti-EGFR antibody therapy. Retrospective analyses of the PRIME study showed that patients with KRAS mutation (mut) in not only exon 2, but also in exons 3 or 4, or mutations in the NRAS gene had worse outcome with the addition of panitumumab than patients who did not receive anti-EGFR inhibitor[113]. An absolute gain of 5.8 months in OS was seen with addition of panitumumab to FOLFOX in the non-mutated group. The observations were supported by retrospective analyses of the OPUS[114] and CRYSTAL[115] studies that investigated the addition of cetuximab to doublet chemotherapy.

A systematic meta-analysis and review of nine randomised trials have corroborated the findings[112]. Mutations of KRAS in exon 2 (codons 12/13), and in exons 3 and 4 (codons 59/61 and 117/146 respectively) and mutations in specific oncogenic codons of NRAS in exon 2 (codon 12/13), exon 3 (codon 59/61) and exon 4 (codon 117) are currently established as negative predictive markers for the

effect of anti-EGFR antibodies in CRC[9]. Any of these RAS mutations occur in approximately half of the patients with mCRC. Tumours not harbouring any of these mutations are denoted RAS-wild-type (wt). Thus, only patients with RASwt mCRC are eligible for treatment with cetuximab or panitumumab.

As previously mentioned, the phase III ATLAS trial investigated the additional benefit of erlotinib to bevacizumab maintenance treatment after response to first line chemotherapy plus bevacizumab in NSCLC, and as such this study was very similar with our first Nordic ACT trial in mCRC (paper I). Although preliminary data from ATLAS suggested a negative predictive value of KRAS mutation for the erlotinib effect[97], the final results of the biomarker analysis could not confirm the initial findings[116].

EGFR

The first clinical studies of cetuximab in mCRC were performed in patients with tumours expressing EGFR as determined by immunohistochemistry[66, 68]. This biomarker was selected as an inclusion criteria based on the assumption that expression of EGFR was important for the benefit of anti-EGFR antibodies. However, this belief was later abandoned after retrospective analyses indicating that this biomarker was poorly associated with response to EGFR inhibiting agents[117]. Nonetheless, 60-80% of colorectal cancers are EGFR positive, and high EGFR expression implicates worse prognosis, but it is not used in clinical practise[79].

Mutations in the EGFR gene are seen in around 15% of NSCLC, but are extremely rare in CRC[116, 118]. In NSCLC, it is well established that the presence of an EGFR mutation in the tumour, increase the sensitivity to anti-EGFR tyrosine kinase inhibitors and EGFR mutation is used as an obligate predictive biomarker for selection of patients to erlotinib treatment in NSCLC.

BRAF

The EGFR downstream signalling pathways involve many proteins coded by proto-oncogenes, *i.e.* genes that have the potential to cause cancer. B-Raf proto-oncogene, serine/threonine kinase (BRAF) codes for the BRAF protein involved in the RAS/mitogen activated protein kinase (MAPK) signalling pathway. The most frequent activating mutation of the BRAF gene in CRC (V600E) is found in approximately 10% of CRC cases[112]. KRAS, NRAS and BRAF mutations are mutually exclusive in CRC, thus BRAF mutations occur almost only in RAS wild type CRC tumours[117]. BRAF mutation is an independent negative prognostic factor in mCRC[119]. The predictive role of BRAF status on EGFR inhibition therapy in mCRC has been a matter of debate, as reflected in two meta-analyses from 2015[120, 121]. Currently there is no clear evidence to generally exclude patients with BRAF mutated mCRC from treatment with cetuximab or panitumumab.

Skin toxicity

EGFR inhibitors can cause dermatological toxicity, typically an acneiform skin rash, which has been shown to associate with a better response to the treatment in many solid tumours[122]. This seems to be a class-effect of the EGFR inhibitors including erlotinib in lung cancer, and cetuximab and panitumumab in mCRC[79, 117, 122]. In a meta-analysis of mCRC trials, the authors concluded that moderate to severe grades of skin toxicity yielded an increased chance of tumour response to EGFR targeted antibodies[123]. Moreover, the presence of any grade of acneiform rash, occurring in more than half of the patients, was confirmed to be a predictor of better survival. However, the predictive value of this biomarker has not been evaluated in prospective trials and the possible prognostic impact of this side-effect remains to be clarified.

Primary tumour location

In recent years, a more basic biomarker has received considerable attention, namely the location of the primary tumour location in the large bowel[124]. It is well known that cancers arising from the right side of the colon have different clinical characteristics than left sided tumours including rectal cancers[125]. Right-sided colon cancers differ also in molecular characteristics from its counterparts, which is thought to be a result from the distinct embryological origin with right colon deriving from the embryonic mid gut and left colon deriving from the hindgut. The right-sided colon cancers, in clinical trials usually defined as carcinomas arising on the proximal side of the splenic colonic flexure, have a worse prognosis than left sided, *i.e.* distal colorectal cancers[124].

Proximal cancers are more common in women, and more often show mucinous histology, and more advanced stage at diagnosis. Tumours in the right colon are more often microsatellite instable (MSI positive), *i.e.* they are predisposed to DNA miss-match repair deficiency (dMMR) and abundant mutations, as well as hypermethylation through the CpG island methylator phenotype (CIMP) pathway. Mutations in BRAF, and PIK3CA genes are more common in proximal tumours, which also show a higher rate of MAPK activity than distal CRC. In contrast, distal carcinomas have a higher rate of chromosomal instability (CIN) and microsatellite stability (MSS), and the BRAF/KRAS wild type distal tumours are associated with EGFR pathway activation and Her2 expression[125].

This knowledge has now translated into results from retrospective analysis of clinical trials demonstrating the predictive value of primary tumour site regarding efficacy of EGFR-targeted drugs. Left sided tumours seem to be more sensitive to EGFR-inhibiting antibodies than right-sided tumours[124]. The molecular biology of the colon is very complex and great effort has been made to classify different subtypes of colorectal carcinomas in terms of gene expression patterns. Four different groups of consensus molecular subtypes (CMS1-4), have been proposed

by the CRC Subtyping Consortium[126]. The sidedness of the colon tumour is most likely a surrogate marker for the molecular properties.

Molecular biomarkers of angiogenesis inhibition

In view of the more widespread use of anti-VEGF(R) inhibiting agents, the quest for predictive biomarkers to angiogenesis targeted agents is essential as an attempt to augment the clinical benefit of these drugs. Many different methods are described to have promising implications for predicting outcome to anti-angiogenic inhibition in cancer treatment[127-130]. Despite extensive work, no clinically useful treatment predictive biomarker to angiogenesis inhibition has yet been validated and established. A full review of this subject is beyond the scope of this introduction and has been covered by others also with special focus on bevacizumab[131-134]. Here I will outline some important findings.

Molecular biomarkers in the tumour tissue

Biomarkers with binary expression on a molecular level, such as mutational status of RAS (mut/wt), or expression of a protein in the tumour tissue, such as Her2(+/-), are examples of convenient molecular biomarkers to predict outcome of targeted cancer drugs. The tissue-based biomarkers can be analysed at baseline before treatment start, on tumour tissue biopsies sampled from diagnostic evaluation or from surgical removal of the cancer. For prediction of response to the anti-angiogenic agent bevacizumab, potential biomarkers have been explored by investigation of the expression of angiogenic factors in tumour tissue including cancer cells and stromal cells[131]. In summary, from reviews of angiogenesis inhibition in CRC, the expression of VEGF or other angiogenesis-related proteins in CRC tumour tissue have not shown to be predictive of the outcome of bevacizumab based treatment[135-137].

In addition to expression of specific protein products, other tumour-associated molecules have been studied, such as MicroRNA (miRNA) expression. MiRNA are small RNA fragments that are not themselves being translated into a protein, but regulate the gene expression process, so called non-coding RNA. Specific miRNA molecules can regulate the expression of VEGF and other proteins of importance for tumorigenesis and angiogenesis[137]. Although distinct miRNA molecules, such as miRNA-126, have been suggested as prognostic markers or predictors for the bevacizumab effect in mCRC, the results are not valid for clinical decision making[138].

Molecular biomarkers in the circulation

Circulating biomarkers are also possible to measure at baseline, *i.e.* before treatment start. Potentially useful blood-borne baseline biomarkers are related to factors regulated by the DNA of the host, which vary by germline polymorphisms. The DNA polymorphisms may affect gene transcription of angiogenesis related proteins in *e.g.* the genetically stable endothelial cells. These pharmacogenomic biomarkers cause inter-individual differences with potential implications for the response to anti-angiogenic therapy. The single nucleotide polymorphisms (SNPs) are variations of single nucleotides that occur normally throughout the germline DNA of an individual and can play functional role if present within genes. Accordingly, SNPs in genes regulating core proteins of the VEGF-pathway have been investigated in mCRC patients[139, 140]. Some results on pharmacogenomic biomarkers in the angiogenesis pathway genes have been promising, but have yielded no validated predictive biomarker for the effect of bevacizumab treatment[131, 132, 135].

Additionally, sequential blood tests are non-invasive and more accessible than repeated tissue biopsies in a clinical setting. This allows for on-treatment assessment of dynamic patterns in biomarkers associated with modulation of a specific drug target, so called pharmacodynamic biomarkers[127]. Several studies have focused on angiogenic factors in serum and plasma. Ideally, VEGF levels in plasma would associate with the effect of VEGF-inhibition by bevacizumab, but reports of this hypothesis are conflicting[135]. Baseline VEGF is elevated in many tumours and has not proven to be a stable predictive biomarker for anti-angiogenic treatment. Circulating VEGF is shown to both decrease and increase in response to VEGF inhibition, most likely due to a lack of standardised assay methodology, reflecting both free and antibody bound-VEGF. Increase in VEGF during bevacizumab treatment has been proposed as a tumour associated escape mechanism associated with resistance to the drug[127]. However, this hypothesis has been rejected through results showing that VEGF accumulate by the blocking of endocytic clearance of the protein- antibody complex in the endothelial cells[141]. This may explain why a bevacizumab-induced change in VEGF levels is not a reliable predictive pharmacodynamic biomarker.

Many trials have investigated the treatment predictive role of changes in circulating angiogenesis related proteins apart from VEGF. Results from the literature are disparate and studies are heterogenic in terms of investigated proteins, tumour types and treatments. For bevacizumab treated CRC, there are reports indicating treatment predictive signatures of circulating factors including *e.g.* soluble VEGFR-2, Angiopoietin-2, Thrombospondin-2, Insulin Growth Factor-1 (IGF-1)[142, 143], basic fibroblast growth factor (bFGF), Hepatocyte Growth Factor (HGF), Placental Growth factor (PlGF), Stromal Derived factor-1,

macrophage chemoattractant protein-3[144, 145], Interleukin (IL)-8, IL-10, Il-6, and EGF[146].

In addition to protein levels, circulating cell elements have been investigated for a predictive potential given their potential association to resistance mechanisms of bevacizumab and other anti angiogenic drugs. Circulating endothelial cells, circulating endothelial progenitor cells and circulating tumour cells are studied as prognostic markers and predictive markers of therapy in mCRC[135]. As methodological technical advancements are made it remains to be elucidated whether these circulating markers, or cell free DNA/RNA reflecting the presence of tumour cells, have a role in prediction of outcome to targeted therapy treatment[137].

In view of the complexity of the angiogenesis pathways and mechanisms of resistance to the angiogenesis inhibiting agents, it is unlikely that the dynamic change in one or a few soluble proteins will reflect and predict treatment outcome. However, by measuring multiple angiogenesis related factors simultaneously, sequential patterns of protein levels could reveal dynamic protein signatures associated with response and/or resistance to the anti-angiogenesis inhibition. The development of multiplex protein arrays has increased the potential to investigate multiple proteins simultaneously from a small volume of plasma or serum, with promising applications in research and in the clinical setting[147].

This background inspired us in the design of the second translational study presented in paper IV of this thesis.

Non-molecular biomarkers of angiogenesis inhibition

Imaging

The response to therapy of solid tumours is standardised by the Response Evaluation Criteria in Solid Tumours (RECIST)[148]. RECIST is based on serial tumour size measurements performed by use of CT or MRI scans. However, efficacy of anti-angiogenic agents does not necessarily lead to tumour shrinkage, why RECIST evaluation of the response to chemotherapy and bevacizumab treatment can be questioned. Functional imaging techniques are proposed to better measure the vascular changes induced by VEGF inhibition, most importantly the reduction of vascular permeability. The dynamic contrast enhanced MRI (DCE-MRI) with its parameters measuring capillary permeability has been recognised as a novel method to assess response to bevacizumab, but is currently not used as a routine imaging biomarker in the clinic[136].

Hypertension

An abnormal raise in blood pressure defined as hypertension, is a toxic class effect of many angiogenesis inhibiting agents, including bevacizumab[149]. Hypertension is a pharmacodynamic biomarker that is shown to be associated with improved anti-angiogenic drug effects in different tumour settings[150-155]. Any grade of hypertension occurs in around 20-50% of patients treated with angiogenesis inhibitors[156, 157]. It should be noted that these event rates could vary depending on the frequency and method of blood pressure measurement[158], which classification system is used to determine the grade[159, 160], and according to tumour type[157]. In clinical trials, moderate to severe hypertension grades are reported in 10-20% of bevacizumab treated patients[149, 161, 162]. In case of high-grade hypertension (≥160/100 mmHg) it is recommended that bevacizumab be halted (see Table 1). Anti-hypertensive medication should be initiated or titrated to maintain blood pressure levels below 140/90 mmHg[156]. The time of onset of hypertension varies but is often seen before the first tumour evaluation within 3 months of treatment with bevacizumab[154, 161].

In mCRC, retrospective studies have reported significant correlations between bevacizumab induced hypertension and efficacy endpoints in terms of response rate and PFS [153, 154, 161, 163]. In a study of 101 consecutive mCRC patients from a Finish centre, any grade of hypertension was associated with better response and survival in comparison with no hypertension on bevacizumab treatment (mOS 25.8 *vs.* 11.7 months, p<0.001)[154]. In line with results from other trials[151, 158], the authors showed that, early hypertension within three months of treatment start, was an independent predictor of survival with HR 0.53 [95% CI 0.34-0.84, p= 0.007] for OS. A meta-analysis by Cai *et al.* investigating over 500 mCRC patients from seven trials, showed that bevacizumab associated hypertension was significantly associated with PFS, OS and response[164].

However, other studies have shown conflicting results[165, 166]. Hurwitz reported a large meta-analysis of seven phase III placebo controlled trials investigating bevacizumab plus chemotherapy in close to 6500 patients with different tumour types including metastatic CRC, breast cancer, NSCLC, pancreas and renal cancer. The endpoint hypertension in this trial was defined as an increase in systolic blood pressure >20mmHg or diastolic blood pressure >10 mmHg during the first 60 days of bevacizumab treatment. In six of the seven studies, early hypertension was not predictive of clinical benefit from bevacizumab.

Several biological mechanisms have been described to clarify the process of anti-angiogenic drug induced hypertension. Angiogenesis and the microcirculation are involved in the pathogenesis of hypertension[167]. Interestingly, there is a close association between VEGF inhibition toxicity and pre-eclampsia, a disease of late pregnancy where hypertension, proteinuria and oedema are main symptoms. This

condition is associated with systemic endothelial dysfunction and high circulating levels of soluble VEGFR-1, which is thought to trap VEGF and inhibit VEGFR signalling similar to bevacizumab[37]. Moreover, the condition is linked with deficient production of Nitric Oxide (NO), a potent vasodilator. In consistence with the fact that VEGF is known to induce NO production through activation of endothelial NO synthase (eNOS) via the VEGFR-2 receptor, bevacizumab has been shown to decrease NO levels *in vitro*. Additionally, the decrease in NO is believed to cause sodium retention in the kidneys, causing a rise in blood pressure. It has also been proposed that dysregulation of Endothelin-1, a potent vasoconstrictor, is implicated[168].

Furthermore, preclinical evidence and investigations in humans support morphological changes in the vascular bed to occur as a response to VEGF-inhibition[45]. It is not clear whether the observed rarefaction of vessels is contributing to the raised blood pressure or if hypertension is causing reduced density of microvessels[168]. Hence, the mechanisms by which hypertension could be linked to a better response to anti-angiogenic drugs is not fully elucidated.

Vasoactive peptides

It is suggested that the vascular normalisation process is of major importance for the synergistic action of chemotherapy and anti-VEGF treatment[41], and given the potential predictive role of hypertension, it is possible that host-related cardiovascular processes may be involved. However, hypertension *per se* does not seem to be a useful biomarker in the clinic and blood pressure measurements are difficult to standardise in a reliable manner. It is also possible, that the rise in blood pressure induced by angiogenesis inhibition is not always depicted in grading of the adverse event. Instead, other factors measurable in the circulation, such as vasoactive peptides, could be superior in attempt to mirror the vascular host-related biological effects that separate the responding patients from the non-responders. Determining factors of vascular normalisation could serve as pharmacodynamic biomarkers to predict the bevacizumab effect, as suggested by Jain *et al.*[127]. This context formed a rationale to explore vasoactive peptides as a potentially novel class of predictive biomarker of the bevacizumab effect, as presented in the third study of this thesis (paper III).

Vasoactive peptides are biologically active peptides with the ability to influence vascular smooth muscle and consequently regulate blood flow and blood pressure. Many of these peptides have additional functions apart from their cardiovascular effects, and some are hormones that are secreted from glands in the body[169]. There are some clinically useful examples of vasoactive peptides that function as drug targets (*e.g.* Angiotensin I-II), therapeutic analogues (*e.g.* Oxytocin,

Somatostatine) or as biomarkers for disease such B-type (brain) natriuretic peptide (BNP) levels in cardiac insufficiency[170]. Other examples are Endothelin-1, Parathyroid Hormone, Bradykinin, Vasopressin, Adrenomedullin and Atrial Natriuretic Peptide[169].

Co-workers in our group have previously explored the precursor fragments of Vasopressin, Adrenomedullin and Atrial Natriuretic Peptide as cancer risk associated biomarkers, based on their link to angiogenesis[171]. A short introduction to the three vasoactive peptides studied in paper III is given as followed.

Copeptin

Vasopressin, also known as Antidiuretic hormone (ADH), is a peptide hormone with many physiological functions including regulation of blood pressure and electrolyte balance of the blood by stimulating the kidneys to retain water in the body. It is produced in the hypothalamus, transported to the pituitary gland and released into the circulation where it acts as a potent vasoconstrictor with an ability to increase blood pressure[172]. Consequently, it is involved in the physiological endocrine response to cardiac arrest and shock. Vasopressin is unstable in plasma and detection methods are technically difficult to use in a clinical setting. Therefore, the analysis of a surrogate marker, Copeptin, has been established[173]. Vasopressin derives from the precursor protein prepro-vasopressin (Figure 10). The C-terminal part of the Vasopressin precursor constitutes the vasoactive peptide denoted Copeptin. High Copeptin levels have in a few publications been associated with hypertension and microalbuminuria[174, 175], which are both side effects of bevacizumab. However, it is mostly investigated as a biomarker for acute myocardial infarction and other conditions associated with cardiovascular distress[172].

MR-proAdrenomedullin

Adrenomedullin (ADM) is a peptide with strong vasodilatory activity. It is derived from the larger precursor molecule preproADM, in a process during which other smaller peptides are generated. One of these smaller fragments of preproADM, the mid-regional (MR) part, is denoted MR-proADM (Figure 10)[176]. The measurement of ADM in the circulation is unreliable due to short half-life. Conversely, the MR-proADM peptide is more stable in human plasma and the levels of MR-proADM is considered to reflect the amount of released ADM. ADM is produced and secreted from a wide variety of tissues including glands, kidneys and blood vessels, by stimulus of different cytokines and hormones and in response to *e.g.* inflammation and hypoxia[177]. It is highly expressed in endothelial cells. The MR-proADM peptide has no intrinsic function, whereas mature ADM has multiple biological effects apart from the ability to dilate vessels. MR-pre-proADM has been investigated as a disease mechanistic biomarker of syncope[178], and was found

associated with albuminuria and high pulse pressure in hypertensive patients, why the release of ADM is thought to serve as a protective mechanism to hypertension-related organ damage[179, 180].

MR-pro-ANP

Atrial Natriuretic Peptide (ANP) is the most abundant natriuretic peptide in the circulation under normal conditions. ANP and BNP promote natriuresis (excretion of sodium in the urine by the kidneys), diuresis and vasodilatation. ANP derives from myocytes in the atrium of the heart and is released to regulate blood pressure and blood volume, in response to atrial stretch[170]. *In vitro* studies have demonstrated that ANP have a complex role in vasculogenesis and angiogenesis, and may act both as stimuli for the regeneration and permeability of endothelial cells, but also as inhibitor of the angiogenic effect of VEGF[170]. The N-terminal portion of the prohormone of ANP (proANP) has a longer half-life than mature ANP, and the mid regional portion MR-proANP is therefore suggested to be a more reliable analyte for measurement in plasma. (Figure 10)[181]. MR-proANP is associated with arterial stiffness and high blood pressure and has been suggested as a biomarker of syncope, heart failure, and hypertension.[178, 182].

Aims of the thesis

The aims of this thesis were:

- To investigate the effect and safety of maintenance therapy with bevacizumab plus/minus erlotinib after response to first line induction treatment with chemotherapy and bevacizumab in mCRC (Paper I).

- To determine whether the addition of erlotinib to bevacizumab is superior to bevacizumab alone as maintenance therapy in KRAS wildtype mCRC, and to explore the effect of low dose metronomic capecitabine as maintenance treatment in patients with KRAS mutant tumours (Paper II).

- To examine circulating vasoactive peptides as potential biomarkers for prediction of response to bevacizumab containing treatment in mCRC (Paper III).

- To study the feasibility of a multiplex protein array method to explore patterns of circulating angiogenesis related proteins during chemotherapy and bevacizumab treatment, and to investigate their possible association with the effect of bevacizumab as maintenance (Paper IV).

Patients

Paper I & paper II

ACT stands for "Avastin and Chemotherapy followed by Avastin alone or in combination with Tarceva as maintenance Treatment of metastatic colorectal cancer".

Patients included in the first Nordic ACT trial (**paper I**) were recruited from nine oncological centers in Sweden (Umeå, Sundsvall, Uppsala, Stockholm, Jönköping, Kalmar, Växjö, Lund, Malmö) and six centers in Denmark (Vejle, Herning, Roskilde, Hilleröd, Esbjerg, Odense).

In the second trial, the Nordic ACT2 (**paper II**), patents were recruited from eleven centers in Sweden (Umeå, Sundsvall, Uppsala, Stockholm, Västerås, Karlstad, Linköping, Jönköping, Kalmar, Växjö, Malmö/Lund) and one unit in Denmark (Odense).

All patients included in ACT and ACT2 were at least 18 years of age with Eastern Cooperative Oncology Group (ECOG) performance status 0-1 and histologic proof of adenocarcinoma of the colon or rectum. In the ACT2 trial, availability of tumour tissue for determination of KRAS mutational status was added to the inclusion criteria. Patients were eligible if they were recommended to start first-line fluoropyrimidine-based combination chemotherapy for colorectal cancer. Thus, all patients started treatment with primarily palliative intent, without previous history of chemotherapy for metastatic disease. Prior adjuvant chemotherapy for CRC was allowed if ended more than six months before study treatment start. All patients had measurable disease according to RECIST v.1.0, and no major deterioration of haematological, renal or hepatic function at inclusion, as specified in the study protocols. Patients with clinically significant and active cardiovascular disease, uncontrolled hypertension or active full dose anticoagulant treatment for thromboembolism were excluded. The full list of eligibility criteria is given in Appendix 1.

The ACT trial included patients from May 2007 to November 2009 whereas the recruitment period for the ACT2 trial was between October 2010 and May 2012.

Paper III & paper IV

The translational studies included patients who were treated in the ACT2 trial as two independent retrospective analyses. These patients were not selected according to KRAS status, thus both KRASwt and mut patients were included in paper III and IV. All patients in the translational trials had signed separate informed consent to participate in the biomarker analysis. In both biomarker studies all patients had ended treatment due to evidence of progressive disease according to RECIST 1.0. Hence, in paper III and IV, patients were excluded if they had stopped treatment in the ACT2 trial for other reasons, *e.g.* due to toxicity, withdrawn consent, death, or surgery.

The biomarker study presented in **paper III**, included patients who had started treatment in ACT2 and who stopped treatment due to progression which could occur *either* within 18 weeks from treatment start with chemotherapy plus bevacizumab in the induction period *or* after randomisation during maintenance treatment within either of the study arms, including bevacizumab single, bevacizumab plus erlotinib or metronomic capecitabine. We applied strict inclusion criteria regarding availability of plasma samples for analysis at both defined time points in the study, *i.e.* at baseline and at approximately six weeks from induction treatment start.

In the second translational study presented in **paper IV**, we only included patients who had proceeded with treatment after randomisation in the ACT2 trial. In this cohort, all patients had responded to induction treatment with chemotherapy and bevacizumab and had been randomised to treatment with bevacizumab alone as maintenance therapy and stopped treatment due progression. Also, the availability of serum samples from three defined time points, *i.e.* at baseline, at start of maintenance treatment and at end of treatment, was obligate for inclusion.

Methods

"I am not an optimist. I´m a very serious possibilist. It´s a new category where we take emotion apart and we just work analytically with the world."

- Hans Rosling

Clinical trial designs and methodology

Randomised Clinical Trials

In the present era of evidenced-based medicine, any new therapy should first be tested through clinical trial phases before replacing the standard treatment. The clinical trial methodology in medicine was first initiated after the Second World War with the first randomised clinical therapy trial in tuberculosis[183]. Early trial phases make sure the treatment is safe to use in humans (phase I) and evaluate doses with regards to toxicity and early efficacy (phase II). Phase III trials are performed to test if the new treatment is better than the standard treatment strategy. The randomised controlled clinical trial (RCT) assigns human participants to random allocation between interventions; with the purpose of statistically compare the effects on given outcomes. Outcome measures, endpoints, regarding *e.g.* safety and survival are pre-defined with the intention to demonstrate or rule out a realistic and clinically relevant improvement by the new treatment. The goals of this method are to eliminate systematic error (bias) and to minimise random error to increase precision and generalizability of the results.

The ACT and ACT2 studies were prospective, multi-national, multicentre randomised controlled phase III trials, based on the designs and the size of the included cohorts. The trials were academically sponsored and coordinated from the study secretariat at the Clinical Research Unit of the Department of Oncology, Skåne University Hospital in Lund, Sweden. Both trials received economic and technical support from Roche through provision of the software program for electronic Case Report Form (SAS® Pheedit) and by partial sponsoring of the study drugs bevacizumab and erlotinib. A representative from Roche Sweden was

involved in the design process of the trials, but Roche had no influence on the collection, analysis or interpretation of the data and results. An independent Contract Research Organisation (CRO) was engaged for the ACT studies to aid with the quality control in data monitoring and data management of the trial.

The randomisation process ensures minimisation of biased results by avoidance of systematic differences in baseline characteristics. In ACT and ACT2 the randomisation was conducted by a central coordinated randomising service provided by the South Regional Cancer Centre (RCC), Lund, Sweden, which ensured allocation concealment. The block randomisation method was used for both trials. The stratification factors were identical in ACT and ACT2, and included treatment response in the induction phase (complete response/partial response *vs.* stable disease) according to RECIST, and use of oxaliplatin in induction treatment chemotherapy schedule (Yes/No). In ACT2 this translates into four strata groups (=2 strata x 2 groups) for each separate randomisation procedure of the KRASwt and KRASmut groups respectively. Four persons in each block, double block size, balanced the strata groups.

Good Clinical Practise and toxicity criteria

The methodology to conduct clinical trials have improved greatly during the last decades, with initiatives aiming to minimise distrust of the trial results, and to increase safety for the patients[183]. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) was founded in 1990 with a mission to ensure worldwide high quality harmonisation in the process of registration of new pharmaceutical compounds[184]. The ICH has developed efficacy guidelines to support the conduct of clinical trials, most importantly the ICH E6 guideline for Good Clinical Practise (ICH-GCP), which was launched in 1996.

ACT and ACT2 were conducted in accordance with the GCP principles. GCP guidelines ensure protection of the rights and well being of trial subjects and that collected and reported data are credible. The guidelines describe standardised principles regarding ethical aspects, investigator and sponsor responsibilities including *e.g.* safety reports, monitoring, data handling, documentation of clinical trial protocol, investigator´s brochure and other essential documents.

During the conduct of ACT and ACT2 we used the standardised definitions of adverse events (AEs) given in the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0, which is provided by the National Cancer Institute (NCI) of the National Institutes of Health (NIH), USA[185]. An AE is defined as any abnormal clinical finding associated with the use of therapy, and can be related to the study compound(s) or not. Toxicity is graded as mild (grade 1), moderate

(grade 2) severe (grade 3) or life threatening (grade 4), in relation to the specific symptom or organ system involved. Grade 5 denotes a fatal AE, *i.e.* death.

Response evaluation

RECIST 1.0 was used for evaluation of tumour response outcome[148]. The RECIST criteria define the anti-tumour efficacy into complete response (CR), partial response (PR), or by stabilisation of disease (SD). In case of no response to treatment the tumour growth cause progressive disease (PD). The sum of the longest diameter for all measurable defined cancer target lesions is calculated at baseline before treatment start. CR is defined as disappearance of all the target lesions, PR means at least 30% decrease in the sum of the target lesions with baseline as reference, PD is defined as at least 20% increase in the sum of the target lesions, taken as reference the smallest sum recorded since treatment start, *or* the appearance of at least one new lesion. RECIST defines stable disease as neither, sufficient shrinkage to qualify for PR, nor sufficient increase to be PD, also taking as reference the smallest sum since start of treatment.

Evaluations of response were performed with radiological assessments by CT or MRI scan of the thorax and abdomen at baseline and every nine weeks during treatment (Figure 9). The radiology scans in both trials were analysed by local radiologists at each participating site. The size of each target lesion and presence of non-target lesions and new lesions were also recorded in the electronic Case Report Form separately for each response-evaluation scan. There was no central radiologist review of the CT scans in either of the trials.

Data management and quality control

Protocol

The protocol document sets the standard for the conduct of the clinical trial and include sections specifying *e.g.* objectives/goals of the study, subject and drug information background, eligibility criteria, stratification factors, treatment schedules, treatment modification plan and safety issues, definitions of end of treatment and endpoints as well as statistical considerations. The ACT and ACT2 studies were registered in the European Medicines Agency´s European Clinical Trials Database (ACT EudraCT no: 2006-002295-18 and ACT2 EudraCTno: 2010-019815-40) as well as in the clinicaltrials.gov registry (ACT: NCT00598156 and ACT2: NCT01229813), which is electronically accessible to the public. According to the ACT2 protocol KRAS status was determined during the

induction treatment period, using a validated and approved assay on each study site, detecting mutations in KRAS exon 2 codon 12/13.

Data Entry and monitoring

We used electronic Case Report Forms (eCRF) for reporting of data at each site and both ACT and ACT2 used the same software program (SAS® Pheedit) for this purpose. Data monitor professionals were appointed by the sponsor in accordance with GCP to ensure that the studies were conducted and documented in compliance with the protocol and regulations.

End of treatment and Follow up

Patients were permitted to withdraw consent to the study participation at any time. The (co-)investigators had the right to withdraw a patient from the ACT or ACT2 trials in the event of disease progression, unacceptable adverse event, pregnancy, non-compliance, serious protocol violation, lost to follow up and study termination. Irrespectively, the date and reason for end of treatment (EOT) were described in the CRF. Patients ending study treatment for any reason without documented progressive disease were to be assessed by radiology every three months until documented PD or start of new anti-tumour treatment. Survival and additional cancer therapies were documented for all included patients every third months until time of death or study end. Follow up of AEs and laboratory abnormalities were specified in the protocol.

Data quality control

During the post-inclusion period, before data analysis, we used a similar approach for the data validation plans in ACT and ACT2. We performed quality control of all patient´s CRF records, by listing of patient record data, and used defined logical checks with regards to variables in the CRF to identify missing or inconsistent data. Re-evaluation of tumour response involved comparison of the given tumour lesions data in CRF with the stated response (CR/PR/SD/PD). Similarly, the eligibility criteria and follow up data were validated, and queries were issued to the respective sites in case of omissions or inaccuracies in the primarily reported dataset. A separate reconciliation of reporting of serious adverse events (SAE) was conducted. At the time of declaration of clean file, the data set was locked before statistical analysis work could begin.

The appointed members of the respective ACT and ACT2 study teams consisted of representatives of the Sponsor (Principal Investigator (PI), central study officer/coordinator and me), the data manager and the biostatistician. At the final classification meetings, each subject was reviewed case by case and classified into the pre-defined analysis populations: intention to treat (ITT), Full Analysis Set (FAS), per protocol (PP) and Safety Analyses Set.

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Design of the ACT trial (paper I)

Primary objective

- o To evaluate maintenance treatment with combined bevacizumab + erlotinib versus bevacizumab alone following response on first line chemotherapy plus bevacizumab, by comparing progression-free survival (PFS).

Secondary objectives

- o To evaluate safety

- o To evaluate efficacy in terms of overall response and overall survival.

- o To perform translational research (by Danish investigator collaboration).

For the ACT study (Figure 7), we used an all-comers (randomise all) design, *i.e.* there was no biomarker guidance in the randomisation process[183]. We used no blinding or placebo allocation. There was an early discussion of including a pause/no treatment arm, but final decision on the design was made based on information that other, larger studies were on-going where bevacizumab alone as maintenance would be compared with treatment pause after induction including bevacizumab[186, 187]. For induction treatment regimens see Supplementary material of Paper I.

Figure. 7
Nordic ACT trial(Paper I). The time-equivalent dose of bevacizumab in the induction phase was 2,5mg/kg i.v. *week. In the maintenance phase dosing of bevacizumab was 7,5mg/kg i.v. q3w, and of erlotinib 150mg p.o. once daily. PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response. R, randomisation.

Design of the ACT2 trial (paper II)

Primary objective

- o To evaluate maintenance treatment with bevacizumab + erlotinib versus bevacizumab alone in patients with KRAS wildtype metastatic colorectal cancer, following response on first line chemotherapy plus bevacizumab, by comparing PFS rate at three months from start of maintenance therapy.

Secondary objectives

- o To explore the activity and toxicity of low dose metronomic capecitabine in KRAS mutant patients

- o To evaluate the efficacy of the KRASwt arms and KRAS mutant arms respectively in terms of PFS and OS.
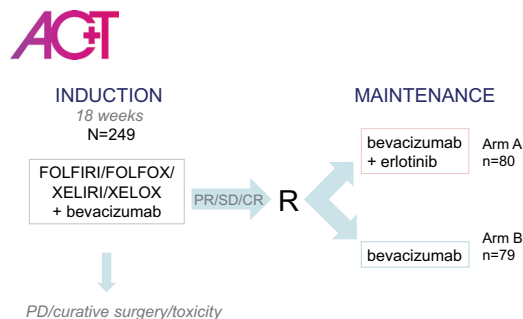
- o To perform translational research
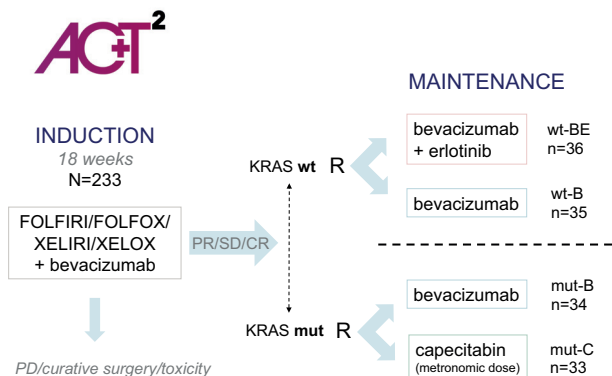
Figure. 8
Nordic ACT2 trial(Paper II). The time-equivalent dose of bevacizumab in the induction phase was 2,5mg/kg i.v. *week. In the maintenance phase dosing of bevacizumab was 7,5mg/kg i.v. q3w, of erlotinib 150mg p.o. once daily and of capecitabine 500mg p.o. bi daily. PD, progressive disease; PR, partial response; SD, stable disease; CR, complete response. R, randomisation.

The ACT2 study (Figure 8) was based on a modified enrichment (targeted) design[188], including KRAS-guided randomisation. Thus, only patients fulfilling the assumption of biomarker predictability (in this case KRAS wildtype) were randomised to receive the study drug schedules to be compared in the primary objectives of the study (bevacizumab versus bevacizumab plus erlotinib). Instead of excluding the KRAS mutant patients from further analysis we extended the

objectives by expansion of the design. This explorative sub-study of the KRAS mutant cohort included a randomised comparison of bevacizumab and metronomic capecitabine as maintenance treatment.

Translational study designs and assay methods

The timing of collection of blood samples and clinical data for the translational analyses are illustrated in Figure 9.
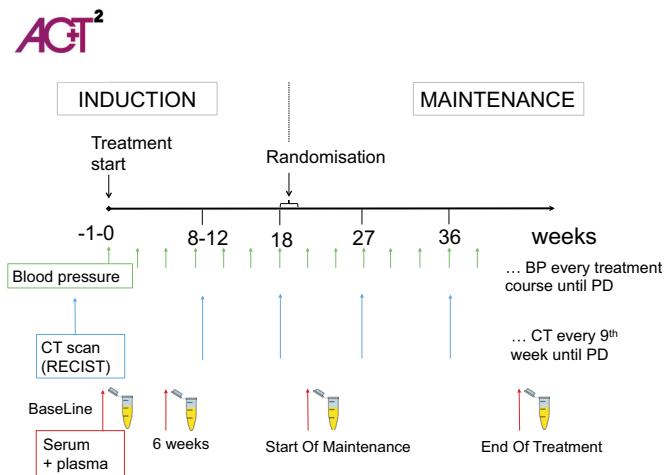
Figure 9.
Data collection for translational studies of the ACT2 trial (paper III and IV). Blood was collected at baseline (sample A) and at approximately 6 weeks from start of treatment (sample B) in paper III, and at baseline (BL), start of maintenance (SOM) and at end of treatment due to progressive disease (EOT) in paper IV. BP, blood pressure was registered before each treatment course (each 2nd or 3rd week); CT, computed tomography of thorax and abdomen.

Objectives of the vasoactive peptides study (paper III)

Primary objective
  o To explore the association between levels of three vasoactive peptides (MR-proADM, MR-proANP and Copeptin) and treatment effect in terms of best objective response during induction phase, and in terms of time to progression (TTP) on first line induction chemotherapy plus bevacizumab followed by maintenance treatment for mCRC.

 o To investigate the association between early hypertension during induction treatment with chemotherapy plus bevacizumab and clinical outcome in terms of TTP during maintenance therapy.

 o To explore the association between early dynamic levels of the three vasoactive peptides and grade of hypertension at approximately six weeks from start of first line induction treatment with chemotherapy plus bevacizumab for mCRC.

Immunoassay of vasoactive peptides

Translational blood samples were frozen on site as specified in paper III, and sent to the sponsor site where plasma samples from two time points were collected from eligible patients; at baseline (sample A) and at approximately 6 weeks (sample B) from start of induction treatment (at time of treatment cycle 2 or 3 depending on cycle length) (Figure 9). The plasma samples underwent two freeze-thaw cycles before analysis due to one alliquoting procedure at the sponsor site before transport to the laboratory.

The peptide analytes were measured using a fully automated immune analyser for serum or plasma, the KRYPTOR instrument, which is manufactured by Thermo Fisher B·R·A·H·M·S Biomarkers. The KRYPTOR instrument uses the TRACE technology, or time-resolved amplified cryptate emission[189]. This method is not dependent on time-consuming washing and separation steps to eliminate background noise from unspecific signals in the biological sample (here plasma). Instead, TRACE isolates the signal of interest by energy transfer from antibody bound europium cryptate to a fluorophore molecule when both are bound to the analyte (peptide) by formation of an immune complex. This lengthens the light emission of the fluorophore molecules that are attached to the peptide detection antibody (Figure 10). The immune complex is isolated by measurement of the intensity of the longer emission signal wavelength, which corresponds to the concentration of the analyte.
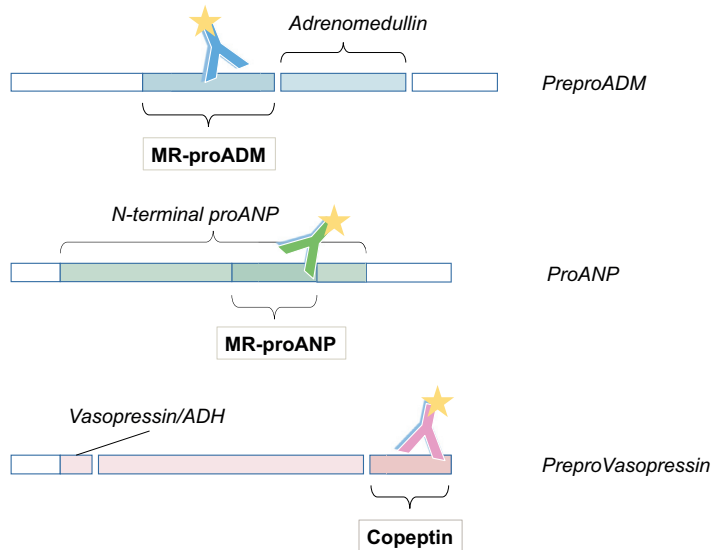
Figure 10.
Vasoactive peptides and their respective pro-molecules. The detection antibodies trace and bind to the respective aminoacid sequence of the analyte, *i.e.* the vasoactive precursor peptide. The yellow stars illustrate the luminescent fluorophore molecules bound to the detection antibodies of the immunoassay. (Author´s own figure, modified from refs[176, 181, 190])

Hypertension grade

The grade of Hypertension was determined by retrospective evaluation using blood pressure measurements recorded at each treatment cycle. Very early hypertension is shown to better predict response to bevacizumab plus chemotherapy than hypertension occurring at any time during the treatment period[158]. Therefore, we measured early changes in vasoactive peptides levels and used the grade of hypertension at six weeks from treatment start in our analysis. This grade was determined by including the sequential blood pressure levels from baseline before treatment start (cycle 1 of induction treatment) until the second or third treatment cycle depending on the chemotherapy schedule (cycle interval of three weeks as XELOX/XELIRI, or two weeks as FOLFOX/FOLFIRI, respectively). The CTCAE version 4.0 (Table 1) was chosen based on reports that CTCAE 3.0 had a risk of underestimating hypertension grade, and as a consequence were recommended for use in trials investigating anti-angiogenic agents and hypertension[160, 191]. Hypertension diagnosis and use of anti-hypertensive drugs at baseline were recorded in the CRF of the ACT2 trial, and additional information regarding any raise in dosing of baseline medication or prescription of any new drug was used for grading.

Table 1.

Hypertension grade according to CTCAE 4.0 Definition according to CTCAE 4.0: A disorder characterized by a pathological increase in blood pressure; a repeatedly elevation in the blood pressure exceeding 140 over 90 mm Hg. Either of the criteria in each column defines the respective grade. BP, blood pressure; WNL, within normal limit. Grade 5 = death is excluded from table.

Hypertension (adults)	GRADE			
	1	2	3	4
	Pre-hypertension	Stage 1 hypertension	Stage 2 hypertension	Life threatening
Systolic BP	120-139	140-159	≥ 160	
or Diastolic BP	80-89	90-99	≥100	
		Medical intervention indicated, monotherapy	Medical intervention indicated, more than one drug	Urgent intervention indicated
		Recurrent or persistent ≥ 24 hrs		Consequences, e.g. malignant hypertension, transient or permanent neurologic deficit, hypertensive crisis
		Symptomatic increase by > 20 mmHg (diastolic), or to >140/90 if previously WNL		

Objectives of the protein array study (paper IV)

Primary objective
   o   To investigate the feasibility and utility of the Proteome profiler Human Angiogenesis Array to study angiogenesis related factors in serum collected from mCRC patients enrolled in the clinical ACT2 trial.

Secondary objectives
   o   To explore relations between angiogenesis linked proteins and clinical outcome in terms of TTP, to search for dynamic patterns of biomarkers with potential to predict the effect of bevacizumab as maintenance treatment.

   o   To explore circulating proteins potentially involved in acquired resistance against bevacizumab.

The Human Angiogenesis Protein Array

For this study we used the Proteome Profiler™ Array manufactured by R&D Systems (Minneapolis, USA). The Human Angiogenesis Array kit was chosen for its capacity to simultaneously determine the relative levels of 55 individual human

angiogenesis-related proteins. The Array method principle is based on the sandwich immunoassay technique (Figure 11). Capture antibodies are fixed on nitrocellulose membranes in duplicate spots representing each corresponding analyte. Each individual serum sample was mixed with a cocktail of biotinylated detection antibodies and incubated with the membrane overnight. During this procedure the immuno-complex of the analyte protein and the detection antibody was bound to the related immobilized capture antibody on the corresponding membrane spot. The membranes were washed to remove unbound material and incubated with Streptavidin–horseradish peroxidase (HRP). Following repeated wash, each membrane was shortly incubated with a chemiluminescent detection substrate. After removal of remaining substrate mix the membranes were coated with plastic film and placed in an autoradiography film cassette. In the presence of the substrate, the HRP enzyme produced a detectable luminescent signal. By the high affinity binding of streptavidin to biotin, a light signal was produced at each spot in proportion to the amount of analyte bound. The membranes were exposed to a light sensitive X-ray film three by three (BL, SOM and EOT samples of each individual). Multiple exposure times were used for each patient´s set of membranes. The different exposure times, adjusted and determined by visual inspection in the dark room, yielded varying signal intensities of the membrane spots.

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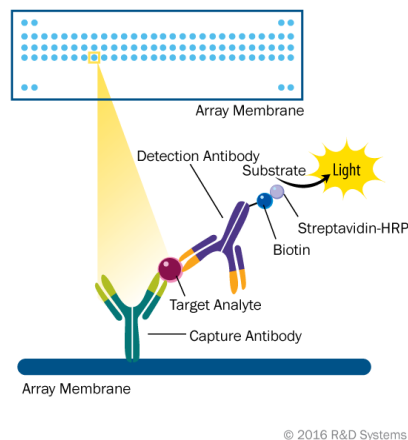
Figure 11.
The Proteome Profiler™ Array sandwich immuoassay principle. The target analye (here, serum protein) is bound between the capture and detection antibodies (like in a sandwich). There are six (2x3) positive reference spots, and two negative reference spots located in the corners of each membrane. (Reprinted with permission, © R&D Systems)

The array films were later scanned, digitally inverted in the software Adobe Photoshop and imported to the free and by public domain accessible image processing program ImageJ. The semi-quantification of protein levels was

performed by determination of pixel intensities in duplicate spots of each target protein, followed by normalisation to the reference spots on each membrane. The dynamic range of each detectable protein was compared between the three sample time points for each patient, *i.e.* at baseline before treatment start, at start of maintenance and at end of treatment (Figure 9).

Statistical considerations

Endpoints and power

The primary objective, *i.e.* the main research question of a clinical trial, needs to be formulated as a hypothesis, using appropriate outcome measures or endpoints, which should reflect a relevant benefit for the patients. Together with an effect size considered clinically relevant to detect, and the chosen level of statistical significance and power, this is used to perform a sample size calculation. Patients with incurable cancer generally expect anti-tumoral treatment to prolong life and decrease symptomatic burden, and so do their oncologists. Thus, for phase III trials with aim to prove superiority of a new treatment, the only valid endpoints should be OS, safety, and/ or quality of life. However, proving gain in OS can be challenging since this requires many participants, and/or long follow up time in the trial. In the palliative treatment setting, OS time is also influenced by effects of post study treatment. In first line randomised trials, varying therapeutic effects of second and later line regimens, can introduce bias in the reporting of OS results. Consequently, in trials evaluating metastatic cancer, progression-free survival (PFS) is often used as a surrogate endpoint[183]. We used a two-sided alfa level of 5% for statistical significance in all studies (paper I-IV).

ACT (paper I)

The primary aim was to evaluate if maintenance treatment with combined bevacizumab and erlotinib (arm A) would have a better effect on PFS than treatment with bevacizumab alone (arm B). It was assumed that the median PFS during maintenance treatment would increase by two months with the addition of erlotinib, from three months in arm B compared to five months in arm A. The study was first designed to detect this difference with a two-sided significance level of 5% and a power of 90%, whereby we would have needed 84 patients in each arm. With an estimated attrition rate of 30% before randomisation, we planned to enrol in total 240 patients. Due to an unexpectedly high rate of exclusion of patients with progression, toxicity or good partial response leading to withdrawal for planned curative surgery during the induction period, we chose at

closure of the trial to investigate the primary endpoint at a power of 80%, for which only 126 events would be required.

The ACT study enrolled 249 patients, and 35% of the patients were excluded before randomisation. An additional three randomised patients were excluded before start of maintenance, and thus 80 (arm A) and 79 (arm B) patients were included in the analysis of primary endpoint. Finally, the statistical analysis was performed when 131 events (progressions or deaths) had occurred. An interim analysis was performed, as planned for safety purposes, when 80 patients had completed six months of study treatment. This analysis did not find any safety concerns that would have to result in study closure.

ACT2 (paper II)

The aim of the ACT2 trial was to evaluate if addition of erlotinib to maintenance treatment with bevacizumab would have a better effect than bevacizumab alone in KRAS wild type patients, estimated in terms of PFS rate at three months. The use of PFS rate as primary endpoint is often appropriate for phase II trials, aiming to determine if there is sufficient evidence of anti-tumour activity to undertake further studies in phase III. In the power calculation for the ACT2 trial we required a rather large treatment gain in PFS rate to be considered a clinically relevant benefit by the addition of erlotinib in a KRAS wildtype population. It was assumed that the PFS rate after three months of maintenance treatment would be 80% in patients treated with erlotinib plus bevacizumab (arm wt-BE) compared to 50% in patients treated with bevacizumab alone (wt-B). We used a significance level of 5% and a power of 80%. To meet these conditions, we would need to analyse 40 patients in each arm. We expected that 60% of the patients included would be KRAS wild type, and with an attrition rate of 30% we initially planned to enrol 181 patients to compensate for included patients not valid for efficacy analysis. In parallel with the first ACT trial, the attrition rate during induction phase was found to be higher than expected also in ACT2. Therefore, in order to reach the calculated size of the randomised groups, we decided, through an amendment to the study protocol, to increase the included study population to 233 patients. Although 146 patients were now randomised, further attrition was seen with in total 40% of patients withdrawn before randomisation, and an additional eight were excluded before start of maintenance treatment. Also, more patients than expected were KRAS mutant, and thus excluded from the primary endpoint analysis of the wildtype cohort. These circumstances reduced the power of the statistical analysis.

Additionally, the pre-planned pooling of data from the KRAS wild type patients in the first Nordic ACT trial added power to the calculation of PFS as a secondary endpoint in ACT2. Even though the surrogate endpoint PFS has advantages in that it can be reported earlier than OS and requires smaller cohorts, it also has

disadvantages, *e.g.* that the time of progression is determined by the frequency of tumour evaluation and by the quality of the radiological examination and RECIST report.

Translational studies (paper III & IV)

In paper III, the main reason for choosing time to progression (TTP) and objective tumour response in induction phase rather than OS as clinical endpoints was to avoid bias by second and third line treatment effects. An alternative could have been to include all patients with available plasma samples, regardless of reason for end of treatment, thus censoring patients ending treatment for other reasons than PD. That would have increased the study population and thereby also the statistical power. However, during the induction treatment with combination chemotherapy-bevacizumab many patients dropped out due to *e.g.* adverse events, intended curative surgery, and withdrawn consent. By including those patients in the analysis we would have faced the risk of obscuring the results by patients in whom the full anti-tumoral effect could not be properly evaluated. Consequently, for the translational studies, we regard TTP as a valid surrogate endpoint for the anti-tumoral effect of the treatment.

The primary aim of this exploratory study was to investigate a potential association between early changes in vasoactive peptide levels and objective response and time to actual tumour progression (according to RECIST criteria). This was based on our original hypothesis that effects of a bevacizumab-containing regimen on the systemic vasculature correlate with treatment effects on the tumour vasculature. Thus, the explorative nature of our hypotheses in both translational trials (papers III and IV) was not dependent on a strong association of our surrogate endpoint and OS. In paper IV the endpoint TTP reflects the time on maintenance treatment, given that the induction period is the same for all patients, *i.e.* approximately 4.5 months (18 weeks). The sample sizes of the translational studies were further determined based on the inclusion criteria necessitating available blood-samples at all defined time points. Due to these considerations, our results must be interpreted with great caution in a clinical perspective, but may still be pertinent in the discussion of pharmacodynamics and associated biomarkers, in that we might gain new insights to biological processes involved in the treatment response.

The adverse event hypertension can be reported in many ways to reflect dynamic changes in blood pressure during anti angiogenic treatment. The argument of choosing CTCAE 4.0 is given in the methods section. To apply the ordinal data from a retrospective grading of hypertension in the statistical analysis has disadvantages, but since it takes into consideration the use of anti-hypertensive treatment, hypertension grade was considered more relevant than use of continuous blood pressure data.

Statistical analysis and reporting of data

ACT and ACT2 clinical trials (paper I & paper II)

A Statistical Analysis Plan (SAP) was presented by the appointed biostatistician of the respective clinical trial based on information given in the study protocols. The SAP was approved by the PI, prior to declaration of clean file for both ACT and ACT2 studies, and the analysis populations were defined similarly and in accordance with the ICH E9 guideline for statistical principles for clinical trials[184]. The ITT populations included all patients registered for treatment start and their data were presented descriptively for OS and to address secondary endpoints defined by questions arising before randomisation, such as overall response rate in induction phase. The FAS population included all patients who had taken at least one dose of maintenance treatment. The ICH guideline for statistical principles proposes the Full Analysis Set to be:" The *set of subjects that is as close as possible to the ideal implied by the intention-to- treat principle. It is derived from the set of all randomised subjects by minimal and justified elimination of subjects."* Further, the circumstances that might lead to exclusion of subjects from the FAS are given as: *"...failure to satisfy major entry criteria (eligibility violations), the failure to take at least one dose of trial medication and the lack of any data post randomization"*. The number of excluded subjects and reasons for exclusion from the ITT and FAS populations were specified in the CONSORT diagrams of the respective studies. To address the primary objectives in both ACT and ACT2, involving only the randomised population, we considered the FAS populations most suitable.

The PP set included all FAS patients compliant with the study protocol. The safety analysis population included all patents that had received at least one dose of study treatment including the induction phase, and this population was used for safety analysis. One of the ICH multidisciplinary guidelines include the Medical Dictionary for Regulatory Activities (MedDRA), which presents a standardised medical terminology for registration, documentation and safety monitoring of medical products[184]. The terms used for reporting of AE data according to CTCAE 3.0 were standardised and mapped to the MedDRA hierarchy of terms, which facilitated reporting of toxicity in the ACT2 trial.

The difference in treatment effect was visualised using Kaplan-Meier survival curves and quantified as a Hazard Ratio (HR) estimated using the Cox proportional hazards regression model. This model is appropriate if the ratio of the mortalities in the two groups is approximately constant over the whole follow-up period, *i.e.* if the relative treatment effect is the same for any given time interval. Many times, this is not the case, as in the ACT2 trial, where the estimated survival curves were overlapping and of different shapes, which reduces the validity of a single HR as an effect estimate. Nonetheless, we chose to report HR in view of the

fact that HR is commonly reported for time to event endpoints in randomised clinical trials communications, irrespective of the validity of the proportional hazard assumption. The estimated HR can be interpreted as a weighted average of the effect over the follow-up time. A statistical comparison between the survival function curves was also made by the log rank test, for which we reported the p-value in association with the Kaplan Meier estimates.

Translational studies (paper III & paper IV)

In explorative studies, weak correlations may be of interest and numerical quantification of effects of less importance. Consequently, we used correlation analysis as statistical approach in paper III.

Calculation of correlation coefficients and evaluation of statistical evidence for non-zero correlations (*i.e.* calculation of the associated p-values) is an appropriate data analysis strategy when studying relationships between two continuous variables like TTP and each of the three peptide ratios (paper III). Pearson correlation is usually the measure of choice for symmetrically distributed variables without outliers, and this was also the first choice in our analysis of these relationships. If the normality assumption is violated for one or both of the variables, it is standard to switch to the rank based Spearman correlation. We calculated also Pearson correlation and found that in comparison with Spearman´s test the two correlation coefficients, and the corresponding P-values, were approximately the same for investigation of TTP *vs.* MR-proADM and Copeptin. For TTP *vs.* MR-proANP the difference between the two correlation coefficients was interpreted as evidence for violation of the normality assumption for one or both the correlated variables. Hence we decided to report the Spearman correlation for MR-proANP *vs.* TTP. For consistency and to simplify data interpretation, this measure of correlation was used also for pro-ADM and Copeptin, respectively, *vs.* TTP.

Since inclusion in the biomarker cohorts were based on documented progressive disease as reason for end of study treatment, survival analysis methods were not appropriate. Kaplan-Meier curves are possible to draw also with this sampling scheme, but since patients who did not have documented progressive disease were excluded, such curves would not reflect the underlying population from which the patients in the study were sampled. Hence, these curves would lack meaningful interpretation.

In the report of protein levels in paper IV, protein assessments that were found to be below the detection limit were not regarded as missing data. Instead of being given a nil value, each of the non-detectable protein levels were set to 50% of the lowest protein level measured in any patient at any of the three time points for that protein. By doing so these superseded non-detectable protein values will still be

given the lowest ranks in the non-parametric methods used for statistical analysis in this study. This is a standard procedure used to minimise bias that could be inflicted by the exclusion of missing data.

In paper IV we analysed dynamic trends in protein levels, by pairwise comparison of the protein values between two time points for each patient. Because of the limited size of the cohort and the skewed distribution of the dynamic ranges of proteins, we chose the Wilcoxon matched-pairs signed ranks test to analyse significant dynamic rise or decline in levels of each protein throughout the cohort.

Ethics

All patients signed informed consent for both the clinical and translational parts of the studies. The ACT and ACT2 trials were conducted in accordance with GCP as previously described, and in accordance with the code of ethics of the Helsinki declaration of the World Medical Association.

The patients enrolled in the ACT and ACT2 trials were given the eligibility criteria scheduled for first line doublet chemotherapy. Hence, the patients that did not enter the trials after screening were anyhow offered the same induction treatment with chemotherapy plus bevacizumab. A separate optional informed consent was given regarding collection of tumour tissue and blood for the translational research questions. In ACT2 this meant sampling of one extra blood test at six weeks from treatment start, apart from this, the scheduled radiological evaluation and blood tests were performed in accordance with routine care during standard treatment.

A KRAS status evaluation before treatment start was not standard at the time of enrolment in ACT and ACT2. Since all patients in ACT2 were planned to start bevacizumab plus chemotherapy as first line induction, we considered it valid to allow time for KRAS testing during the months of induction phase. Evidence of the KRAS biomarker´s ability to predict treatment response was not sufficient enough to justify exclusion from randomisation of the sub-population of patients with KRAS mutant tumours. Therefore, we believe it was ethical to offer these patients randomisation in two explorative treatment arms comparing bevacizumab and metronomic capecitabine.

At the launch of the clinical trials, there was a lack of prior randomised trials evaluating the bevacizumab single or metronomic capecitabine treatment. Hence, a thorough risk/gain evaluation of these maintenance regimens was uncertain. However, in view of the known mild toxicity profile the active maintenance was not believed to increase the risk significantly for the patients in comparison with treatment pause, which was considered a valid alternative option after response to induction treatment.

Results

"The intention to live as long as possible isn't one of the mind's best intentions, because quantity isn't the same as quality."

- Deepak Chopra

The detailed results are presented in the original communications, and the principal findings are therefore only briefly summarised and compared here.

Paper I & paper II

Demography

In both of the ACT trials 15% of the total population had received adjuvant chemotherapy, whereas adjuvant oxaliplatin was more prevalent in the ACT2 patients (10% *vs.* 4 % in ACT). The distributions of induction treatment regimens in the ACT and ACT2 trials are presented in Figure 12. A larger proportion of patients in ACT2 had the primary tumour left in situ (52% *vs.* 35% in ACT). In ACT the baseline characteristics were evenly distributed between arms, whereas in ACT2, there were some discrepancies worth noticing. In the wt-BE arm of ACT2, rectal cancers were under-represented compared to the other arms, with 19% compared to 54% in arm wt-B. Despite a higher rate of colon cancers in wt-BE, the rate of previous adjuvant treatment was lower compared to the wt-B (6% *vs.* 21%) in ACT2.

Figure 12.
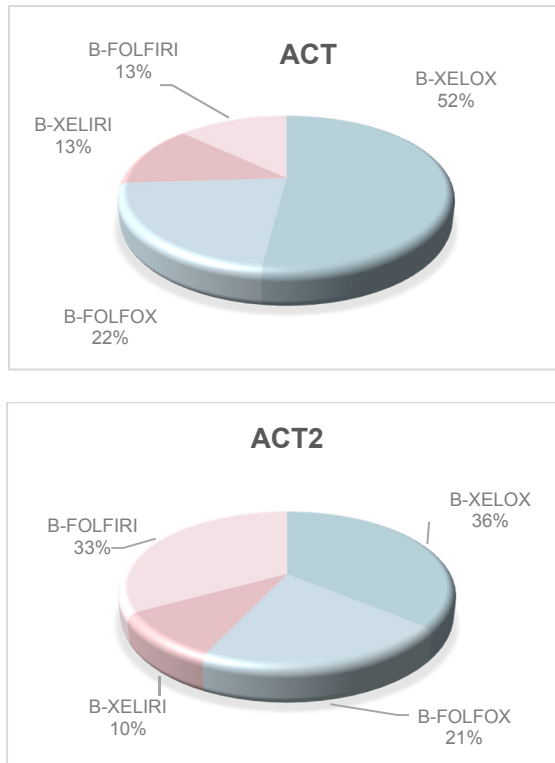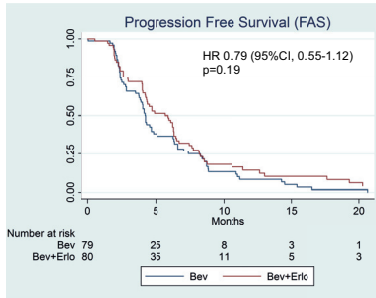**Investigator´s choice of induction therapy regimens in the ACT and ACT2 trials.** In ACT2 there was a more even allocation between oxaliplatin and irinotecan (blue vs. pink) as well as between capecitabin and infusional 5FU based chemotherapy (dark vs. light coulors). Rates are given in safety analysis set from ACT2 (N=229), and from evaluable patient population in ACT (N=232). B, bevacizumab.

A)

ACT — all KRAS population

ACT² + ACT — KRAS wildtype population

Progression Free Survival (FAS)
HR 0.79 (95%CI, 0.55-1.12) p=0.19

Number at risk
Bev 79 ... 25 ... 8 ... 3 ... 1
Bev+Erlo 80 ... 35 ... 11 ... 5 ... 3

Progression Free Survival – KRAS wt (pooled FAS)
HR 1.03 (95%CI, 0.70-1.50) p=0.87

	mPFS from R months (95% CI)
B	**4.2** (3.7-4.8)
B+E	**5.7** (4.2-6.4)
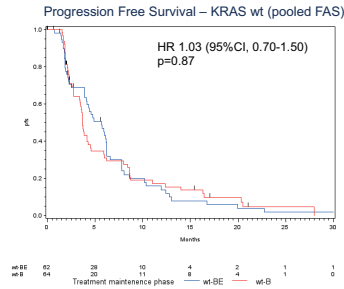
	mPFS from R months (95% CI)
wt B	**3.7** (3.4-4.5)
wt B+E	**5.7** (3.9-6.2)

B)

ACT — all KRAS population

ACT² + ACT — KRAS wildtype population

OverallSurvival (FAS)
HR 0.88 (95% CI, 0.61-1.27) log rank p=0.51

Number at risk
Bev 79 ... 64 ... 42 ... 16
Bev+Erlo 80 ... 68 ... 44 ... 19

Overall Survival – KRAS wt (pooled FAS)
HR 0.76 (95% CI, 0.51-1.13) log rank p=0.20
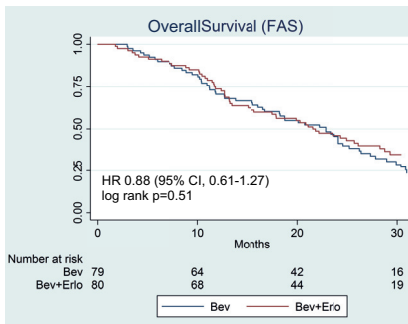
	mOS from R months (95% CI)
B	22.8 (16.6-25.3)
B+E	21.5 (15.4-28.3)
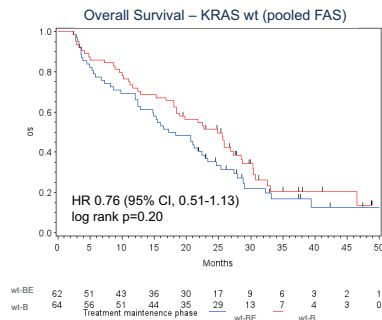
	mOS from R months (95% CI)
wt B	24.9 mån (18.0-28.5)
wt BE	17.8 mån (12.5-23.0)

Figure 13A and B.
13A Comparison of Progression-Free Survival (PFS) and 13B Overall Survival (OS) for bevacizumab alone versus bevacizumab plus erlotinib in the non KRAS guided ACT population (PFS as primary and OS as secondary outcome) and in the pooled KRAS wildtype population of the ACT2 trial (PFS and OS secondary outcomes). The effect of erlotinib was not improved in a selected KRASwt population. NB the inverse colour code of the arms inbetween the two Kaplan-Meier graphs. mPFS, median Progression-Free Survival; wt, wildtype; B, bevacizumab; E, erlotinib; R, randomisation; HR, Hazard Ratio; CI, confidence interval.

Survival and biomarker effect

The trials presented in paper I and II did not meet their respective primary endpoint. In the ACT study the mPFS was not statistically superior when erlotinib was added to bevacizumab, and in ACT2 there was no significant gain in PFS-rate at three months with the addition of erlotinib to bevacizumab in a KRASwt cohort. The survival results are presented and compared in Figure 13. Median PFS for bevacizumab plus erlotinib was numerically exactly the same in the pooled KRASwt (N=62) as in the all RAS patients from first ACT (N=80). Although a small numerical gain in mPFS was demonstrated in both cohorts compared to bevacizumab alone (pooled KRASwt, N=64 and ACT all RAS, N=79), the curves are overlapping and log rank comparisons are non-significant in both trials (Figure 13A). Thus, the benefit of adding erlotinib to bevacizumab seems not to increase in KRASwt compared to the all RAS patients. In contrast, a numerically large, albeit not significant, drop in mOS was demonstrated for the erlotinib combination compared to bevacizumab alone, in the ACT2 trial KRASwt cohort, as demonstrated in paper II. When the KRASwt population from ACT was added in the pooled dataset, presented here as OS from randomisation, the survival curves are closer, and again no statistically significant difference is seen (Figure 13B). However, the mOS for the erlotinib combination arm is only approximately 18 months, which is lower than expected in a first line mCRC trial, and certainly in a KRASwt population.

Safety and metronomic treatment effect

Almost half of the patients had at least one grade 3 or 4 adverse event during the chemotherapy induction phase in both ACT and ACT2. Thus, moderate to serious toxic effects in general was not altered by the slightly diverging choices of induction chemotherapy backbone regimens. Strikingly, in both trials, more than half of the patients experienced at least one grade 3 or 4 adverse event during maintenance treatment with bevacizumab plus erlotinib (Figure 14). This clearly illustrates some safety concerns of adding erlotinib to bevacizumab. However, this did not correspond to an unexpectedly high number of patients that were withdrawn from study due to adverse events in the erlotinib combination maintenance arms, albeit it was slightly more common than during treatment with bevacizumab alone (Figure 15). Most probably, this demonstrates that the toxic skin effects of EGFR inhibition can be managed by supportive treatment and/or dose reduction. During the induction phase in ACT and ACT2, respectively, 9% *vs.* 11% of the safety populations were withdrawn due to an adverse event.
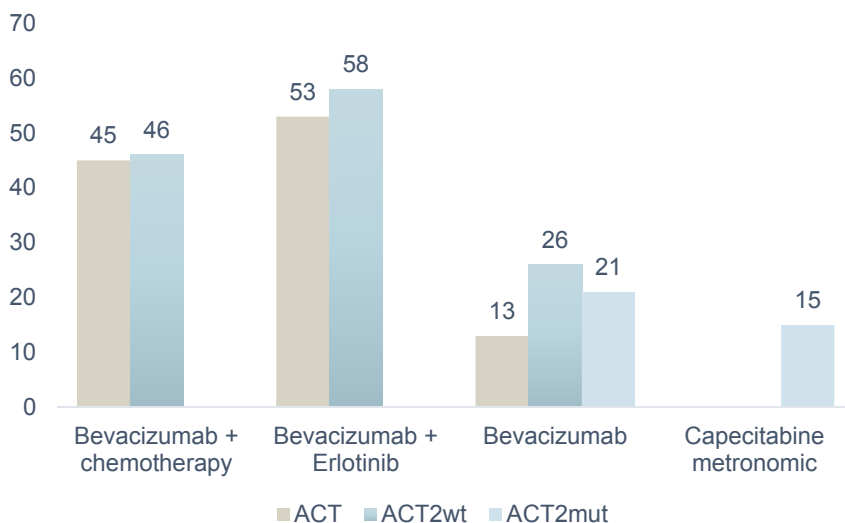
Figure 14.
Proportion (%) of patients with at least one grade 3/4 toxic event in the respective treatment regimen groups of the ACT and ACT2 trials. Bevacizumab + erlotinib is the most toxic regimen. Toxicity rates presented for Bevacizumab + chemotherapy were seen in the induction phase of the respective trial, thus for ACT2 including both wt and mut patients. Wt, KRASwildtype; mut, KRASmutant.

Figure 15.
Distribution (%) of reasons for end of treatment, as percentage of patients in each of the three maintenance treatment regimens summarized from both ACT and ACT2. The largest proportion of patients who were withdrawn from study due to toxicity was seen in the Bev+Erlo arms. Bev+Erlo, bevacizumab + erlotinib (arms A and wtBE, N=115); Bevacizumab (arms B, wt-B and mut-B, N=149); Capecitabine (arm mut-C, N=33).

The randomised comparison between bevacizumab single (N=34) and metronomic capecitabine (N=33) yielded no large or unexpected differences in terms of safety as reported in the original ACT2 publication (paper II). The efficacy was very similar as illustrated by the survival curves (Figure 16), with no statistically significant difference between the survival functions. Due to the small sample size large confidence intervals are seen for mOS, and the study was not powered for survival endpoints comparisons. Nevertheless the mPFS times of the mutant patients were close to the numerical median PFS values of the bevacizumab alone arms in ACT and in the ACT2 wildtype cohort (Figures 13A and 16).
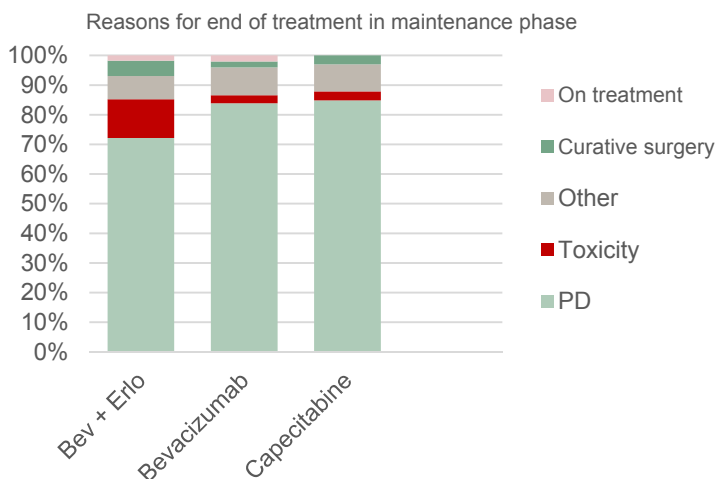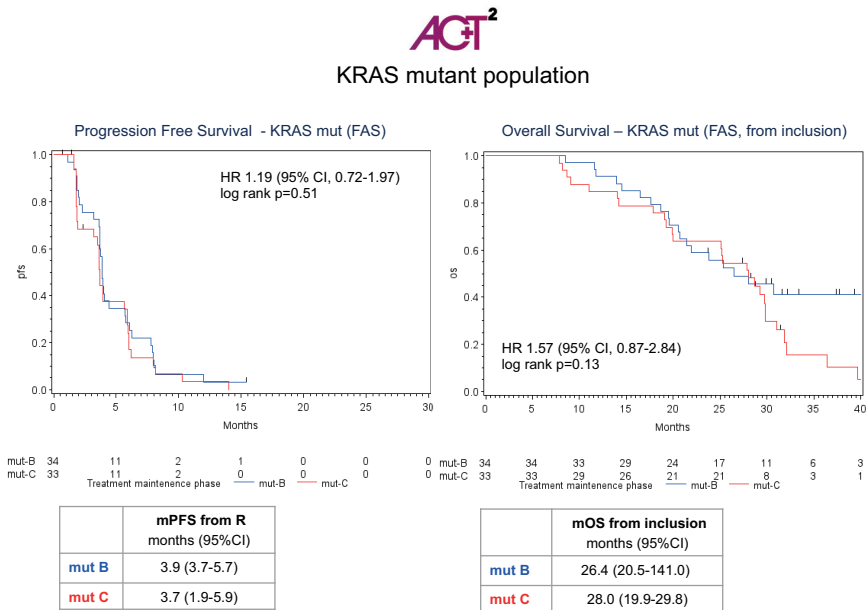

Figure 16.
Survival of the metronomic capecitabine maintenenance cohort. mPFS, median Progression-Free Survival; wt, wildtype; B, bevacizumab; E, erlotinib; C, capecitabine; R, randomisation; HR, Hazard Ratio; CI, confidence interval.

Paper III

For the study presented in paper III, 97 patients fulfilled the inclusion criteria and had evaluable plasma samples. Within this cohort we demonstrated a relationship between blood levels of three vasoactive peptides and the effect of treatment with chemotherapy plus bevacizumab, *i.e.* clinical outcome in terms of TTP and ORR.

The strongest relation was seen for MR-proADM; increasing levels of the peptide correlated with better objective response during induction phase. However, the statistically significant correlations were in general weak for each individual peptide (Figure 17). In a combined, dichotomised peptide ratio score we found that outcome was improved in the presence of a parallel rise of all three peptide levels measured early in the induction treatment phase with chemotherapy and bevacizumab. In the group of 28 patients who had rising levels of all three peptides during the first six weeks of treatment, median (m)TTP was significantly longer compared to mTTP in the 59 patients who had rising levels of only one or two of the peptides (284 *vs.* 225 days, p=0.02).

There was no significant correlation between the grade of early hypertension according to CTCAE 4.0 and clinical outcome in terms of TTP. Nor did we find any association between dynamic vasoactive peptide levels and higher grades of hypertension, as illustrated in Figure 17.

Peptide ratio	HT grade at 6w	
	r_s	p
MR-proADM	0.005	0.96
MR-proANP	-0.03	0.74
Copeptin	-0.04	0.66

Hypertension	TTP	
	r_s	p
BL diagnosis	-0.05	0.64
HT grade at 6w	-0.18	0.07

Peptide ratio	PR/SD/PD		TTP	
	r_s	p	r_s	p
MR-proADM	-0.36	0.0003	0.20	0.05
MR-proANP	-0.20	0.05	0.22	0.03
Copeptin	-0.17	0.10	0.23	0.02
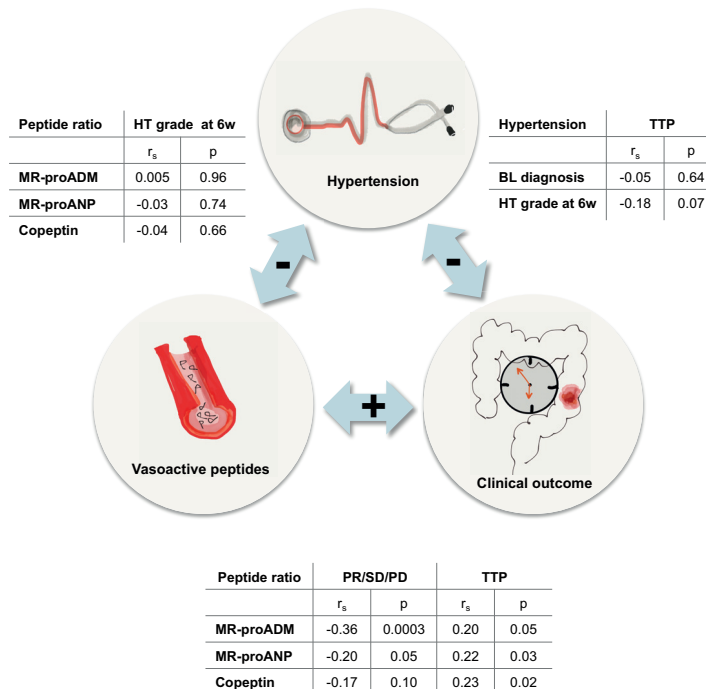
Figure 17.
Results from correlation analyses investigating relations between hypertension, vasoactive peptides and clinical outcome (paper III). A statistically significant (**+**) week correlation between rising vasoactive peptides and better clinical outcome was demonstrated. There were no significant (**-**) association between vasoactive peptide levels and hypertension, or between hypertension and clinical outcome. r_s, Spearman´s rang correlation coefficient; HT grade at 6w, hypertension grade according to CTCAE 4.0 at six weeks from start of induction treatment (0-1<2<3); BL, baseline; TTP, time to progression, PR, partial response; SD, stable disease; PD, progressive disease.

Paper IV

In the 22 patients who fulfilled the pre-defined inclusion criteria presented in paper IV, we investigated an array of 55 angiogenesis-related proteins from serum collected at three time points (BL, SOM, and EOT). Between baseline (BL) and start of maintenance (SOM), these patients received doublet chemotherapy plus bevacizumab for 18 weeks (4.5 months). All eligible patients were evaluated with at least stable disease according to RECIST, and were then given maintenance therapy with bevacizumab alone until end of treatment (EOT) due to progression. Eight of the 55 array proteins were non-detectable in all samples from the cohort, whereas nine were detectable in all patients at all time points. We found a statistically significant decrease in protein levels with mostly pro-angiogenic properties during response to the chemotherapy-containing induction treatment. Other, mostly anti-angiogenic proteins showed increased levels during the same treatment phase (BL to SOM) (Figure 18). From the time of response until the time of progressive disease (SOM to EOT), during treatment with bevacizumab alone, we found a significant rise in mostly pro-angiogenic factors. There was a significant positive correlation between rising protein levels during induction treatment and TTP2, *i.e.* time on response to the maintenance treatment, for three proteins: Insulin Growth factor binding protein (IGFBP)-2, Interleukin (IL)-8 and Activin A. These findings must be interpreted with caution, particularly owing to the very low number of patients with detectable levels of Activin A and IL-8, and the number of statistical tests performed.
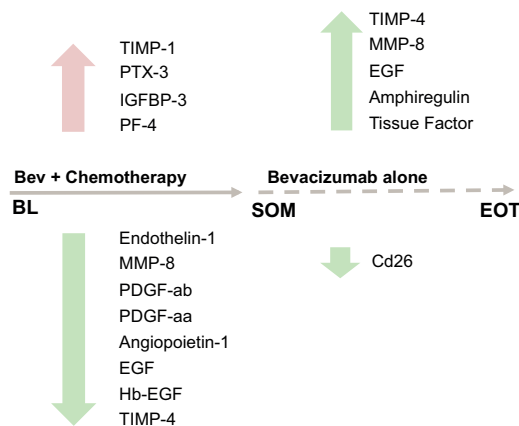
Figure 18.
Angiogenesis related proteins with significant dynamic level changes. Statistically significant (p<0.05) changes in serum levels during induction treatment phase (BL to SOM), and during maintenance treatment with bevacizumab alone (SOM to EOT), respectively. Arrows illustrates the trend of the dynamic change (up/down). Proteins with anti-angiogenic properities (pink colour) rise, whereas levels of factors with mostly pro-angiogenic functions (green colour) decrease during response to chemotherapy + bevacizumab (Bev).

Discussion and future perspectives

"Out beyond the ideas of wrongdoing and right doing there is a field. I will meet you there"

- Jalal al-din Rumi

Treatment of metastatic colorectal cancer (mCRC) is a field of medicine that is constantly facing practice-changing progress. Palliative systemic anti-tumoral treatment is offered to most patients during the course of the disease. The goal is to prolong survival by reducing tumour burden and postponing both tumour progression and deterioration of symptoms. Maintenance therapy aims at improving the oncological result by maintenance of the tumour response induced by induction treatment, while optimising quality of life by reducing toxicity.

With the introduction of targeted therapy agents in this setting, promising strategic treatment options have evolved, as investigated in this thesis project. In parallel to our publications, other clinical studies have provided important results to this field. Nonetheless, a major limitation of many targeted agents is that biomarkers to predict treatment effects are still lacking.

Maintenance treatment: why, when, how, to whom?

Methodological considerations papers I & II

Although planned chemotherapy free intervals and maintenance treatment are widely used treatment concepts, they are subject of debate. In conjunction with the "collective wisdom" theme of the ASCO congress in 2016 I picked up a citation by architect Frank Lloyd Wright: "Less is only more if more is no good". But how do we know if more treatment is no good? And if so, can we be sure that less treatment will provide more days and/or more quality to life? What if less is not more, only "good enough"?

As previously mentioned, any new treatment concept should preferably aim to prolong survival in comparison with current standard. PFS is well established as a

surrogate endpoint for OS in mCRC, also in the era of anti-angiogenic treatment[192]. Even so, it takes substantial endeavour time and structural resources to run first line randomised clinical trials with PFS as primary endpoint. Taking into consideration the negative results from the ACT and ACT2 trials, we are well aware that the design of the studies may be criticized. At the time of the planning of the ACT study protocol, the study steering committee was not confident enough in the possibility to recruit the double number of patients within a reasonable time limit, why the proposal of a third control arm with either 5FU or treatment pause was rejected. Also, as previously mentioned, other much larger European trial initiatives were on-going to investigate the effect of single bevacizumab as maintenance compared to treatment pause[186, 187]. However, as the ACT trial moved on, we found that the inclusion pace was reassuring and that it would be relevant to take the research question further with a biomarker-guided extension of the trial. The translational approach of this project is also important to consider in support of the relevance of the both Nordic ACT trials.

A general problem in many randomised oncological clinical trials is the rapid development in the field of tumour biology, biomarkers and cancer treatment. The time frame from planning a trial until it is ready for publication is usually more than five years, during which a lot of new knowledge is being generated. Therefore, a study should be judged according to what we knew when the study was launched, rather than when it was published.

For mCRC maintenance treatment, Tabernero *et al.* presented the preliminary results from the MACRO TTD trial at the ASCO congress in 2010 (abstract #3501). The authors concluded that treatment with bevacizumab as maintenance after response in the induction period seemed not much inferior to continuous chemotherapy plus bevacizumab, which was consistent with the final results[95]. Additionally, preliminary data for ovarian cancer, presented at the same ASCO meeting by Burger *et al.* (abstract #LBA1) showed that maintenance treatment with bevacizumab alone seemed better than placebo controlled pause of treatment. This was later confirmed in the final publication[193]. Thus, in view of the available promising data at the time of planning and launch of the ACT2 trial, the concept of prolonged angiogenesis inhibition with bevacizumab as a single maintenance strategy was still relevant to study in mCRC.

Furthermore, in support of the erlotinib combination, the ATLAS trial in NSCLC, had reported preliminary results of improved PFS for bevacizumab plus erlotinib compared to bevacizumab + placebo as a maintenance switch strategy[97]. Also, as previously mentioned, the phase II XELOBER trial [96] presented preliminary data in 2010 showing that this combined EGFR + VEGF inhibition approach was also feasible and active as maintenance in mCRC. During the conduct of the first ACT

trial, around 2008 we had also learned that patients with KRAS exon 2 mutated colorectal tumours do not benefit from EGFR-inhibiting antibodies[67].

On the other hand, there were many questions still unanswered. In 2009-2010 we did not have the final results from the first ACT trial, and the larger but similar, GERCOR DREAM (OPTIMOX-3) trial was recruiting patients to find out if the addition of erlotinib would be superior to bevacizumab alone as maintenance in mCRC. The importance of performing extended RAS analysis before giving EGFR-inhibiting antibodies was not yet established, and we did not know whether the efficacy of erlotinib in mCRC was dependent on the KRAS status of the tumour.

Since the early results from the ATLAS trial in NSCLC had suggested that erlotinib was more efficient in KRASwt patients[97], our hypothesis was that this could be the case also in mCRC. This was the rationale behind the design of the ACT2 trial.

In retrospect one may argue that the primary endpoint assumption of ACT2 was optimistic, but we did not consider it unrealistic. The three months PFS rate of 65% in the comparator arm bevacizumab alone, turned out higher than the expected 50%. The groups are small and considering the risk of type II error, no firm conclusions can be made from this KRAS wildtype selected comparison. Instead, another important aim with ACT2 was to perform a pooled analysis of PFS, using the KRASwt patients from ACT + ACT2, in all 126 patients. Given the identical inclusion criteria we believe that the pre-determined pooling of these data sets was a valid approach, not inferior to meta-analyses that mix data from several different studies.

Another maintenance strategy that had caught our interest was the possibility of using capecitabine in a metronomic dosing schedule. We saw an opportunity to investigate this option in the KRASmut patients within the ACT2 study. To compare low dose metronomic capecitabine with bevacizumab in a randomised way would give us a sense of feasibility, and the proposed anti-angiogenic effects of metronomic chemotherapy also supported this exploratory design in view of possible retrospective translational efforts. We chose not to generate a statistical hypothesis, *i.e.* no formal sample size calculation was done for this cohort, which of course is a drawback. The number of patients was limited but it is the first report on a randomised controlled comparison of this explorative medication schedule in mCRC.

Interpretation and future directions

Is maintenance treatment better than treatment pause?

As given in the background chapter, for a majority of mCRC patients, introduction of a less intense treatment period is a valid option to non-stop continuous first-line chemotherapy. What is then the best strategy for "less treatment": maintenance or pause?

The OPTIMOX-2 trial published in 2009[88] compared maintenance treatment with 5-FU to treatment pause and found that the treatment free interval had a small negative effect on PFS when introduced after only three months of induction chemotherapy. Three studies published in 2015 have investigated this question further in mCRC by comparing bevacizumab-based maintenance with a treatment pause, after first line induction with chemotherapy plus bevacizumab[105, 186, 187]. As in the Nordic ACT trials, the SAKK41/06 study used backbone doublet chemotherapy with either irinotecan or oxaliplatin, whereas the two others used oxaliplatin based doublet plus bevacizumab in the induction period ranging from four to six months.

The CAIRO3 trial compared observation, *i.e.* a treatment pause arm to maintenance with metronomic scheduling of capecitabine plus bevacizumab[105].

The SAKK41/06 trial compared observation to maintenance with bevacizumab alone[187].

Finally, the AIO0207trial randomised into three arms and compared observation/treatment pause to fluorouracil plus bevacizumab or bevacizumab alone as maintenance strategies[186].

The CAIRO3 study reported positive results from a randomised superiority analysis with PFS2 as primary endpoint (second progression after re-start with induction treatment after first progression). This trial demonstrated a statistically significant gain in PFS and PFS2 with maintenance capecitabine plus bevacizumab compared to pause.

The SAKK41/06 study explored the non-inferiority of pause versus bevacizumab alone with TTP as primary endpoint, and the primary objective was statistically not met. The numerical mPFS was somewhat better in the bevacizumab arm, however the difference was not judged clinically meaningful and according to the authors maintenance with bevacizumab alone is not a better option than pause.

In the AIO0207 trial the primary endpoint time to failure of strategy (TTFS) was compared between three arms in a randomised non-inferiority design. The conclusions drawn from the results were well in line with CAIRO3 in that observation was *not* non-inferior, *i.e.* pausing treatment could actually be worse,

than continuing 5-FU + bevacizumab as maintenance. Secondly, bevacizumab was shown non-inferior to 5-FU+ bevacizumab, *i.e.* addition of 5-FU did not yield a large difference in effect. Notably, quality of life was equal in the three arms.

In a review by Stein and co-workers (2015) a meta-analysis of the three studies was presented. This analysis showed a significantly improved PFS in favour of bevacizumab with or without a fluoropyrimidine as maintenance compared to pause after response to induction treatment (HR 0.57, [95%CI 0.43-0.75], p=0.0004). More recently Tamburini *et al.* (2016) published a review of this subject. In this meta-analysis including five trials, the authors found a very similar significant improvement in PFS (HR 0.56, [95%CI 0.44-0.71], p<0.00001) for the active maintenance strategies. Both publications demonstrated a trend for OS gain in favour of bevacizumab containing maintenance contra treatment pause after first-line induction, although this was not statistically significant.

Let us get back to the question of how less can become more: It is evident that for many patients more treatment (non-stop continuous chemotherapy) is no good due to accumulated and potentially harmful toxicity. Instead it has been demonstrated that less treatment is better, since it does not compromise survival.

On the other hand, maintenance therapy obviously means more treatment than pausing treatment. Consequently, in some situations maintenance therapy is no good due to more toxicity and more costs than treatment pause. Even if less treatment, *i.e.* pause, does not compromise overall survival it does lead to shorter progression-free survival. Hence, maintenance treatment is too good to be rejected. One may state that pausing treatment is not of more value since maintenance treatment is "good enough".

We must not forget that the first line maintenance studies in mCRC were not designed to find a statistical gain in OS, and later lines of treatment may bias this endpoint. Caution should also be taken not to overuse these de-escalation treatment strategies in clinical practise. While maintenance treatment has been widely implemented in first line, the effect of repeated de-escalation strategy during first and later lines of therapy has not been thoroughly investigated. Also, the exact accumulated impact of repeated treatment pauses during the continuum of care in mCRC is not known, although often used. Further studies on validated clinical trial registries for metastatic disease could be useful to address these questions.

In conclusion, since the launch of our Nordic ACT trials, we have now learned that after response to a first-line induction period, maintenance treatment is believed to be more effective than pause in terms of efficacy. However, it should only be used if it does not compromise quality of life. Some patients have more indolent cancer, low tumour burden and minimal or no symptoms. In these cases,

maintenance treatment is more likely to increase symptomatic burden than the disease itself, why pause is a relevant option to discuss to optimise quality of life. On the other hand, for many patients with a large tumour burden involving abdominal organs, liver and/or other vital organs, or with evidence of aggressive histology there is a great risk of fast tumour re-growth and progression leading to symptomatic deterioration if treatment is discontinued. These patients are most likely to benefit from the PFS gain of a maintenance strategy rather than from treatment pause.

Who may benefit from switch maintenance with targeted therapy?

Even though there seem to be a small benefit from bevacizumab alone as maintenance compared to pause, the conclusion from gathered data is to recommend 5FU or capecitabine with or without addition of bevacizumab as the standard maintenance regimen of choice, as it provides the best potential for a gain in PFS.

Our Nordic ACT studies have contributed to the field by investigating, as an option, combined targeted agents in a switch maintenance strategy. Following our ACT trial publication (paper I), the GERCOR group presented results from a similar much larger cohort of 701 mCRC patients[194]. In this DREAM OPTIMOX-3 trial 452 patients were randomised to bevacizumab with or without addition of erlotinib as maintenance, using the same doses as in our ACT studies. The induction period was six months compared to our 4.5 months. The addition of erlotinib increased mPFS from 4.9 to 5.4 months (unstratified HR 0.78 [95% CI 0.68–0.96], p=0.019), which is numerically very close to our results. Furthermore, they reported a mOS of 24.9 months in the bevacizumab plus erlotinib group and 22.1 months in the bevacizumab group (unstratified HR 0.79 [95%CI 0.64–0.98], p=0.035). DREAM OPTIMOX-3 is the first maintenance trial to demonstrate significantly superior OS, with a gain of almost three months, for an active maintenance strategy. The DREAM trial obviously had more power than our Nordic ACT study to detect this difference in effect.

The interpretation of DREAM data in addition to preclinical data from the GERCOR group, was that erlotinib is also effective in KRAS mutant patients[194]. Interestingly, the final results from the ATLAS trial in NSCLC did demonstrate a better outcome of erlotinib in the KRASwt group than in the mutant patients. However, this was actually driven by another more important biomarker shown to be predictive for the erlotinib response, namely EGFR mutation. EGFR mutations are associated with KRASwt but not with KRAS mutant tumours, these mutations are mutually exclusive. As previously mentioned, EGFR mutation is much more rare in mCRC than in NSCLC; in one report it was found in less than 0,5% of CRC tumours[118]. Thus the EGFR mutations would not have the same potential impact on the erlotinib effect in mCRC. However, knowledge of the EGFR

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mutation status in colorectal cancers will probably be more available to clinicians in the future, with the introduction of large-scale molecular profiling techniques, such as next generation sequencing. It could be relevant to investigate the outcome of erlotinib according to EGFR mutation status also in mCRC, which would require a retrospective analysis in a large cohort of erlotinib treated patients.

Thus, together with the ACT2 report, we can conclude that KRAS status is not a useful predictive biomarker for the effect of erlotinib.

In Nordic ACT2, the visually worse outcome for OS in the KRASwt cohort by the addition of erlotinib was somewhat bewildering. In line with the DREAM trial, we found no large differences in the rate of established treatment drugs post-study between the arms, *e.g.* for the EGFR inhibiting antibodies. It is optimal to randomise the closest time as possible to the start of the treatments that the trial aims to compare, which is why we chose to randomise patients after the induction treatment period. This also allowed time for KRAS mutation assessment in the ACT2 trial. Thus, any loss of patients with skewed patient characteristics during induction cannot explain the slight variations in baseline characteristics reported between the patient arms in ACT2. Previous adjuvant treatment was less frequently reported in the patients´ medical history of the bevacizumab + erlotinib arm than in the bevacizumab alone arm. This could mirror more synchronously detected mCRC in this group, but other unknown patient related factors influencing the adjuvant treatment decision could also matter.

Additionally, there were more patients with colon cancers than rectal cancers in the ACT2 trial KRASwt combination arm. Here, the current debate about the prognostic and treatment predictive effect of primary tumour sidedness should be kept in mind. It is possible that not only the difference in rates of rectal and colon cancers, but also an unidentified skewed distribution of right and left sided colon cancers between the ACT2 arms could introduce a bias. This may explain the inferior OS of 18 months in the bevacizumab + erlotinib KRASwt arm of the ACT2 trial. Also, it is not known whether EGFR inhibition per se has worse outcome in right-sided colon-cancers, or if it only applies to the EGFR inhibiting antibodies. Future randomised studies on mCRC should definitely stratify for sidedness.

What is the best combination maintenance regimen?

There is no randomised comparison between bevacizumab plus 5-FU or erlotinib as maintenance, and although between-trial comparisons are hazardous, some important aspects are interesting to discuss.

In the CAIRO3 trial there was no clinically meaningful difference in quality of life (QoL) between pause and maintenance with capecitabine plus bevacizumab (QoL reported in 88% of patients)[105]. Nevertheless, 60% had any grade 3/4 toxicity

during maintenance, versus 34% in the observation arm (the authors comment on this is that it could be remaining toxicity from the induction). The OPTIMOX-3 DREAM investigators did not report the total grade 3/4 toxicity, but the most frequent grade 3/4 events in the erlotinib plus bevacizumab arm were skin rash (21%), diarrhoea (10%) and asthenia (5%)[194]. It should also be noted that the majority of patients in the DREAM trial had doublet oxaliplatin chemotherapy backbone for 3 months followed by de-escalation to 5FU+ bevacizumab for 3 months as induction. This could affect the general toxicity in the experimental maintenance phase to the better. Furthermore, the QoL was equal between the arms, although reported in only less than a quarter of the patients in DREAM. The percentage of patients that ended treatment in maintenance due to toxic events in our ACT studies were well in line with the DREAM and CAIRO3 doublet maintenance arms (8 and 10% respectively).

It is a drawback that we did not have QoL data to report from the ACT studies, since we cannot rule out a deterioration of some aspects of QoL by the added toxicity of erlotinib. Lessons from other trials on EGFR inhibition, however, and from clinical experience, are that the skin toxicity is often possible to control with local and systemic therapy, through active patient involvement. Better insight to a pro-active approach with up-front systemic tetracycline treatment could also improve compliance and QoL during EGFR inhibition since this approach is shown to reduce the incidence or severity of rash significantly[195]. However the use of antibiotics for long-term prophylaxis has disadvantages both for the patients and for the community.

In conclusion, combining bevacizumab and erlotinib might not be the most optimal maintenance regimen to the non-selected mCRC population since maintenance should aim to ameliorate symptom burden while minimizing medicament use. Most probably the negative results from our ACT trials have raised some caution and diminished the risk of over-interpretation of the survival gain of the succeeding DREAM trial.

Obviously there are patients, like Lars in my preface, who might benefit from erlotinib in combination with bevacizumab, but our means to find them in the process of treatment decisions still remain very limited. Targeted agents need personalised targeted strategies to maximise the therapeutic effect of the compound and minimise toxicity. We can foresee better applications of molecular pathology techniques in the report of tumours, but we will also need a better clinical translation of this down to our MDT boards, much as we have incorporated imaging methods. Compared to the phase II and III clinical trials, innovative and adaptive trial designs probably have better potential to explore the complex applications of genomic technology results in a faster and more targeted fashion.

To sum up, de-escalation to 5-FU or capecitabine remains the standard maintenance option, with optional addition of bevacizumab in those patients who received bevacizumab as part of the induction treatment.

What is the future role of metronomic chemotherapy?

The CAIRO3 trial reported the longest mPFS for an active maintenance regimen, with 8.5 months for bevacizumab plus metronomic normal-dose capecitabine. The next step would be to investigate a more optimal dosing of metronomic capecitabine in combination with bevacizumab, in order to minimise toxicity.

Metronomic chemotherapy has been investigated in clinical trials of many solid tumours, and the area was covered in a review investigating the impact of dosing of metronomic therapy on response[196]. In this trial 80 clinical and retrospective studies including over 3000 patients were found (number of patients >20 in each individual study). Only four studies were performed in CRC. No randomised reports were seen demonstrating effect in an early treatment setting and the authors found no association between dose intensity and any specific choice of drug and tumour response. In general, the toxic effects of metronomic chemotherapy dosing are minimal, and ranges from 0-5% grade 3-4 toxicity rate in the reviewed trials. This is in line with our findings in ACT2.

For a general mCRC population, in the absence of predictive biomarkers, bevacizumab *alone* does is not deemed "good enough" to use as an anti-angiogenic maintenance strategy. However there seems to be some patients that benefit from this strategy, and the same could hold for metronomic low dose capecitabine.

The tumour dormancy thesis of metronomic chemotherapy scheduling (Figure 6) remains to be explored in future trials also in relation to predictive biomarkers, *e.g.* circulating endothelial cells, circulating cancer cells or proteomics. I also believe metronomic capecitabine could be considered for CRC trials in the adjuvant setting, as proposed by Loven *et al.*[103] and by Prof David Kerr in a commentary on our findings published in the interactive community of Medscape Oncology (Metronomic Capecitabine: mCRC Maintenance Strategy Worth Assessing, *Medscape* Jan 25, 2016).

Angiogenesis inhibition: where are the biomarkers?

Methodological considerations papers III & IV

The design of the first translational study of this research project (paper III) was justified by the mechanistic discussion on bevacizumab-induced hypertension as an elusive predictive biomarker, together with the known potential association of vasoactive peptides with the cardiovascular response of the host.

There are potential confounders to the results in this small cohort. Both individual patient related factors, the varying induction chemotherapy regimens and the maintenance regimens could have diverging influence on the vasoactive peptide concentrations and TTP. Also, we have no data for vasoactive peptides in a control group treated without bevacizumab. One could argue that the patients treated with metronomic capecitabine (randomised in ACT2 to arm mut-C) should not be included in the analysis, since they did not get bevacizumab in the maintenance phase. Consequently, we performed statistical analyses without this group and despite reduced power we found no significant changes that would alter our conclusions (data not presented).

In the original publications evaluating measurement of the stable fragments of the three vasoactive peptides, they are reported to be highly stable in plasma, after storage in different temperatures and after repeated freeze thaw cycles[176, 181, 190]. In healthy subjects, Copeptin values are reported to be higher in men than in women. For MR-proADM and MR-proANP there are no gender differences, although there is a trend for rising values of these two peptides by age. Considering the use of intra-individual dynamic trends for the peptide levels in the analysis this is not likely to introduce bias to our results in paper III.

The study and interpretation of serum proteomics by microarray analysis (paper IV) is challenging since the dynamic range of proteins in the circulation is extremely wide, and the turnover times vary substantially between proteins. In paper IV, pre-analytical confounders related to sample handling procedures, which could diminish the quality of the sample, may also influence the results. Nevertheless, the sandwich antibody array is considered to be a reproducible and highly sensitive and specific method[147]. Semi-quantitative analysis reduces the applicability in a clinical setting, but the antibody array method is fairly easy to use and showed a robust pattern of signals for many proteins, which is consistent with findings from other reports using the same assay in cancer patients[197]. In order to improve the assay performance we made an attempt to standardise the array method as described in paper IV.

Interpretation and future directions

We are aware that the weak correlations between the individual vasoactive peptides and clinical outcome presented in paper III is not enough to draw firm conclusions on the predictive role of these dynamic biomarkers in relation to the bevacizumab effect (see also Figure 17). The association could also exist for patients who were not treated with bevacizumab. Nonetheless, the gathered results support our hypothesis that a host-dependent vascular effect linked with tumour angiogenesis and angiogenesis inhibition is associated with better response to bevacizumab containing treatment. Others have also proposed this link in relation to anti-angiogenic TKI treatment[150]. Various mechanisms are described to explain the synergism between bevacizumab and chemotherapy, such as vascular normalisation, decreased hypoxia, enhanced intra-tumoral drug delivery, and suppression of progenitor endothelial cells[37]. However, the paradox of how the tumour-starving anti-VEGF treatment at the same time can enhance the efficacy of chemotherapy is not fully understood. As described in the introduction chapter, the rise in blood pressure by anti-angiogenic treatment is believed to play a role in the equilibrium of events leading to an optimal tumour response.

One could also speculate on the role of anti-hypertensive medication in this context. In renal cancer, there are reports that lowering of raised blood pressure by anti-hypertensive treatment has no detrimental impact on the anti-angiogenic treatment effect of bevacizumab or sunitinib[150, 198]. Preclinical data supports this notion[199]. In fact, in the study by Rini *et al.* it was demonstrated that treatment with anti-hypertensive treatment at baseline had a substantial positive impact on PFS and OS[150]. Intriguingly, reports in the literature have shown that certain anti-hypertensive drug compounds such as Angiotensin II receptor blockers could exert an anti-tumoral effect *per se*, independent of the anti-angiogenic treatment. A study on human colon cancer cells demonstrated anti-proliferative effects of telmisartan[200], and a case report on mCRC demonstrated anti-tumoral response to irbesartan[201]. Improved clinical outcome including tumour response has been demonstrated in mCRC patients exposed to anti-hypertensive drugs[202], and recently another retrospective study reported that use of antihypertensive drugs were shown to increase the pCR rate in patients exposed to neoadjuvant radiotherapy for rectal cancer[203].

One explanation to the conflicting data on hypertension as a predictive biomarker for effect of bevacizumab could be the heterogenic definitions of blood pressure elevation. Another confounding factor could be the variety of use of anti-hypertensive drugs. One can speculate that some of the anti-hypertensive compounds may exert better synergism with anti-VEGF treatment than others. In fact, use of Angiotensin II type-1 receptor blockers were shown to be associated with better survival outcomes in mCRC patients treated with bevacizumab plus

oxaliplatin based chemotherapy in first line[204]. These results may be attributed to an anti-angiogenic effect of these drugs but it is also possible that anti-hypertensive treatment may contribute to the counterbalancing process of tumour vasculature normalisation seen with the anti-VEGF treatment. Anti-hypertensive treatment may interrupt the vicious circle of hypertension and vessel rarefaction as suggested by Battegay *et al.*[205]. In support of our results in paper III, the same effect could be facilitated by host-mediated release of other vasodilatory factors like the vasoactive peptide ADM in response to VEGF inhibition. In all, this might counterbalance the hypoxic state in the tumour during anti-VEGF treatment, with positive consequences on the chemotherapy potentiating effects of bevacizumab, or the accessibility of the small TKI compounds to the cancer cells. A schematic presentation of the study concept presented in paper III is shown in Figure 19.

We do not have fully validated data on the exact anti-hypertensive treatment used in patients from our Nordic ACT trials. I believe it would be of interest to perform a retrospective review of Nordic mCRC cohorts treated with chemotherapy plus bevacizumab, to explore the hypothesis of an association with use of different classes of anti-hypertensive medicaments and treatment effect. Depending on the results, a prospective approach could be discussed, together with further evaluation of vasoactive peptides as potential pharmacodynamic response biomarkers.
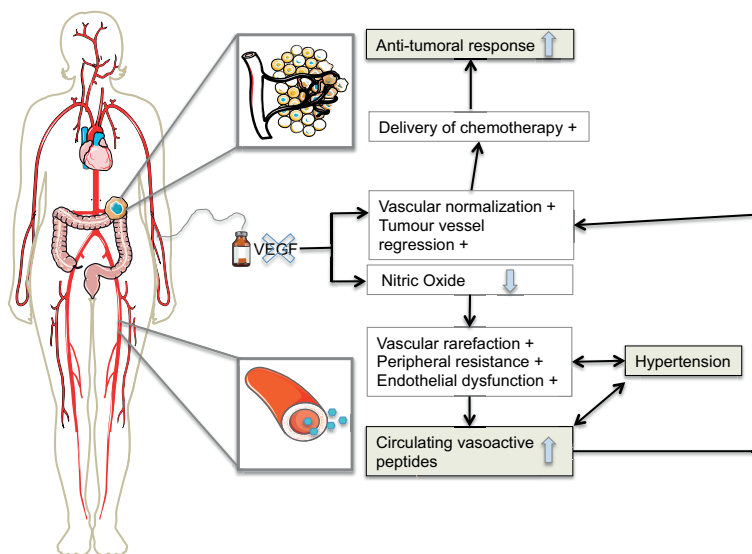
Figure 19.
Study concept paper III. Associations between tumour vascular effects (upper part of picture) and the host´s vascular response (lower part) during anti-angiogenic treatment, *e.g.* inhibition of VEGF by bevacizumab. VEGF, vascular endothelial growth factor.

In paper IV we studied angiogenesis related protein levels during response to chemotherapy plus bevacizumab and explored the association with duration of response to bevacizumab as maintenance therapy. The presented dynamic variations indicate that there is clearly detectable activity in the angiogenesis related signalling cascades during bevacizumab-based treatment, but with considerable inter-individual variations. Due to the small sample size and the exploratory nature of the study, the results must be interpreted with caution but some of our findings still deserve to be commented.

None of the proteins measured at baseline correlated with TTP. This was hardly surprising, given the many previous efforts that have failed to identify any clinically useful biomarkers for up-front prediction of anti-angiogenic drug efficacy.

In the serial analyses we found that increasing levels of three proteins (IL-8, Activin A and IGFBP-2) during chemotherapy plus bevacizumab were significantly correlated with a prolonged effect of bevacizumab maintenance. The

detectability of IL-8 and Activin A was very low in the assay, whereas IGFBP-2 was much more abundant.

As the name implies, IGFBP-2 binds the tumour associated growth factors IGF-1 and IGF-2, involving a system that contributes to the pathogenesis of CRC[206]. High levels of IGFBP-2 are associated with poor prognosis, whereas a decrease in IGFBP-3 is reported in patients during progression to chemotherapy. This may support our findings of rising levels of IGFBP-3 during treatment response. Our results for IGFBP-2 were more ambiguous. We found a weak significant correlation between rising levels of IGFBP-2 and longer response to maintenance therapy. Most likely the up regulation of IGFBP-2 mirrors the hypoxic state of the tumour since IGFBP-2 is up regulated by Hypoxia Inducible Factor -1α, i.e. a master transcriptional regulator of the hypoxic response[207]. Thus, increased secretion of IGFBP-2 may be interpreted as a surrogate marker of effective vascular regression. It has also been suggested that IGFBPs can act as negative regulators of IGF activity or influence tumour growth independent from IGFs[206]. Further studies are needed to clarify the role of the IGF cascades in relation to the effects of bevacizumab.

Besides looking for predictive biomarkers, we also wanted to study the dynamic changes during bevacizumab treatment, which could increase the knowledge on the mechanisms of action of the drug, but also to get a better understanding of molecular changes leading to resistance against bevacizumab.

The complex events induced by VEGF inhibition may vary depending on tumour type and tumour stage, and may be influenced by different combinations and sequencing of anti-tumoral treatments[47, 61]. Our analyses of protein changes during response to induction treatment showed decreasing levels of eight proteins and increasing levels of four proteins. Interestingly, almost all proteins that decreased had mostly proangiogenic properties, whereas those with most significant increase were primarily anti-angiogenic. This suggests that during successful treatment with chemotherapy and bevacizumab the effects on angiogenesis are not limited to the direct blockage of VEGF-A, but may include other collateral mechanisms that enhance the effect of angiogenesis inhibition.

One of the proteins that showed significant increase during response to induction treatment was Pentraxin-related protein (PTX)-3. PTX-3 is an extra-cellular matrix associated molecule that like C-reactive protein (CRP) belongs to the pentraxin-family. Numerous cell types including smooth muscle cells and endothelial cells synthesise PTX-3, and it is up regulated by hypoxia. Elevated circulating PTX-3 levels are seen in cardiovascular and inflammatory disease such as myocardial infarction, pre-eclampsia and infection[208]. PTX-3 inhibits the pro-angiogenic effects of the FGF-2/FGFb, and it has low affinity for VEGF[209]. It is possible that our findings of a rise in PTX-3 during response to treatment with bevacizumab

could be involved in the rise in blood pressure during bevacizumab treatment. In fact, rising PTX-3 levels have been suggested as a novel biomarker of hypertension[208], and as previously discussed hypertension is associated with improvement of anti-angiogenic treatment effect. In our small study the increase in PTX-3 during chemotherapy plus bevacizumab did not predict the response to treatment including maintenance bevacizumab single therapy, and it is possible that a rise in PTX-3 levels occurs in response to chemotherapy alone. Nevertheless, it merits further investigation as a potential biomarker for treatment effect in patients receiving anti-VEGF treatment.

By use of a multiplex protein array method we made an attempt to discern a pattern in the complex regulation of angiogenesis also with focus on resistance to treatment with bevacizumab alone. Multiple mechanisms of resistance to anti-angiogenic therapy have been described in comprehensive reviews on this subject[127, 210-212]. Cancers can either be intrinsically resistant to the therapy at start of treatment, or develop acquired resistance during VEGF/VEGFR-inhibition. In tumours, new vessels can grow from the existing vasculature and be protected by pericytes by mechanisms that are not VEGF dependent. Furthermore, tumour hypoxia can induce recruitment of endothelial progenitor cells from the bone marrow, induce local tissue invasion programmes in cancer cells, and select more aggressive cancer cell clones. These adaptive resistance mechanisms are likely to be orchestrated by the cancer cells and their microenvironment including fibroblasts, vascular cells and cells of the immune system. Increased signalling of pro-angiogenic factors is thereby a way to escape the VEGF/VEGFR blockade. Following a significant decrease in the levels of MMP-8, TIMP-4 and EGF during tumour inhibition, we found a corresponding significant increase in these factors during progression in maintenance phase. These are factors involved in cancer cell survival, proliferation and migration[197]. In addition, rising levels of tissue factor (TF) were demonstrated at the time of progressive disease. TF is a major activator of intra-tumoral coagulation, which has been strongly associated with angiogenesis[213]. The large inter-individual variation in protein alterations by the time of tumour progression supports the view that mechanisms of resistance differ between patients.

Stimulated by our results we believe that multiplex protein assays would be convenient to use in future trials on angiogenesis inhibitors and other targeted agents, both in the search for predictive biomarkers and to further increase our knowledge of mechanisms of resistance to personalise anti-angiogenic treatment in the later treatment lines. A logical next step will be to further explore angiogenesis-related dynamic proteomic changes in larger cohorts, including non-responding patients and patients treated with chemotherapy alone, but also in remaining patients from the ACT2 trial, that received erlotinib in addition to bevacizumab, or metronomic capecitabine as maintenance treatment.

For the anti-angiogenic agents to maintain their position in the arsenal of targeted agents, it is of crucial importance to gain better knowledge of the host-specific response to these drugs. Here, pharmacodynamic biomarker studies are valuable in order to find out how hard the targeted agent hits its goal, how fast the target is moving, where it is taking cover and who its allies are. Additionally, we need to understand under which conditions the anti-angiogenic agents exert the most optimal and durable effect on the cancer. This requests further exploration of the functional synergism with other biologics or non-targeted drugs. It could be that the most powerful partners to bevacizumab remains to be found.

Strengths and limitations

"For things to reveal themselves to us, we need to be ready to abandon our views about them"

- Thích Nhất Hạnh

This thesis project as a whole has limitations in the design and limited sizes of the studies as discussed above. The inclusion rates in the clinical trials were satisfying. However, due to the fast change in clinical praxis during the recruitment period of ACT, the larger proportion of patients withdrawn *e.g.* due to liver resection could not be anticipated. This forced us to decrease power in the ACT trial, which is of course a limitation, since the results turned out negative. The results from the ACT trial were the first to be presented on the addition of erlotinib to bevacizumab in a randomised mCRC cohort. Furthermore, a randomised comparison between bevacizumab and metronomic capecitabine, as in the KRASmut group of the ACT2 trial, had never been published before. Despite some concerns regarding the internal validity of the ACT and ACT2 trials, a fair external consistency in terms of numerical survival data is seen in comparison with later publications. In paper III and IV the cohort sizes were restricted owing to plasma sample availability. A rather large proportion of plasma and serum samples had to be excluded from the analysis, which was not expected. A shortcoming of academic sponsorship is limited resources for very frequent monitoring and quality control of blood and tissue sampling during the conduct of the trial. Some clinical trial centers depend on a larger group of nurses to deliver chemotherapy study drugs, and staffing resources in the out-patient wards vary, why the sampling of blood at some occasions might be missed by lack of time or experience. Furthermore, the unintentional disappearance of samples during the alliquoting and handling process on site is a possible explanation. The vasoactive peptide and hypertension theme is relevant, and the results from paper III plausible from a biological perspective, although the findings of the translational studies have not lead to any firm conclusions about new enough clinically relevant predictive biomarker candidates for anti angiogenic treatment.

Notwithstanding, clear strengths in the conduction of the project are seen. First, it is a multicentre collaboration between Swedish and Danish research groups, which has resulted in additional fruitful translational publications from the Danish group

using the ACT cohort[138, 139]. The use of multiple induction treatment schedules strengthens the generalizability of the results. Additionally, the clinical trials were recruiting in a fairly high pace in a consecutive order, which strengthens the quality of the pooled dataset in ACT2. The translational research questions of the ACT2 trial were discussed and planned through a protocol amendment before the launch of the trial to secure additional blood sampling for the vasoactive biomarker analysis. Moreover, the translational approach and biomarker guided randomisation in the ACT2 is in harmony with efforts in oncology to accelerate progress in precision medicine. Finally, the ACT trial was presented at the oral poster CRC session ASCO meeting in 2011 and the ACT2 trial was presented with an abstract poster at the ESMO congress 2014, which ensured early access to the preliminary clinical data.

Conclusions

- The effect of adding erlotinib to bevacizumab was not improved compared to bevacizumab alone as maintenance therapy in our cohort of mCRC patients following response to first line induction treatment with chemotherapy and bevacizumab.

- The toxicity of adding erlotinib to bevacizumab was substantial, why this targeted combination as maintenance strategy is questionable.

- The addition of erlotinib to bevacizumab was not superior to bevacizumab alone as maintenance therapy in a pooled randomised cohort of KRAS wildtype mCRC patients.

- KRAS status does not seem to be a strong predictive biomarker for the effect of erlotinib in CRC.

- Low dose metronomic capecitabine as maintenance treatment was not significantly different compared to bevacizumab alone in terms of effect and toxicity profile, in a small cohort of mCRC patients with KRAS mutant tumours.

- Rising levels of circulating vasoactive pro-peptides associated with blood pressure regulation (MR-proADM, MR-proANP and Copeptin) was weekly correlated with better response to bevacizumab containing treatment in mCRC.

- Use of the multiplex protein array method for dynamic semi-quantification of multiple circulating angiogenesis related factors was feasible in a small cohort of mCRC clinical trial participants.

- We found no specific dynamic protein pattern with clear predictive potential for effect of bevacizumab single maintenance treatment.

Populärvetenskaplig sammanfattning

Cancer i tjock- eller ändtarm, s.k. kolorektalcancer, är i Sverige den tredje vanligaste cancersjukdomen hos båda könen, efter bröstcancer och prostatacancer. Dottersvulster i andra organ, så kallade fjärrmetastaser påträffas i ca en fjärdedel av alla fall vid diagnostillfället. Ungefär hälften av de patienter som genomgår behandling i botande syfte, får senare återfall. För de flesta av dessa patienter erbjuds någon form av palliativ onkologisk behandling, t ex cytostatika, i syfte att bromsa upp cancerns tillväxt. Målet är att förlänga överlevnaden för individen, samt att lindra sjukdomsrelaterade symtom. Genom att cancerläkemedel kombineras i olika behandlingsregimer, har överlevnaden och nyttan med behandling för denna patientgrupp ökat i en stadig takt. I aktuella behandlingsstudier visar resultaten att hälften av patienterna med mCRC lever längre än cirka 2,5 år från onkologisk behandlingsstart.

Detta avhandlingsarbete studerar nya behandlingsstrategier i syfte att ytterligare förbättra dessa resultat. Målet är också att hitta bättre metoder som förenklar behandlingen för patienten, och som samtidigt minskar de biverkningar som uppstår under behandling med kemoterapi, vilket kan optimera patientens livskvalitet.

Behandlingen vid mCRC inleds ofta med cytostatikakurer innehållande läkemedlen 5-fluorouracil (5-FU) i kombination med oxaliplatin eller irinotekan. Det är möjligt att i vissa fall lägga till så kallad målstyrd behandling. De målstyrda läkemedlen angriper särskilda molekyler på tumörcellen eller i blodbanan, vilka har en avgörande roll för tumörens utveckling, överlevnad och förmåga att sprida sig i kroppen. Exempel på sådana molekyler är den epidermala tillväxtfaktorn (EGFR) och vaskulär endotelial tillväxtfaktor, VEGF. Bevacizumab (Avastin®) är en målstyrd antikropp riktad mot VEGF. För att cancer ska utvecklas och sprida sig i kroppen behöver tumörerna bilda nya kärl som kan försörja tumörerna med syre och näringsämnen. Genom att binda upp VEGF i blodbanan kan detta hämma kärlnybildningen, dvs. angiogenesen. Bevacizumab ökar framför allt effekten av kemoterapin, och kan på så vis ytterligare förlänga överlevnaden för en andel av mCRC patienterna. Erlotninb (Tarceva®) är ett annat målstyrt läkemedel som verkar på insidan av EGFR receptorn som sitter på ytan av cancercellen. Genom att hämma EGFR kan erlotinib stoppa den signalkaskad som leder till att cellen kan överleva och dela sig ohämmat. Erlotinib har visat effekt för t ex patienter

med icke-småcellig lungcancer, men det saknas studier som visat tillräckligt god effekt av erlotinib vid mCRC. När detta avhandlingsprojekt inleddes verkade resultaten lovande från de tidiga studier som undersökt möjligheten att kombinera dessa två målstyrda läkemedel med varandra.

Cytostatika ger med tiden upphov till svåra biverkningar, så som trötthet, illamående, diarréer, viktnedgång, infektionskänslighet och nervpåverkan. En fördel med bevacizumab är att det inte ger upphov till så många symptomgivande biverkningar som cytostatika. När behandling med cytostatika +/- bevacizumab ges brukar de patienter som har god tumör-respons och god behandlingstolerans antingen få fortsätta behandling till dess att effekten avtar, eller till dess att biverkningarna blir oacceptabla. Det har visat sig att man, utan att äventyra överlevnaden, kan trappa ner intensiteten av behandlingen innan sådana biverkningar uppstår genom att t ex sätta ut oxaliplatin eller irinotekan. Denna strategi kallas underhållsbehandling (maintenance treatment), och ges ofta med enbart 5-FU + /- bevacizumab för att minska biverkningarna och hålla sjukdomen i schack. Man kan också göra en fullständig behandlingspaus, men studier har visat att underhållsbehandling istället förlänger den tid som patienten lever utan att sjukdomen växer till, d.v.s. den progressionsfria överlevnaden (PFS).

I det första delarbetet (**paper I**) presenteras den första studie som publicerade data för tilläggseffekten av erlotinib till bevacizumab som underhållsbehandling i mCRC, ACT studien. Vi inkluderade 249 patienter med tidigare obehandlad mCRC. Patienterna fick inledningsvis cytostatika med 5FU+ antingen oxaliplatin eller irinotekan plus bevacizumab under 4,5 månader. De patienter som inte hade gått ur studien under denna tid, och som hade tillräckligt god tumör respons, lottades då till att få underhållsbehandling antingen med bevacizumab enbart, eller med bevacizumab plus erlotinib. Behandlingen gavs tills röntgenkontroller visade tecken på signifikant tumörtillväxt, d.v.s. progress, eller till dess att behandlingen behövde avbrytas p.g.a. andra skäl, t ex biverkningar.

ACT studien visade att de patienter som fick tillägget av erlotinib till bevacizumab hade en median PFS på 5,7 månader från start av underhållsbehandling, jämfört med 4,2 månader för de som fick enbart bevacizumab. Skillnaden var dock inte statistiskt signifikant, och den totala överlevnaden skiljde sig inte heller åt. Tillägget av erlotinib gav upphov till något mer biverkningar, t ex trötthet och acneliknande hudutslag.

Det verkar dock vara så att en liten andel av patienterna kan dra nytta av denna behandlingskombination. För att bättre kunna förutse effekten av en behandling önskar vi hitta något mätbart tecken i patienten eller i tumören, som kan associeras med ett bättre behandlingssvar, sk.prediktiva biomarkörer. En biomarkör kan vara en blodanalys, ett symtom, eller en genetisk förändring, s.k. mutation i ett vävnadsprov från tumören.

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I avhandlingens andra delarbete (**paper II**) har vi undersökt mutationer i genen som kodar för ett protein, KRAS, som en möjlig biomarkör för effekt av erlotinib. Vi misstänkte att om KRAS genen är muterad i tumören (KRASmut) skulle stimulering av EGFR på cellens yta innebära ohämmad signalering och celldelning oavsett om man ger erlotinib i syfte att försöka hämma EGFR. Vi ville alltså studera om erlotinib hade en bättre effekt i de patienter som inte har mutation i KRAS, vilket kallas KRAS vildtyp (KRASwt). I detta delarbete, ACT2 studien, inkluderades 233 patienter, och nu lottades endast patienterna med KRASwt tumör till behandling på samma sätt som i första ACT studien. Vi fann att erlotinib-effekten inte var bättre i denna patientgrupp och har därmed dragit slutsatsen att KRAS mutation inte är någon lämplig prediktiv biomarkör för effekten av erlotinib. Andra studier som publicerats på senare år stödjer också detta fynd.

För de patienter som hade en KRAS mutation valde vi att studera en annan typ av strategi som kallas metronomisk cytostatikabehandling. Cytostatika ges vanligen puls-vis i maximal tolererad dos för bästa effekt. Detta ger biverkningar på frisk vävnad, vilket gör att man måste ha kortare pauser mellan behandlings kurerna. Istället har prekliniska studier på celler och djur visat att metronomiska doser, d.v.s. låga doser dagligen eller flera gånger per dag kan hålla tumörer i schack. Detta sker delvis genom mekanismer som hämmar angiogenesen. Metronomisk cytostatikabehandling med 5-FU i form av capecitabin är lämpligt då capecitabin ges i tablettform. I ACT2 jämförde vi bevacizumab behandling med låg daglig dos (metronomisk) capecitabin behandling som underhållsbehandling, och fann ingasignifikanta skillnader i effekt eller biverkningar. Vi drar slutsatsen att metronomiskt capecitabin är en behandling som förtjänar att studeras vidare.

En vanlig och tidig biverkan till bevacizumab är förhöjt blodtryck, hypertoni. Vissa studier visar att de patienter som får hypertoni under behandlingen verkar ha en bättre nytta av bevacizumab-tillägget. Anledningen till detta är inte fullständigt klarlagd. Tyvärr fungerar det inte i praktiken att använda blodtrycks-mätning som prediktiv biomarkör, och trots intensiv forskning saknas ännu metoder för att förutse effekten av angiogenes-hämmande läkemedel. Detta innebär att många patienter får bevacizumab i onödan, vilket inte bara ökar risken för biverkningar för patienterna utan också ökar kostnaderna för samhället.

I det tredje delarbetet (**paper III**) undersökte vi tre vasoaktiva peptider som visat sig vara kopplade till blodtrycksreglering och angiogenes; Adrenomedullin (ADM), Atrial Natriuretic Peptid (ANP) och Copeptin. Vi analyserade dessa peptidnivåer i 97 patienter från ACT2 studien. Stegring av peptidnivåer kunde inte kopplas till en tidig hypertoniutveckling och hypertoni var inte signifikant associerat till bättre behandlingssvar. Vi fann däremot att stegring av dessa peptider under de första sex behandlingsveckorna med cytostatika +bevacizumab var signifikant associerat med bättre behandlingsrespons. Detta stärker teorin om

att effekter i patientens kärlsystem bidrar till behandlingssvaret i tumörerna. Vasoaktiva peptider kan därmed vara av intresse att undersöka vidare som möjliga dynamiska prediktiva biomarkörer vid angiogeneshämmande behandling.

I den fjärde studien (**paper IV**) undersökte vi multipla angiogenes-relaterade proteiner i blodprov från 22 patienter som lottats i ACT2 till underhållsbehandling med enbart bevacizumab. Vi samlade prover vid tre tillfällen under behandlingstiden. Proteinerna analyserades med så kallad multiplex immuno-array metodologi där 55 olika protein undersöks samtidigt i ett och samma blodprov. Vi beskriver i studien hur metoden kan standardiseras. Fyra av proteinerna ökade signifikant under respons på cytostatika plus bevacizumab, och flertalet av dessa proteiner hade angiogenes-hämmande funktion. Samtidigt minskade åtta protein med angiogenes-stimulerande egenskaper. För tre av proteiner associerades stegring av nivån under denna del av behandlingen med längre behandlingssvar på underhållsbehandling med bevacizumab. Resultaten går inte att översätta till några kliniskt signifikanta slutsatser, p.g.a. den lilla grupp patienter som undersöktes. Däremot är protein-array analys en praktiskt genomförbar metod som kan användas till att vidare undersöka proteinmönster under behandling med målstyrdaläkemedel och/eller metronomisk behandling. Detta kan i förlängningen leda till ökade kunskaper om dynamiska skeenden i patienten som kan relateras till effekt och resistensutveckling av angiogenes-hämmande läkemedel vid cancersjukdom.

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- Eckhart Tolle in A New Earth

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> "Relationships are laboratories of the spirit, they are hospitals of the soul."

> - Marianne Williamson

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STUDY POPULATION ACT and ACT2

Patient Inclusion Criteria

1. Patients with histological confirmed diagnosis of adenocarcinoma of the colon or rectum, untreated yet with chemotherapy for metastatic disease (prior adjuvant chemotherapy for CRC allowed if ended >6 months before treatment start), and who are scheduled to start first line fluoropyrimidine-based chemotherapeutic treatment.
2. Tumour tissue available for determination of KRAS mutational status (only applicable in ACT2)
3. Age ≥18.
4. Measurable disease according to RECIST criteria.
5. Blood sample and paraffin embedded tumour material for translational research.
6. ECOG/WHO performance status 0 or 1.
7. Life expectancy more than 3 months.
8. Haematology and blood chemistry including haemoglobin >90 g/L (may be transfused to maintain or exceed this level), absolute neutrophil count ≥1,5 x 109/L, platelets ≥100 x 109/L, bilirubin ≤ 1.5 ×ULN, ALAT ≤ 2.5 × ULN (<5 ×ULN if liver metastases), creatinine ≤ 1.5 ×ULN, PK ≤ 1.5; APTT<1.5 ULN.
9. Urine dipstick of proteinuria <2+. Patients discovered to have ≥2+ proteinuria on dipstick urinalysis at baseline, should undergo a 24-hour urine collection and must demonstrate ≤ 1 g of albumin/24 hr.
10. Fertile men and women of childbearing potential (<2 years after last menstruation in women) must use effective means of contraception (oral contraceptives, intrauterine devices, barrier method of contraception in conjunction with spermicidal jelly or surgically sterile).
11. Signed written informed consent to the clinical study and translational research according to ICH/GCP and the local regulations (approved by the Institutional Review Board [IRB]/Independent Ethics Committee [IEC]) will be obtained prior to any study specific screening procedures.
12. Patient must be able to comply with the protocol.

Patient Exclusion Criteria

1. Previous treatment with first-line chemotherapy for metastatic CRC.
2. Previous adjuvant therapy within 6 months.
3. Major surgical procedure, excision biopsy or significant traumatic injury within 28 days prior to Day 0 (Patients must have recovered from any major surgery), or anticipation of need for major surgical procedure during the course of the study.
4. Planned radiotherapy against target lesions.
5. Clinical or radiological evidence of CNS metastases.
6. Past or current history (within the last 5 years) of malignancies except for the indication under this study and curatively treated basal and squamous cell carcinoma of the skin or in-situ carcinoma of the cervix.
7. Serious non-healing wound or ulcer.
8. Evidence of bleeding diathesis or coagulopathy.
9. Uncontrolled hypertension despite poly-pharmacological antihypertensive treatment.
10. Clinically significant (i.e. active) cardiovascular disease for example cerebrovascular accidents (≤6 months) , myocardial infarction ≤ 6 months, New York Heart Association (NYHA) grade II or greater congestive heart failure, serious cardiac arrhythmia requiring medication.
11. Current or recent (within 10 days prior to study treatment start) use of full dose oral or parenteral anticoagulants for therapeutic purposes i.e. except anticoagulation for maintenance of patency of permanent indwelling IV catheters.
12. Participation in another investigational study within 30 days prior to enrolment.
13. Ongoing treatment with aspirin (>325 mg/day) or other medications known to predispose to gastrointestinal ulceration.
14. Any significant ophthalmologic abnormality, especially severe dry eye syndrome, keratoconjunctivitis sicca, Sjögren's syndrome, severe exposure keratitis or any other disorder likely to increase the risk of corneal epithelial lesions. The use of contact lenses is not recommended during the study. The decision to continue to wear contact lenses should be discussed with the patient's treating Oncologist and the ophthalmologist.
15. Pregnancy (positive pregnancy test) and lactation.
16. Known hypersensitivity to any contents of the study drugs.
17. Any other serious or uncontrolled illness, which, in the opinion of the investigator, makes it undesirable for the patient to enter the trial.

A randomized phase III trial on maintenance treatment with bevacizumab alone or in combination with erlotinib after chemotherapy and bevacizumab in metastatic colorectal cancer: the Nordic ACT Trial

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Background: The main objective was to study the effect on progression-free survival (PFS) of adding erlotinib to bevacizumab as maintenance treatment following chemotherapy and bevacizumab as first-line treatment of metastatic colorectal cancer (mCRC).

Patients and methods: Patients with untreated mCRC received doublet chemotherapy + bevacizumab during 18 weeks and those without tumor progression were eligible for randomization to bevacizumab + erlotinib (arm A) or bevacizumab alone (arm B), until progression or unacceptable toxic effect.

Results: Of the 249 patients enrolled, 80 started maintenance treatment in arm A and 79 in arm B. The rate of any grade 3/4 toxic effect was 53% in arm A and 13% in arm B. Median PFS was 5.7 months in arm A and 4.2 months in arm B (HR = 0.79; 95% confidence interval 0.55–1.12; $P = 0.19$). Overall survival (OS) from start of induction chemotherapy was 26.7 months in the randomized population, with no difference between the two arms.

Conclusions: The addition of erlotinib to bevacizumab as maintenance treatment after first-line chemotherapy in mCRC did not improve PFS significantly. On-going clinical and translational studies focus on identifying subgroups of patients that may benefit from erlotinib in the maintenance setting.

Clinical Trials number: NCT00598156.

Key words: metastatic colorectal cancer, bevacizumab, erlotinib, maintenance treatment

introduction

A majority of patients with metastatic colorectal cancer (mCRC) suffer from incurable disease, where the treatment goal is to prolong survival and improve quality of life. Most of these patients receive combination chemotherapy up-front. Addition of bevacizumab, an antibody directed against the vascular endothelial growth factor (VEGF) has been shown to improve outcome [1, 2].

During palliative chemotherapy, patients develop side-effects that sooner or later will lead to modification or interruption of the treatment. Finding a maintenance treatment that could prolong the progression-free interval without serious side-effects would be clinically useful. One option is to continue with bevacizumab during the chemotherapy-free period [3].

Preclinical studies have indicated that dual targeting of both epidermal growth factor receptor (EGFR) and VEGFR pathways may result in supra-additive antitumor effects[4, 5]. In nonsmall-cell lung cancer (NSCLC) bevacizumab combined with the EGFR tyrosine kinase inhibitor erlotinib has shown to be an effective maintenance treatment [6]. In mCRC, this maintenance strategy was tested in a phase II trial [7], with promising results.

The present phase III trial was designed to compare the efficacy and toxic effect of maintenance treatments with bevacizumab + erlotinib and bevacizumab after induction treatment with first-line chemotherapy plus bevacizumab in patients with mCRC.

patients and methods

patient population

Patients ≥18 years of age were eligible for the study if they had mCRC, histologically confirmed adenocarcinoma, not previously treated with

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chemotherapy for metastatic disease, ECOG performance status 0–1, measurable disease according to RECIST v. 1.0, adequate hematological, renal, and hepatic functions. Prior adjuvant chemotherapy for CRC was allowed if ended >6 months before inclusion.

Patients were excluded in case of major surgery within 28 days before treatment start, CNS metastases, other malignancies within 5 years, serious nonhealing wound or ulcer, bleeding diathesis or coagulopathy, significant ophthalmologic abnormality, uncontrolled hypertension, clinically significant cardiovascular disease within 6 months, or start of anticoagulants for therapeutic purposes within 10 days.

study design

This open-label phase III randomized clinical trial recruited patients from 15 sites in Denmark and Sweden between May 2007 and November 2009. All patients signed written informed consent before start of induction treatment. The study was approved by ethics committees and medical products agencies in both Sweden and Denmark, and was conducted in accordance with the International Conference of Harmonization guideline for Good Clinical Practice and with the Declaration of Helsinki.

This was an investigator sponsored trial. Roche supported the study. A representative from Roche (DB) took part in designing the study protocol but Roche had no role in interpretation of the data.

induction phase

Enrolled patients started induction treatment according to investigator's choice with six cycles of XELOX/XELIRI or nine cycles of FOLFOX/ FOLFIRI in combination with bevacizumab (for chemotherapy schedules see supplementary material, available at *Annals of Oncology* online). Patients who had at least stable disease after completion of induction treatment were eligible for randomization to one of the maintenance treatments.

maintenance phase

Randomization was carried out in a 1:1 ratio to maintenance treatment with either bevacizumab 7.5 mg/kg every 3 weeks + erlotinib 150 mg daily (arm A) or bevacizumab 7.5 mg/kg as a single agent every 3 weeks (arm B). Stratification was carried out according to best response to induction therapy (complete or partial response, CR/PR, versus stable disease, SD) and to whether an oxaliplatin-containing induction regimen had been used or not.

Maintenance treatment was given until disease progression (PD), intolerable toxic effect, withdrawn consent, planned surgery, noncompliance, serious protocol violation, or lost to follow-up. After withdrawal from the study, further antitumoral therapy was allowed at the

Table 1. Patient characteristics at baseline

Patient characteristics						
			FAS[b] (N = 159)			
			Arm A (n = 80) bevacizumab + erlotinib		Arm B (n = 79) bevacizumab	
	ITT[a] (N = 249)					
	No.	%	No.	%	No.	%
Age, years						
Median	64		64		65	
Range	26–82		48–80		43–82	
Gender						
M/F		58/42		66/34		54/46
ECOG[c]						
0/1		67/33		73/27		67/33
Primary tumor site						
Colon	150	60	44	55	53	67
Rectum	83	33	30	38	19	24
Both	16	6	6	8	7	9
Primary tumor *in situ*						
Yes	88	35	31	39	26	33
Previous adjuvant chemo						
Yes	38	15	13	16	15	19
Previous oxaliplatin						
Yes	9	4	4	5	3	4
Metastatic site						
Liver	185	74	62	78	58	73
Lung	103	41	33	41	37	47
Lymph	91	37	35	44	28	35
Other	56	22	5	6	9	11
Liver only	78	31	29	36	18	23
Number of metastatic sites						
1	117	47	39	49	34	43
>1	132	53	41	51	45	57

[a]Intent-to-treat population.
[b]Full analysis set population.
[c]Eastern Cooperative Oncology Group Performance status.

investigator's discretion. Survival and additional cancer therapies were documented for all patients every third month until time of death or end of study.

dose modification of study drugs

Bevacizumab was permanently discontinued in case of gastrointestinal perforation, arterial thromboembolic event, symptomatic grade 4 venous thromboembolism, grade 3/4 hemorrhagic event, grade 4 hypertension, or grade 4 proteinuria. Bevacizumab treatment could be temporarily interrupted in the event of lower grade toxic effect or wound healing complication.

Reduction of the erlotinib dose to 100 mg daily was allowed in case of intolerable toxic effect. If toxic effect did not resolve to ≤grade 2 within 2 weeks, a second dose reduction to the minimum dose of 50 mg was carried out. Doses were not re-escalated except in the event of rash resolving to grade ≤2. A dose interruption of more than 3 weeks led to discontinuation of erlotinib and withdrawal from the study.

evaluation of response and toxic effect

Tumor response was evaluated according to the RECIST v.1.0 criteria with a CT scan of thorax and abdomen within 28 days before enrollment, after 8–12 weeks of induction treatment, and before randomization to
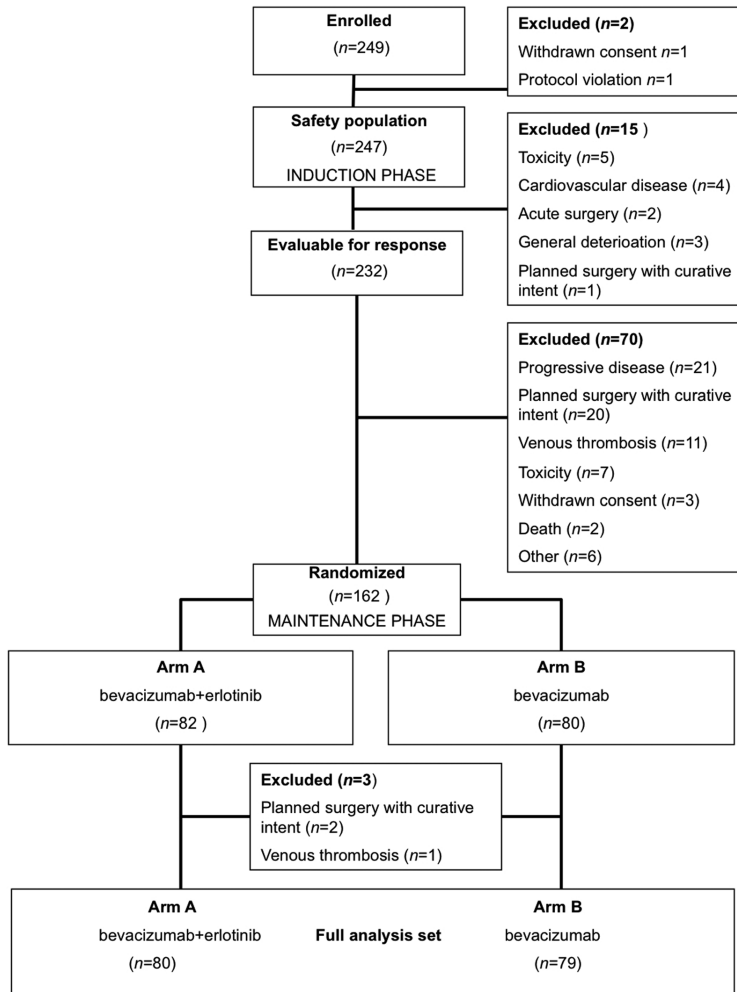

Figure 1. Consort diagram 190 × 254 mm (150 × 150 DPI).

maintenance treatment. No independent radiology review was done. During the maintenance phase, CT scans were carried out every 9 weeks. Toxic effect was recorded according to NCI-CTCAE version 3.0.

statistical analysis

The primary end point was to assess the effect on progression-free survival (PFS) when adding erlotinib to bevacizumab (arm A) compared with treatment with bevacizumab alone (arm B) in a maintenance setting after chemotherapy and bevacizumab in the first-line treatment of mCRC. The study was designed to detect a difference in PFS between 5 months in arm A and 3 months in arm B. In order to detect this difference at a two-sided significance level of 5% and a power of 90%, 168 randomized patients were needed. With an estimated attrition rate of 30% inclusion of 240 patients was planned. Owing to a higher than expected attrition rate, the statistical analysis plan was amended to investigate the primary end point at a power of 80%, for which 126 events were required.

Toxic effect in the induction phase was studied in the safety analysis population, defined as all patients who had received at least one dose of induction treatment. Response on induction treatment was analyzed in the assessable patients in the intention-to-treat (ITT) population. Toxic effect in the maintenance phase was analyzed in the full analysis set population (FAS), i.e. randomized patients who had received at least one dose of maintenance treatment. PFS and overall survival (OS) from start of maintenance treatment were analyzed in the FAS population.

Comparisons in PFS and OS between treatment arms were made with a two-sided log-rank test, and Kaplan–Meier estimates were calculated. Follow-up time was calculated as Kaplan–Meier estimated potential follow-up [8].

results

patient characteristics

A total of 249 patients were enrolled in the study. There were no major differences in demographic and baseline disease characteristics of the patients between the two treatment arms (Table 1). Two patients never started treatment and the safety population consisted of 247 patients (Figure 1). Median follow-up from time of randomization in the FAS population was 36.8 [95% confidence interval (CI) 32.2–41.8] months.

efficacy and safety

induction treatment. Among the 232 patients assessable for response, 48% had PR, 43% SD, and 9% PD. Divided according to chemotherapy regimen, the PR rate was 52% with FOLFOX ($n = 50$), 44% with XELOX ($n = 121$), 50% with FOLFIRI ($n = 30$), and 55% with XELIRI ($n = 31$). The PR rate on induction treatment of the FAS population was 59% of the patients subsequently randomized to arm A and 54% of patients randomized to arm B.

The most common grade 3/4 toxic effects during induction treatment were diarrhea (10%), nausea/vomiting (9%), venous thromboembolic events (8%), infection (6%), fatigue (5%), neuropathy (5%), and hypertension (3%). Forty-five percent of the patients had at least one grade 3/4 toxic effect during induction therapy. Serious gastrointestinal toxic effect, considered at least possibly related to bevacizumab, occurred in seven patients during the course of the study. One patient without primary tumor left *in situ* had a fatal bowel bleeding during induction treatment. Six patients had intestinal perforations of which two occurred in or adjacent to a primary

tumor. Five of the perforations occurred during the induction phase and one during maintenance. Three of the six perforations were fatal.

maintenance treatment. In the full analysis set population, 159 patients were included (Figure 1). The primary end point analysis was carried out when 131 events had occured. Median PFS was 5.73 months in arm A and 4.23 months in arm B. This difference was not statistically significant, HR = 0.79; 95% CI 0.55–1.12; $P = 0.19$ (Figure 2A).

The median duration of maintenance treatment was 5.5 months in arm A and 5.2 months in arm B. The main reasons for ending maintenance treatment were PD, 68% versus 82% in arms A and B, respectively, and toxic effect in 13% versus 4%. Four patients in arm A and two in arm B were withdrawn due to planned surgery with a curative intent.

Generally, there were more side-effects in arm A than in arm B, especially skin rash, diarrhea, and anorexia (Table 2). There were two treatment-related deaths in maintenance phase;
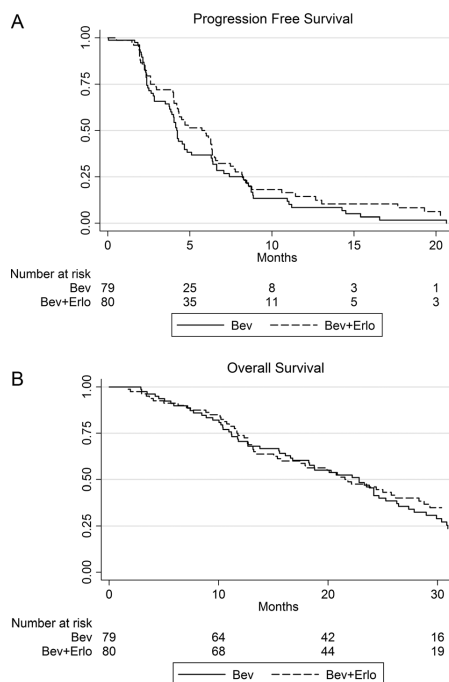

Figure 2. Progression-free survival (A) and overall survival (B) from start of maintenance phase treatment in the full analysis set population. Median PFS was 5.73 months in arm A (bevacizumab + erlotinib) and 4.23 months in arm B (bevacizumab). HR = 0.79; 95% confidence interval (CI) 0.55–1.12; $P = 0.19$. Median OS was 21.5 [15.4–28.3] months in arm A and 22.8 [16.6–25.3] months in arm B, HR = 0.88; 95% CI 0.61–1.27; $P = 0.51$ 1411 × 1058 mm (72 × 72 DPI).

one case of liver failure regarded possibly related to erlotinib in arm A and one intestinal perforation in arm B. The frequency of any grade 3/4 toxic effect was 53% in arm A and 13% in arm B. The dose of erlotinib was reduced in 34 (42.5%) patients in arm A. In 24 of these, the daily dose was decreased to 100 mg, whereas 8 and 2 patients had further dose reductions to 50 and 0 mg, respectively.

At the time of analysis, 118 patients of the FAS population had died. Median OS from start of maintenance treatment was 21.5 months in arm A and 22.8 months in arm B, HR 0.88; 95% CI 0.61–1.27; $P = 0.51$ (Figure 2B). The OS from start of induction chemotherapy was 24.7 months in the ITT population ($n = 249$) and 26.7 months among the 162 randomized patients.

poststudy treatment. Most patients (86%) received further anticancer drugs after the termination of maintenance treatment, with no difference between the treatment arms (S1).

The proportions of patients that received any type of EGFR-inhibiting agent in subsequent lines of therapy were also similar, 25% and 23% in arm A and B, respectively.

discussion

This study does not show a statistically significant gain in PFS when erlotinib is added to bevacizumab as maintenance treatment of mCRC, after first-line chemotherapy combined with bevacizumab, although a numerical increase in median PFS from 4.2 to 5.7 months is noted (HR 0.79, $P = 0.19$).

In recent years, increasing efforts have been made to identify convenient maintenance strategies that could prolong the time to progression with limited toxic effect and thereby optimizing the quality of life for mCRC patients. One strategy is to use targeted agents, which generally are less toxic than chemotherapy. The efficacy of continuous bevacizumab monotherapy after chemotherapy cessation in mCRC is yet to

Table 2. Toxic effect in maintenance phase

	Arm A ($n = 80$) bevacizumab + erlotinib				Arm B ($n = 79$) bevacizumab			
	Grade (%)				Grade (%)			
	1	2	3	4	1	2	3	4
Hypertension	6	3	3		3	6	3	
Fatigue	13	14	6	1	13	6		
Fever	5	8	1		4	1		
Infection total	8	18	8		5	9	1	
Hand-and-foot syndrome	3	6	1		4			
Nail disorder	6	5	3		4			
Dry skin	9	1						
Pruritus	15	4						
Rash	24	30	20		5		1	
Skin ulceration	3		1			1		
Anorexia	9	4	1		4			
Diarrhea	23	13	4		10	3		
Constipation	5	1	3		5			
Oral mucositis	9	3			3			
Nausea or vomiting	14	4	1		9	4		
Lower GI hemorrhage	8	1			3	3	1	
Nasal hemorrhage/epistaxis	16				10	1		
Hemorrhage/bleeding other	16						1	
Proteinuria	63	10	3	1	53	6	5	
Albumin low	30	13	1		35	9		
Hemoglobin	43	5			42	4		
Neutrophils	16	8	5		23	6	4	
Platelets	28	1		1	33	5		
ALP	28	18	11		43	10	5	1
ALAT	23	8	1		20	5	3	3
Bilirubin	15	6	3		10		3	1
Creatinine	16	1			15	1	1	
Peripheral motor neuropathy	5	8	1		3			
Peripheral sensory neuropathy	14	5	3		11	10		
Pain total	40	24	8	3	24	19	5	
Ocular/visual total	15	6	1			1		
Pulmonary/upper resp total	6	11	1		14	3		
Thrombosis total		1	1	1				

Adverse events according to NCI-CTCAE version 3.0 (MedDRA terminology) with a frequency of ≥5% in any of the treatment arms, or with special interest to the study drugs.

be determined, but randomized trials are ongoing [9, 10]. The MACRO trial [11] suggested that single bevacizumab be a valid maintenance option.

Addition of erlotinib to bevacizumab resulted in a significantly improved PFS in the maintenance setting for patients with NSCLC [6]. There are still no published data on this maintenance strategy in mCRC, although preliminary results were recently presented from the GERCOR DREAM trial with a study design very similar to ours [12]. In their study population of 700 mCRC patients, a statistically significant improvement in PFS was found with the addition of erlotinib to bevacizumab as maintenance treatment after first-line chemotherapy plus bevacizumab (HR 0.73; 95% CI 0.59–0.91; $P = 0.005$). The median PFS on maintenance treatment increased from 4.6 to 5.8 months, i.e. similar to the present study (4.2 versus 5.7 months). The reason for lack of significance in our study could be the lower sample size.

The median OS in the present study was 24.7 months in the ITT population and 26.7 months among randomized patients, which compares favorably to other recent first-line chemotherapy studies including biologicals in mCRC, with OS typically ranging from 18 to 24 months [1, 13–15]. Previous studies have suggested that prolonged bevacizumab exposure beyond first-line chemotherapy may have a positive impact on survival [16, 17]. Whether the long OS in our study was due to the treatment or to a positive patient selection cannot be determined.

A general purpose with maintenance therapy is the prospect of offering a prolonged tumor control with limited toxic effect. In the present study, there was clearly more toxic effect with the addition of erlotinib to bevacizumab (Table 2). This was mainly due to a higher frequency of fatigue, diarrhea, rash, and other skin/nail disorders. Most of them are well-known erlotinib-related side-effects that are usually easily managed, and the proportions of patients that stopped maintenance treatment due to side-effects were limited, 13% and 4% in arm A and B, respectively. Serious gastrointestinal toxic effect, at least possibly related to bevacizumab, occurred in seven patients. Six of these had GI-perforations (2%), which is well in line with previous studies [1, 12, 15, 17].

For the EGFR-inhibiting antibodies cetuximab and panitumumab, which have an established role in the management of mCRC, the effect is mainly seen in KRAS wild-type (wt) tumors [14]. Whether this is true also for erlotinib is not known. We have recently finished recruitment of 232 patients in a follow-up study, ACT2 [18], in which patients with KRAS wt tumors are randomized to bevacizumab ± erlotinib and patients with KRAS-mutated tumors to bevacizumab or metronomic capecitabine as maintenance treatment. The outcome of the two ACT studies, along with ongoing translational studies focusing mainly on proteins and genes involved in the EGFR and VEGF cascades, will hopefully help us identify subgroups of patients that benefit the most from maintenance treatment.

acknowledgements

funding

disclosure

AJ has received presentation honoraria from Genentech. DB is employed by Roche Sweden. RDC has received consultant and advisory honoraria from Roche. All remaining authors have declared no conflicts of interest.

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Supplementary material papers I and II

Chemotherapy regimens used in the induction phase
Nordic ACT and ACT2 trials

Enrolled patients started induction treatment with combination chemotherapy according to investigator's choice plus bevacizumab. Allowed chemotherapy-regimens were: **XELOX** (oxaliplatin130 mg/m^2 on day 1, capecitabine 1000 mg/m^2 bid on days 1-14, q 3 weeks), **XELIRI** (irinotecan 180 mg/m^2 on day 1, capecitabine 1000 mg/m^2 bid on days 1-14, q 3 weeks). Dose reductions of irinotecan to 150 mg/m^2 and capecitabine to 750 mg/m^2 bid were recommended for patients >65 years, **FOLFOX (**oxaliplatin 85 mg/m^2 + leucovorin 200 mg/m^2 for 2 hours on day 1; followed by 5-FU 400 mg/m^2 iv bolus, and 5-FU 2400 mg/m^2 continuous infusion over 44 hours, q 2 weeks**)**, **FOLFIRI** (irinotecan 180 mg/m^2 + leucovorin 200 mg/m^2 for 2 hours on day 1; followed by 5-FU 400 mg/m^2 iv bolus, and 5-FU 2400 mg/m^2 continuous infusion over 44 hours, q 2 weeks). Bevacizumab was administered at a dose of 7.5 mg/kg every three weeks (XELOX / XELIRI) or 5.0 mg/kg every two weeks (FOLFOX / FOLFIRI), intravenously over 30 minutes or according to local practice. Standard dose modification schedules for chemotherapy-related toxicity were applied. In case of extensive oxaliplatin toxicity, shift to an irinotecan-based regimen was allowed.

S1. Nordic ACT trial
Subsequent anti-cancer treatments in second or later lines

POST STUDY TREATMENT		
	Arm A (*n*=80) bevacizumab +erlotinib	Arm B (*n*=79) bevacizumab
	No.	No.
Any Fluoropyrimidine	58	58
Oxaliplatin	27	27
Irinotecan	50	52
Other chemotherapy	4	2
Bevacizumab	25	28
Cetuximab	16	13
Panitumumab	2	6
Erlotinib	2	1
Temsirolimus	2	2
No further anti-cancer agents	11	11

S2. Nordic ACT2 trial
Anti-cancer medical treatment after end of treatment in ACT2

N=FAS	total N=138	%	wt-B N=35	%	wt-BE N=36	%	mut-B N=34	%	mut-C N=33	%
NO	15	11%	4	11%	5	14%	1	3%	5	15%
YES	123	89%	31	89%	31	86%	33	97%	28	85%
cetux/pani	24	17%	13	37%	11	31%	0	0%	0	0%
bevacizumab	55	40%	12	34%	12	33%	14	41%	17	52%
irinotecan	104	75%	27	77%	28	78%	27	79%	22	67%
oxaliplatin	50	36%	14	40%	10	28%	11	32%	15	46%
5FU	106	77%	24	69%	25	69%	32	94%	25	76%
capecitabine	34	25%	8	23%	7	19%	6	18%	13	39%
aflibercept	4	3%	1	3%	0	0%	2	6%	1	3%
regorafenib	1	1%	0	0%	1	3%	0	0%	0	0%
S1/Teysuno	2	1%	1	3%	0	0%	1	3%	0	0%
Mitomycin	1	1%	1	3%	0	0%	0	0%	0	0%
Other/ study drug/ placebo	21	15%	8	23%	2	6%	5	15%	6	18%

Full analysis set =FAS; cetuximab=cetux; panitumumab=pani; study drug=known
drug or placebo according to other clinical study protocol. Definition of arms:
KRASwild type=wt; KRAS mutated=mut; bevacizumab= B; bevacizumab+erlotinib=
BE; metronomic capecitabin=C

Paper II

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A randomized study of KRAS-guided maintenance therapy with bevacizumab, erlotinib or metronomic capecitabine after first-line induction treatment of metastatic colorectal cancer: the Nordic ACT2 trial

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Background: Maintenance treatment (mt) with bevacizumab (bev) ± erlotinib (erlo) has modest effect after induction chemotherapy in metastatic colorectal cancer (mCRC). We hypothesized the efficacy of erlo to be dependent on KRAS mutational status and investigated this by exploring mt strategies with bev ± erlo and low-dose capecitabine (cap).

Patients and methods: Included patients had mCRC scheduled for first-line therapy, Eastern Cooperative Oncology Group (ECOG) 0–1 and no major comorbidities. Treatment with XELOX/FOLFOX or XELIRI/FOLFIRI + bev was given for 18 weeks. After induction, patients without progression were eligible for randomization to mt; KRAS wild-type (wt) patients were randomized to bev ± erlo (arms wt-BE, $N = 36$ versus wt-B, $N = 35$), KRAS mutated (mut) patients were randomized to bev or metronomic cap (arms mut-B, $N = 34$ versus mut-C, $N = 33$). Primary end point was progression-free

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survival (PFS) rate (PFSr) at 3 months after start of mt. A pooled analysis of KRAS wt patients from the previous ACT study was performed.

Results: We included 233 patients. Median age was 64 years, 62% male, 68% ECOG 0, 52% with primary tumor *in situ*. A total of 138 patients started mt after randomization. PFSr was 64.7% versus 63.6% in wt-B versus wt-BE, $P = 1.000$; and 75% versus 66.7% in mut-B versus mut-C, $P = 0.579$, with no significant difference in median PFS and overall survival (OS). In the pooled cohort, median PFS was 3.7 months in wt-B ($N = 64$) and 5.7 months in wt-BE ($N = 62$) (hazard ratios 1.03, 95% confidence interval 0.70–1.50, $P = 0.867$). The frequency of any grade 3/4 toxicities during mt was: 28%/58%/18%/15% (wt-B/wt-BE/mut-B/mut-C).

Conclusions: Addition of erlo to bev as mt in KRAS wt mCRC did not significantly improve PFS or OS, but it did increase toxicity. KRAS status does not seem to influence the outcome of treatment with erlotinib. Metronomic cap warrants further investigation in mt strategies, given our explorative results.

ClinicalTrials.gov: NCT01229813.

Key words: metastatic colorectal cancer, maintenance treatment, bevacizumab, erlotinib, capecitabine, metronomic chemotherapy

introduction

The therapeutic mainstay in the management of incurable metastatic colorectal cancer (mCRC) is combination chemotherapy, with or without targeted agents [1].

In recent years, efforts have been put into establishing more tolerable maintenance strategies to be initiated before the dose-limiting toxicity of combination chemotherapy occurs. The aim is to prolong survival with sustained quality of life. Low-dose continuous capecitabine, i.e. metronomic chemotherapy, has only been described in retrospective, nonrandomized studies in this setting, e.g. by Sun et al. [2], whereas targeted therapies have been investigated in several randomized mCRC maintenance trials [3–6]. A combination of the antiangiogenic antibody bevacizumab and erlotinib, a tyrosine-kinase inhibitor (TKI) of the epithelial growth factor receptor (EGFR), has shown synergistic effects in preclinical tests and promising results in clinical trials on nonsmall-cell lung cancer (NSCLC) and in mCRC [7–9].

However, not all patients benefit from this treatment and predictive markers are needed. At the time of initiation of the present study, mutation in KRAS exon 2 had been identified as a negative predictive factor for the efficacy of EGFR-inhibiting antibodies in mCRC [10], but also for the efficacy of EGFR TKIs (gefitinib and erlotinib) in NSCLC [11]. This study was designed to investigate whether addition of erlotinib to bevacizumab leads to improved outcome compared with bevacizumab alone as maintenance treatment in mCRC patients with KRAS wild-type (wt) tumors. In patients with KRAS mutated (mut) tumors, metronomic capecitabine was explored as maintenance in comparison with bevacizumab.

patients and methods

patient population

Eligible patients were ≥18 years of age, Eastern Cooperative Oncology Group (ECOG) 0–1, with histologically confirmed untreated mCRC and paraffin-embedded tumor tissue available for KRAS mutation analysis. Other inclusion and exclusion criteria were equally consistent with the preceding Nordic ACT trial and included standard criteria for first-line mCRC trials involving bevacizumab as study treatment [3]. Prior adjuvant chemotherapy for CRC was allowed if ended at least 6 months before inclusion.

study design

The ACT2 study was an open-label, phase III, randomized clinical trial recruiting patients at 11 sites in Sweden and one in Denmark between October 2010 and May 2012. The study was approved by ethics committees and medical products agencies in both countries and was conducted in accordance with the International Conference of Harmonization guideline for Good Clinical Practice and with the Declaration of Helsinki. All patients signed written informed consent. The trial was investigator sponsored with financial support from Roche. A representative from Roche took part in designing the study protocol but Roche had no role in validation or analysis of the data.

induction treatment

First-line induction treatment was given with XELOX/XELIRI or FOLFOX/FOLFIRI (investigator's choice) plus bevacizumab (for treatment schedules, see supplementary Material S1, available at *Annals of Oncology* online). After 18 weeks of induction treatment, patients without progressive disease (PD) were eligible for randomization to maintenance treatment. Patients were divided by KRAS mutational status and in the randomization process stratified by best response in induction, i.e. partial response (PR) versus stable disease (SD), and to whether or not oxaliplatin had been used in induction. Mutational analyses were carried out with validated standard assays at each study site. Tumors were classified as KRAS mut if any mutation was identified in codons 12 or 13 of exon 2.

maintenance treatment

Patients with KRAS wt tumors were randomized (1:1) between bevacizumab 7.5 mg/kg i.v. once every 3 weeks alone (arm wt-B) or in combination with oral erlotinib 150 mg once daily (arm wt-BE). Patients with KRAS mut tumors were randomized (1:1) to bevacizumab alone (arm mut-B), or oral capecitabine 500 mg twice daily continuously (arm mut-C). Maintenance therapy was given until PD, intolerable toxicity, planned surgery, noncompliance, serious protocol deviation, consent withdrawn or lost to follow-up.

dose modification of study drugs

Dose modifications of bevacizumab and erlotinib during maintenance phase were allowed as previously described in the Nordic ACT trial [3]. In case of capecitabine-related toxicity grade ≥2, maintenance treatment was interrupted until toxicity resolved to grade ≤1, other dose adjustments were not allowed. If interruption of dosing was required by more than 3 weeks for treatment with any study drug, the patient was withdrawn from the study.

evaluation of response and safety

Tumor response was evaluated according to RECIST 1.0 with a computed tomography scan of the thorax and abdomen within 28 days before enrollment, after 8–12 weeks of induction treatment, before randomization and every 9 weeks during the maintenance phase. Toxic effects were recorded according to National Cancer Institute Common Toxicity Criteria for Adverse Events version 3.0. Follow-up was documented every third month until death or study data cutoff (14 November 2014).

statistical methods

The aim of the study was to evaluate whether maintenance treatment with erlotinib plus bevacizumab (wt-BE) increases the progression-free survival (PFS) compared with bevacizumab alone (wt-B) in a mCRC KRAS wt population. The study was designed to detect a difference in 3-month PFS rate (PFSr) from 50% in arm wt-B to 80% in arm wt-BE at a two-sided significance level of 5% and a power of 80%, requiring 40 patients in each arm. It was estimated that 60% were KRAS wt and that 70% would be randomized. Accordingly, inclusion of 181 patients was planned. During the course of the study, an unexpectedly high attrition rate was observed, why the study population was increased to 233 patients by a protocol amendment in January 2012. The primary end point (PFSr) was analyzed within the KRASwt and the KRASmut populations, respectively, by a two-sided Fisher's exact test. Subjects censored before 3 months were excluded from the primary end point analysis.

Secondary end points included PFS, defined as time from start of maintenance treatment until first occurrence of PD or death from any cause, overall survival (OS) from study inclusion and safety. PFS and OS were calculated in the full analysis set (FAS), defined as all randomized patients that started treatment in maintenance phase, and in the per-protocol (PP) population, including all FAS patients compliant with the protocol. Median OS was also analyzed in the intention-to-treat (ITT) population defined as all included patients who started treatment in induction phase with the intention to be evaluated for maintenance treatment if eligible for randomization. Pooled analyses of PFS and OS were carried out according to protocol including arms wt-B and wt-BE from the current study and data from KRAS evaluable wt patients from our first Nordic ACT trial [3]. This was justified by the identical eligibility criteria and treatment design.

For the survival analyses, the Kaplan–Meier method was used and hazard ratios (HRs) were calculated by the Cox regression model. A two-sided log-rank test was used for comparison between study arms. The median follow-up-time was calculated as Kaplan–Meier estimate of potential follow-up. Toxicity in the induction phase was listed for the safety analysis population (SAP), defined as patients who had received at least one dose of induction treatment, and in the maintenance phase for the FAS population. Analyses were done with SAS (version 9.2).

results

patient characteristics

The study enrolled 233 patients. Two patients were withdrawn from the study before any data were recorded and were excluded from the ITT population (Figure. 1A). The baseline characteristics were similar between treatment arms, but some differences were noted (Table 1). In the wt-BE arm, a smaller proportion of patients (19%) had rectum as primary cancer location compared with 54% in the wt-B arm and fewer patients had received previous adjuvant treatment in wt-BE (6% versus 21%).

efficacy and safety

induction treatment. In the ITT population ($N = 231$), the frequencies of each induction chemotherapy backbone used were XELOX (36%), FOLFIRI (33%), FOLFOX (21%) and XELIRI (10%). Response rates in induction phase among assessable patients were PR (43%), SD (51%) and PD (6%). Best response on induction for FAS populations is presented in Table 1, with no statistical differences between the study arms (χ^2 test). In the safety population, 104 patients (45.5%) presented with at least one grade 3/4 adverse event (AE) during induction therapy. There were four cases of gastrointestinal perforation reported in induction phase; three were grade 3 and one was fatal.

maintenance treatment. Of the 146 randomized patients, 138 started treatment in maintenance phase (FAS) (Figure. 1A). Owing to failure of performing obligatory laboratory tests at inclusion, 11 patients were excluded from the PP population. Since the outcome in the PP population did not differ significantly from that in FAS, only results from the FAS population will be presented.

The PFSr at 3 months was 63.6% in the wt-BE arm ($N = 33$) compared with 64.7% in the wt-B arm ($N = 34$), with no statistically significant difference ($P = 1.000$). The median PFS was 5.7 months in wt-BE and 3.6 months in wt-B [HR 0.93, 95% confidence interval (CI) 0.56–1.56, $P = 0.787$] (Figure. 2A). The 3-month PFSr was 75% in mut-B ($N = 32$) and 66.7% in mut-C ($N = 30$) ($P = 0.579$). The median PFS was 3.9 months in mut-B and 3.7 months in mut-C (HR 1.19, 95% CI 0.72–1.97, $P = 0.501$) (Figure. 2B).

The median duration of maintenance treatment was 4.7 months (wt-BE), 4.1 months (wt-B and mut-B) and 3.9 months (mut-C). 94.4% of the patients in wt-BE had at least one AE of any grade during maintenance treatment, compared with 88.6% in wt-B, 82.4% in mut-B and 66.7% in mut-C, respectively. AEs grade 3/4 in the maintenance phase are presented in Table 2. Three patients had intestinal perforations during maintenance phase; one grade 4 included in Table 2 and two additional patients had fatal perforations (grade 5), one in mut-B and one in mut-C. One patient in arm wt-B died of cerebral infarction, considered unlikely related to study drug.

Maintenance treatment was discontinued due to toxicity in a total of five patients (4%) in FAS, three of them were in wt-BE. Other reasons of end of treatment in FAS were PD (86%), death (2%), intended curative surgery (2%) and withdrawn consent (1%).

overall survival. With a median follow-up time of 34.5 months (95% CI 32.3–37.7), 184 patients in the ITT population and 101 in the FAS population had died. Median OS from date of informed consent was 19.5 months in the ITT population and 25.3 months in the FAS. Within the FAS randomized populations, median OS from date of informed consent was 20.6 months in wt-BE and 30.7 months in wt-B (HR 0.58, 95% CI 0.34–1.01, $P = 0.0510$) (Figure. 2C). In mut-B, the median OS was 26.4 months and in mut-C 28.0 months (HR 1.57, 95% CI 0.87–2.84, $P = 0.128$) (Figure. 2D).

pooled analyses. Data from the KRAS wt FAS population of the present trial and our first Nordic ACT trial [3] were evaluated in a combined analysis (Figure. 1B). Median PFS from start of
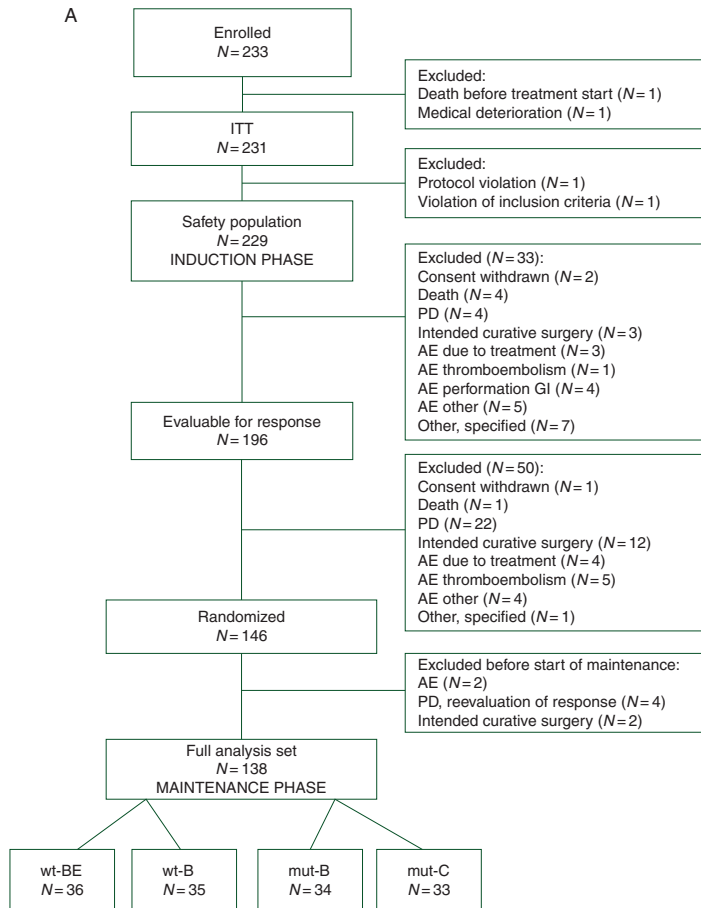
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Figure 1. CONSORT diagrams of (A) the present ACT2 trial and (B) the pooled population KRAS wild-type (wt) patients from the Nordic ACT and ACT2 trials. ITT, intent-to-treat population; PD, progressive disease; AE, adverse event. Definition of arms: B, bevacizumab; BE, bevacizumab+erlotinib; C, metronomic capecitabin.

maintenance treatment was 3.7 months in the pooled wt-B group compared with 5.7 months in the pooled wt-BE group (HR 1.03, 95% CI 0.70–1.50, $P = 0.867$) (Figure. 2E). The median OS from informed consent was 29.4 months in the pooled wt-B group and 23.3 months in the pooled wt-BE group, with no statistically significant difference (HR 0.76, 95% CI 0.51–1.14, $P = 0.197$) (Figure. 2F).

post-study treatment. Most patients (89%) in the FAS population received further anticancer drugs after termination of the maintenance treatment (supplementary Material S2, available at *Annals of Oncology* online). In the KRAS wt cohort, the use of

an EGFR-inhibitor in subsequent treatment lines was similar, 37% and 31% in wt-B and wt-BE, respectively.

discussion

According to our results, maintenance treatment with bevacizumab plus erlotinib does not improve PFS significantly compared with bevacizumab alone in mCRC KRAS wt patients.

A potential criticism of this trial could be its limited size. If erlotinib is to gain wide acceptance as a maintenance treatment, then the efficacy has to be substantial. We decided that a rather large increase in 3-month PFS from 50% to 80% in the KRASwt

cohort would be clinically meaningful to detect. Consequently, the sample size could be limited. The final shortage of assessable patients (71 versus estimated 80) is explained by a higher than expected attrition rate before randomization (40% versus

Fig. 1 *Continued*

predicted 30%) and more (49%) KRAS mut tumors than the expected 40%. Despite an increase of the study population, the high dropout rate was unfortunately not fully compensated for. To increase the power, we carried out a preplanned pooled analysis with data from KRAS wt patients in the preceding Nordic ACT trial. No significant difference between the pooled population arms was found, but there was a numerical increase in median PFS from 3.7 to 5.7 months favoring the addition of erlotinib (Figure. 2E). This is in the same order of magnitude as seen in non-KRAS selected patients both in Nordic ACT [3] (HR 0.79, $P = 0.19$) and in the similar GERCOR DREAM study (HR 0.77, 95% CI 0.62–0.94; $P = 0.012$) [9]. These findings, supported by previous preliminary results from the GERCOR group [12], indicate that KRAS exon 2 mutation is not a good predictor for the efficacy of erlotinib in this setting, as opposed to NSCLC in which KRAS wt patients in the ATLAS trial were more likely to benefit from the addition of erlotinib to bevacizumab, at least in terms of PFS [13].

If a maintenance treatment is to gain wide acceptance, it should preferably also affect OS. Preliminary results from the DREAM trial showed a statistically significant OS gain of 3 months with the addition of erlotinib to bevacizumab whereas, in our first Nordic ACT study, no significant difference in OS was seen. In the present study, there is a somewhat surprising tendency for worse OS in the combination arm compared with
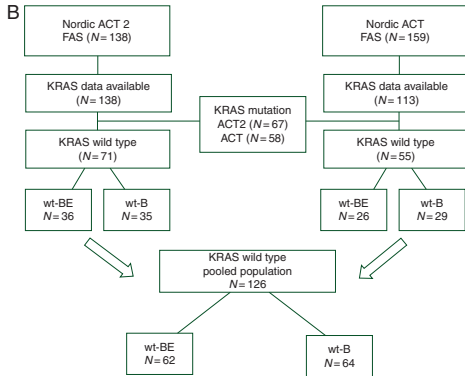
Table 1. Patient characteristics at baseline					
	ITT	Full analysis set			
	Total	KRAS wild type		KRAS mutated	
		wt-B	wt-BE	mut-B	mut-C
	$N = 231$	$N = 35$	$N = 36$	$N = 34$	$N = 33$
Age, years					
Median (range)	64 (32–83)	61 (32–76)	65 (38–74)	65 (44–75)	63 (45–79)
Gender					
M/F	62/38%	66/34%	64/36%	53/47%	70/30%
ECOG					
0/1	68/32%	77/23%	67/33%	82/18%	61/39%
Primary tumor site					
Colon	56%	46%	75%	50%	50%
Rectum	41%	54%	19%	44%	47%
Both	3%	0%	6%	6%	3%
Primary tumor *in situ*	52%	43%	58%	56%	49%
Metastatic sites					
1	39%	34%	50%	35%	33%
>1	61%	66%	50%	65%	67%
Liver metastases					
Total	75%	81%	83%	62%	76%
Liver mets. only	23%	20%	39%	12%	15%
Previous adjuvant treatment					
Total	15%	21%	6%	24%	15%
Oxaliplatin	10%	17%	6%	15%	12%
Best response induction					
PR	n.a.	46%	61%	41%	55%
SD	n.a.	54%	39%	59%	45%

ECOG, Eastern Cooperative Oncology Group; ITT, intention-to-treat population; PD, progressive disease; SD, stable disease; n.a., not applicable (not all patients of the ITT population were evaluable for response); definition of arms: B, bevacizumab; BE, bevacizumab+erlotinib; C, metronomic capecitabine; wt, KRAS wild type; mut, KRAS mutated.
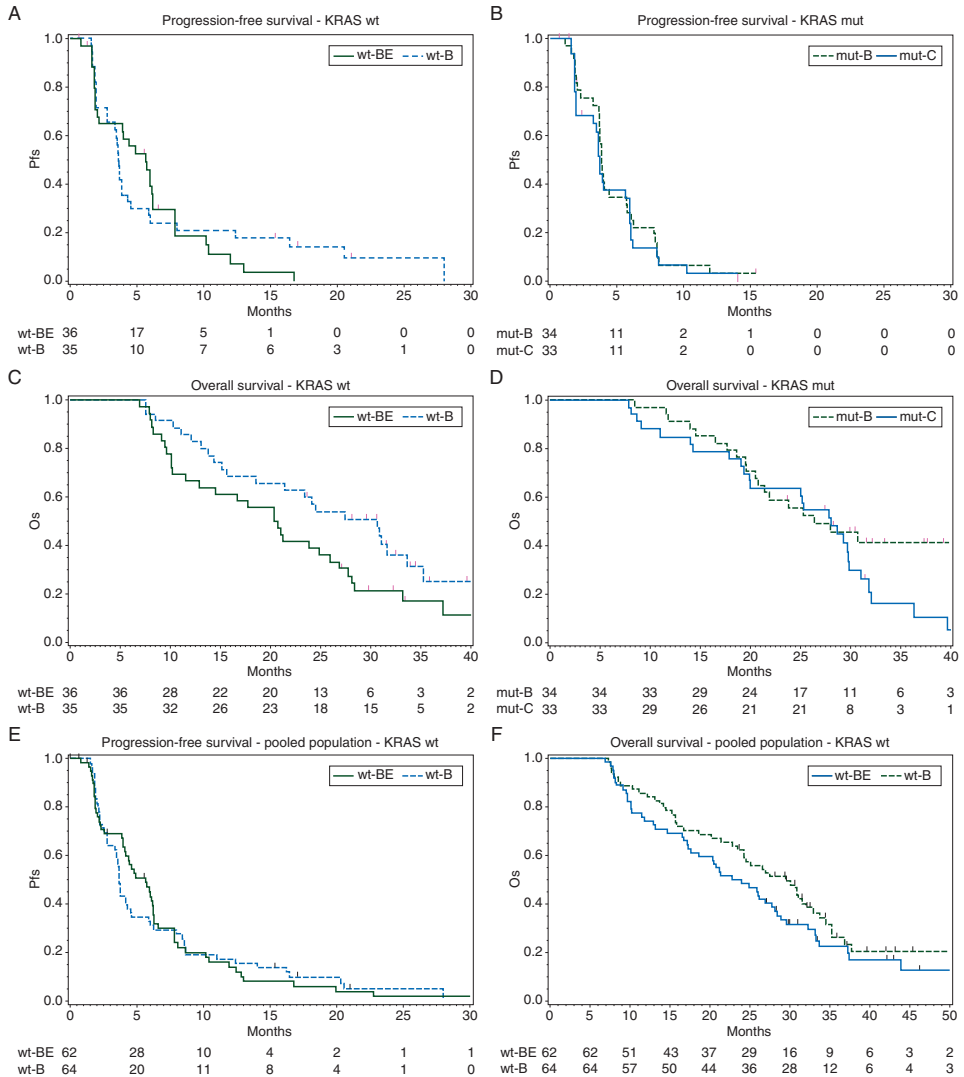
Figure 2. Progression-free survival (PFS) of (A) the KRAS wild-type (wt) population and (B) the KRAS mutated (mut) population from start of maintenance treatment in the ACT2 full analysis set (FAS) population. Overall survival (OS) of the KRAS wt (C) and the KRAS mut (D) patients from start of induction treatment. Corresponding PFS (E) and OS (F) in the pooled KRAS wild-type FAS population of the ACT and ACT2 trials. Definition of arms: wt, KRAS wild type; mut, KRAS mutated; B, bevacizumab; BE, bevacizumab+erlotinib; C, metronomic capecitabin.

the bevacizumab single arm (Figure 2C and F). The reason for this is unclear. Subsequent anticancer treatments were well balanced between the arms (supplementary Material S2, available at *Annals of Oncology* online). Differences in baseline features, such as age, ECOG status, primary tumor location

and history of adjuvant treatment, may have influenced the OS (Table 1).

The design of the study may be criticized due to lack of comparison with a 'standard maintenance' or observation arm. The ACT2 trial was launched as an extension of and in direct

Table 2. Adverse events grade 3/4 in the maintenance phase

	wt-BE N = 36		wt-B N = 35		mut-B N = 34		mut-C N = 33	
	Grade 3 *n*	Grade 4 *n*	Grade 3 *n*	Grade 4 *n*	Grade 3 *n*	Grade 4 *n*	Grade 3 *n*	Grade 4 *n*
Appetite disorders	1		1					
Asthenic conditions/fatigue	2		2					
Disturbances in consciousness	1						1	
Hypertension	1		1		3			
Thrombosis and pulmonary embolism					1	1		
Gastrointestinal ulcers and perforation								1[a]
GI stenosis and obstruction	1	2					1	2[a]
Diarrhea	3						2	
Nausea and vomiting			1					
Gastrointestinal disorders other	3		3					
Infections	3				1			1[a]
Skin and subcutaneous tissue disorders	5							
Peripheral neuropathies			1		1			
Hepatic and hepatobiliary disorders	2							
Hypokalemia								
Renal and urinary disorders (ureteric obstruction)	1						1	
Musculoskeletal and connective tissue disorders	1		1		2			
Any AE grade 3/4	21 (58.3%)		9 (25.7%)		7 (20.6%)		5 (15.2%)	

Adverse events grade 3 or 4 according to NCI-CTCAE version 3.0 [adverse events were categorized according to the Medical Dictionary for Regulatory Activities (MedDRA) system organ classes and preferred terms].
[a]Three of the grade 4 toxicities were related to the same medical event in one patient.
AE, adverse events; definition of arms: wt, KRAS wild type; mut, KRAS mutated; B, bevacizumab; BE, bevacizumab+erlotinib; C, metronomic capecitabin.

succession to the first Nordic ACT trial, which justifies analyses of a pooled dataset. Recent studies have shown that maintenance treatment with bevacizumab alone in mCRC is of limited value, whereas capecitabine + bevacizumab has shown to be an active maintenance strategy [4–6]. Whether metronomic capecitabine has a future role in this setting and what doses should be used is unclear.

In the current study, capecitabine was administered at a dose of 500 mg twice daily, i.e. much lower than the conventional dose, based on a retrospective study exploring fixed low doses of capecitabine to facilitate maintenance treatment and limit toxicity [2]. In an early randomized phase II trial, a continuous capecitabine dose of 625 mg/m^2 twice daily was found almost as effective and less toxic compared with the intermittent schedule with 1250 mg/m^2 twice daily for 2 weeks of 3, that later became the preferred standard [14]. The capecitabine dose of 625 mg/m^2 twice daily was later used as maintenance in the CAIRO3 trial, in combination with bevacizumab [5], but to our knowledge we are first to present a randomized comparison between bevacizumab and single metronomic capecitabine.

The results on metronomic capecitabine in our trial must be interpreted with caution due to the exploratory nature and small sample size, but PFS and OS were not clearly inferior to bevacizumab, and given the limited toxicity, simple administration and low cost, metronomic capecitabine could be of interest to explore in future maintenance trials, including identification of optimal doses. In summary, this study shows that KRAS status does not seem to have an important role in the selection of mCRC patients for treatment with erlotinib. In light of our negative results, including increased toxicity, the combination of erlotinib and bevacizumab is not yet to be broadly implemented as maintenance treatment in mCRC. However, subsequent research should focus on exploring other possible biomarkers to identify subgroups that may benefit from the addition of erlotinib in this setting.

disclosure

The authors have declared no conflicts of interest.

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Paper III

Vasoactive Peptides Associate with Treatment Outcome of Bevacizumab-containing Therapy in Metastatic Colorectal Cancer

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Running title: Exploring vasoactive peptides as biomarkers of anti-angiogenic therapy

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ABSTRACT

Background: Hypertension is a common early adverse event of anti-angiogenic treatment of cancer and may associate with treatment response. However, blood pressure measurement as a surrogate response biomarker has methodological limitations, and predictive biomarkers of angiogenesis inhibitors are lacking. In disease associated with hypertension, vasoactive peptides have been linked to cardiovascular pressure load. Here, we have explored potential associations between circulating levels of vasoactive peptides and tumour response during bevacizumab-containing treatment of colorectal cancer.

Material and Methods: Metastatic colorectal cancer (mCRC) patients with available best objective response (ORR) and time to tumour progression (TTP) data were included from a randomized clinical trial investigating maintenance therapy after first line chemotherapy plus bevacizumab. Midregional-pro-adrenomedullin (MR-proADM), midregional-pro-atrial-natriuretic-peptide (MR-proANP), and C-terminal-prepro-vasopressin (Copeptin) vasoactive peptide concentrations were measured in plasma at baseline and after six weeks of chemotherapy and bevacizumab treatment (n=97). We determined associations between clinical outcome (ORR and TTP), peptide levels, and hypertension (NCI-CTCAE 4.0 criteria), using Spearman´s test, multiple linear regression and Mann-Whitney´s test.

Results: Increased levels of vasoactive peptides from baseline and after six weeks of treatment were associated with improved treatment outcome (MR-proADM: ORR, p=0.0003; TTP, p=0.05; MR-proANP: ORR, p=0.05; TTP, p=0.03; Copeptin: ORR, p=0.10; TTP, p=0.02). Patients with increasing levels of all three peptides (n=28) *vs.* increasing levels of one or two peptides (n=59) showed a median TTP of 284 and 225 days, respectively (p=0.02).

Conclusion: Our results suggest that increased systemic levels of vasoactive peptides associate with improved tumour response and TTP in mCRC patients treated with a bevacizumab-containing regimen. These findings support the proposed link between the tumour vasculature and the cardiovascular system of the host. This should motivate further studies that investigate the potential role of vasoactive peptides as a novel class of dynamic biomarkers in the treatment of cancer.

INTRODUCTION

Angiogenesis, *i.e.* the formation of new blood vessels, is a hallmark and requirement of tumour development and metastasis. The concept of anti-angiogenic treatment of cancer has been extensively investigated for almost half a century. Bevacizumab, an antibody targeting the vascular endothelial growth factor (VEGF), is commonly used in combination with chemotherapy in several advanced cancer types including metastatic colorectal cancer (mCRC)[1]. However, a significant limitation of anti-angiogenic drugs is the current lack of biomarkers to predict treatment response[2]. Therefore, the identification of biomarkers that could separate responding patients from patients with no clinical benefit of anti-angiogenic drugs remains a challenge of high clinical relevance.

Angiogenesis and blood pressure are interconnected, and hypertension is a common adverse event of bevacizumab and other anti-angiogenic agents. It has been proposed that VEGF inhibition by bevacizumab increases peripheral vascular resistance through down-regulation of vasodilators, *e.g.* nitrous oxide, and through a functional decrease of arterioles and capillaries, together resulting in increased cardiovascular pressure load and hypertension[3]. Importantly, some studies have shown an association between the anti-tumoral effect of bevacizumab treatment and increased blood pressure[4, 5, 6, 7, 8, 9]. However, a large comprehensive analysis found that in six out of seven studies, hypertension did not associate with improved clinical benefit from bevacizumab treatment[10]. These discrepancies probably reflect the intrinsic limitations of blood pressure measurement as a surrogate biomarker due to *e.g.* diurnal variation, white coat effect, and the methodological variability of the test.

Several vasoactive peptides that reflect cardiovascular pressure load and blood pressure have been identified [11, 12, 13]. Whether this class of peptides respond to angiogenesis inhibitors and associate with their anti-tumoral activity has never been studied. We have investigated stable fragments of three different vasoactive peptide hormones: Midregional-pro-adrenomedullin (MR-proADM), midregional-pro-atrial-natriuretic-peptide (MR-proANP), and C-terminal-prepro-vasopressin (Copeptin) that were selected on basis of their link to angiogenesis, cardiovascular stress, microalbuminuria, hypertension and diseases characterized by blood pressure instability, such as syncope and sepsis [11, 12, 13, 14, 15, 16]. The study was designed to explore the association between vasoactive peptide levels and efficacy of bevacizumab-containing first line treatment of mCRC patients included in a clinical trial.

MATERIAL AND METHODS

Patient population

Patients were treated within the randomized clinical trial Nordic ACT2 (ClinicalTrials.gov: NCT01229813)[17]. This study was performed in accordance with the Declaration of Helsinki and all patients signed separate written informed consent to be part of the biomarker study. Main inclusion criteria were: Untreated mCRC, ≥ 18 years of age, performance status ECOG 0-1, and adequate haematological, hepatic and renal function. Uncontrolled hypertension, significant active cardiovascular disease, and active use of anticoagulants for therapeutic purpose were not allowed. For the present study, patients were selected for biomarker analyses based on two well defined, pre-determined criteria: 1) Reason for end of treatment (EOT) in ACT2 specified as progressive disease (PD) according to Response Evaluation In Solid Tumours (RECIST) 1.0, and 2) Available plasma samples at baseline before initiation of treatment and at approximately six weeks from treatment start. At the time of the plasma sample inventory, eight patients who fulfilled the above mentioned second criteria were still on treatment in the ACT2 study and had not yet reached EOT. These patients' samples were included for vasoactive peptides measurements in order to maximize the final biomarker cohort. In time for the statistical analysis, all of these patients had reached EOT, however, six of them for other reasons than tumour progression, and thus did not fulfil the first pre-determined inclusion criteria mentioned above (see Figure 1). Accordingly, the sample size was determined by a clear-cut definition of the endpoint time to tumour progression (TTP), and by our aim to minimize any exclusion of subjects due to lack of necessary data.

Anti-tumoral treatment regimens

First line induction treatment was given for a maximum of 18 weeks with a fluoropyrimidine in combination with oxaliplatin or irinotecan (XELOX/ XELIRI or FOLFOX/ FOLFIRI according to investigator´s choice) plus standard dosing of bevacizumab (equal to 2.5 mg/kg body weight per week)[17]. Patients without PD by the second tumour evaluation after 18 weeks of induction treatment were then eligible for randomization to maintenance treatment. Thus, tumour response evaluation was performed twice during the induction treatment for all patients that started maintenance phase. Patients with Kirsten rat sarcoma oncogene (KRAS) wild type (wt) tumours were randomized between bevacizumab alone (7.5 mg/kg) once every three weeks (arm wt-B) or in combination with oral erlotinib 150 mg once daily continuously

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(arm wt-BE). Patients with KRAS mutated (mut in codons 12 or 13 of exon 2) tumours were randomized to bevacizumab alone (arm mut-B), or metronomic capecitabine 500 mg twice daily (arm mut-C).

Tumour evaluation and clinical data

A computed tomography (CT) scan of the thorax and abdomen was performed within 28 days before enrolment in the ACT2 trial as baseline assessment. According to the ACT2 protocol two CT evaluations were planned in the induction treatment phase: One after 8–12 weeks of induction treatment, and for patients that did not have PD and continued treatment in the study a second CT scan was performed after a total of 18 weeks of induction treatment. At this time point, patients without PD were eligible for randomization to maintenance treatment. CT scans were performed every nine weeks during the maintenance treatment phase (see Supplementary Figure 1 for a schematic presentation of interventions and assessments).

Blood pressure (BP) was measured with the patient in resting position for at least five minutes at the start of each treatment course. Verification of BP by repeated measurement should be undertaken if systolic BP ≥140 and/or diastolic BP ≥90 was recorded. Patient specific BP data were used to grade hypertension retrospectively according to the National Cancer Institute Common Toxicity Criteria for Adverse Events (CTCAE), version 4.0[18].

Vasoactive peptide analyses

Blood was collected at baseline before initiation of induction treatment with chemotherapy and bevacizumab (sample A), and approximately after six weeks of induction treatment (sample B), *i.e.* prior to cycle three or four depending on the chosen induction treatment regimen schedule (Supplementary Figure 1). Blood (4-7 ml) was collected in an EDTA tube, and centrifuged after resting for 30 minutes, aliquoted into 1.5 ml cryovials and stored at -70° C until assayed. Absolute levels (pmol/l) of stable fragments of the vasoactive peptides MRpro-ANP, MR pro-ADM, and Copeptin were determined in EDTA plasma using a standardized, commercial fully automated immunoassay (KRYPTOR, Thermo Fisher Scientific, Hennigsdorf/Berlin, Germany) involving the Time Resolved Amplified Cryptate Emission (TRACE) technology, which has been evolved from the originally described immunoassays [19, 20, 21]. The assays were performed and reported by assisting personnel blinded to the patient clinical data.

Statistical methods

TTP was defined as the time in days from start of first treatment cycle in the induction phase until the date of PD recorded in the ACT2 study, either in the induction treatment phase or for the randomised patients during maintenance treatment. The change in blood pressure (with baseline measurement as reference) was expressed as the grade of hypertension (0-1, 2, or 3) according to CTCAE 4.0 before the third or fourth cycle of induction treatment, approximately six weeks from treatment start. Due to the low threshold for grade 1 hypertension, also denoted "pre-hypertension" in the CTCAE 4.0 document, patients with grade 0 hypertension (blood pressure below 120/80 mm Hg, n=3) were pooled with patients classified with hypertension grade 1 into one group. Hypertension grade 4 or 5 was not observed. Changes in peptide concentrations from baseline (sample A) to approximately six weeks from treatment start (sample B) were analysed as log concentration ratios (B/A), base 2. Spearman´s rank correlation test was used to investigate the associations between peptide concentrations at baseline and TTP, hypertension and TTP, and hypertension grade versus peptide concentration ratios. In addition to Spearman´s test, simple linear regression was used to describe peptide concentration ratios versus clinical outcome in terms of TTP and objective tumour response (ORR). ORR was defined as the best objective tumour response, PR (partial response), SD (stable disease) or PD (progressive disease) (according to RECIST 1.0), observed during induction treatment with bevacizumab and chemotherapy. The relationship between TTP and the three peptide concentration ratios, dichotomized at 1.00, *i.e.* increasing (B/A >1.00) *vs.* non-increasing (B/A ≤1.00) concentrations, was studied with t-test and analysed simultaneously using a multiple linear regression model. The patients were then classified into three groups depending on number of peptides with increasing concentrations: 0, 1 to 2, or 3. The TTP-distributions for these three groups were compared overall using the 2-df Kruskal-Wallis test and pairwise using the Wilcoxon-Mann-Whitney rank sum test. All statistical analyses were done with STATA, version 14. A two-sided p-value of <0.05 was considered statistically significant.

RESULTS

Patient characteristics of the biomarker cohort

Of the 196 patients evaluable for response in the ACT2 study, 147 were potentially eligible for vasoactive peptide analyses according to pre-determined selection criteria, and from this group 113 full sets of plasma samples were identified. The main reason for exclusion was missing plasma sample after six weeks of treatment. To minimize the risk of pre-analytical

bias, the study group decided to exclude two patients at one specific Swedish study site before statistical analysis due to improper validation of sample handling. At the time of the statistical analysis, six of the selected patients were excluded due to reason for EOT specified as other than PD, *i.e.* they did not fulfil the criteria for biomarker measurements. The peptide analyses from this biomarker cohort (n=107) yielded reliable data for all three peptides, MR-proADM, MR-proANP, and Copeptin, at both time-points (A and B) for 97 patients, who were included in the statistical analyses (Figure 1).

Patient characteristics of the final biomarker cohort are presented in Table 1. Approximately 81% (n=79) of the patients were randomized after induction therapy, the majority of which continued on bevacizumab as part of their maintenance treatment (n=58), whereas the remaining patients received metronomic capecitabine as anti-angiogenic maintenance treatment until progression (n=21). Ten patients had either SD (n=8) or PR (n=2) as best response at first CT evaluation but later progressed at the second evaluation in induction phase, and thus were not randomized to maintenance treatment.

The median TTP of the biomarker cohort was 238 days (range 57-643 days) from start of induction treatment. For the eight patients of the biomarker cohort that had PD at first CT evaluation, the TTP range was 57-65 days with a median of eight weeks and four days. Importantly, the time interval from start of induction treatment to the first CT evaluation (8-12 weeks) as pre-specified in the protocol to allow for expected treatment cycle delays, thus had only marginal effects on TTP results. The baseline plasma sample A was taken within seven days before start of induction treatment according to the ACT2 study protocol. The median time from start of first cycle of induction treatment to date of sample B (at start of treatment cycle three or four) was 42 days (total range 35-75 days, inter quartile range 42-49 days). The main reason for delay of induction treatment cycles was toxicity of chemotherapy, accounting for the longer interval between induction treatment start and sample B in some patients.

Peptide concentrations and clinical outcome

Initially, we addressed potential associations between baseline concentrations (sample A) of each peptide and TTP. Negative and non-significant correlations were found for all the three peptides (MRpro-ADM: r_s=-0.08; p=0.42, MRpro-ANP: r_s=-0.05; p=0.60, Copeptin: r_s=-0.06; p=0.56). Thus, none of the peptide marker concentrations measured before treatment start did predict outcome in terms of TTP. Changes in vasoactive peptides, expressed as the ratio (B/A) between the six-week sample (sample B) and baseline sample (A), were then correlated with

clinical outcome in terms of ORR (PR, SD or PD, respectively) (Figure 2). The results revealed negative correlation coefficients between each peptide ratio and the ordered objective response variable, coded 1=PR, 2=SD, and 3=PD, *i.e.* an increased peptide concentration was associated with a better ORR. The rank correlation was statistically significant for MRpro-ADM (r_s=-0.36; p=0.0003, Figure 2A) and MRpro-ANP (r_s=-0.20; p=0.05, Figure 2B). A slightly weaker and non-significant association was observed for Copeptin (r_s=-0.17; p=0.10, Figure 2C). In accordance with these results, we found a positive association between an increased peptide concentration and prolonged TTP for all three peptides (MRpro-ADM: r_s=0.20; p=0.05; MRpro-ANP: r_s=0.22; p=0.03; and Copeptin: r_s=0.23; p=0.02) (Figure 3). To better illustrate a possible clinical impact of these associations, we also used t-tests to study relationships between dichotomized peptide ratios (above *vs.* below 1.00) and TTP. Patients with increasing values of MRpro-ADM (B/A ratio >1.00, n=53) had on average 45 days longer TTP than patients with decreasing levels of this peptide (B/A ratio <1.00, n=44), (mean TTP 269 vs. 224 days, 95% CI: 0.6 to 90, p=0.05). Similar data for increasing MRpro-ANP (B/A ratio >1.00, n=65) were 41 days longer mean TTP (262 vs. 221 days, 95% CI: -6.6 to 88, p=0.09), and for Copeptin (B/A ratio >1.00, n=57) 39 days longer mean TTP (265 vs. 225 days, 95% CI: -6.1 to 85, p=0.09) than patients with decreasing peptide levels for the respective peptide.

A multiple linear regression model was then fitted for prediction of TTP using peptide ratios (B/A) for MR-proADM, MR-proANP and Copeptin dichotomized at 1.00, *i.e.* increasing *vs.* non-increasing concentrations. According to this model, the expected TTP is 192 days if all the three peptide ratios are decreasing. The expected TTP increase is 35 days [95% CI; -11 to +80] if MR-proADM increases, adjusted for the status of the other two peptides in the model. Similarly, the adjusted expected TTP increase is 30 days [95%CI; -18 to +78] if MR-proANP increases, and 30 days [95% CI; -15 to 76] if Copeptin increases, summing to 35+30+30 = 95 days longer TTP for patients with increasing levels of all the three peptides compared to patients with decreasing levels. To further illustrate the impact of simultaneous change in peptide concentrations on TTP, patients were divided into three groups: Non-increasing concentrations for all three peptides (n=10), increasing concentrations for only one or two of the peptides (n=59), and increasing concentrations for all the three peptides (n=28). The median TTP for these three groups were 222, 225, and 284 days, respectively (p=0.04, Kruskal –Wallis test) with a stronger evidence of different TTP distributions for the two latter groups (p=0.02, Mann –Whitney test) (Table 2).

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These data suggest that increasing levels of three separate vasoactive peptides between baseline and approximately six weeks of treatment with a bevacizumab-containing chemotherapy regimen were associated with improved outcome in terms of TTP and ORR. .

Hypertension and clinical outcome

To address whether the above data were independent on associations between patient outcome and hypertension, we next correlated available blood pressure data with TTP. The median TTP for patients with and without diagnosis of hypertension at baseline (yes/no) was 239 and 238 days, respectively, with a rank correlation close to zero between TTP and the binary hypertension variable (r_s=-0.05; p=0.64, Spearman´s test) (Supplementary Figure 2A). A non-significant trend towards shorter TTP with increasing grade of hypertension at six weeks was observed (median TTP: grade 0-1: 251 days, grade 2: 243 days and grade 3: 194 days; r_s=-0.18; p=0.07) (Supplementary Figure 2B). Accordingly, no association could be found between an early rise in blood pressure and increased TTP in this cohort.

Peptide concentrations and hypertension

Finally, we investigated a possible link between increasing levels of vasoactive peptides and hypertension. However, we found no evidence of an association between peptide ratios and hypertension grade at approximately six weeks in our cohort (MRpro-ADM: r_s=0.005; p=0.96, MRpro-ANP: r_s=-0.03; p=0.74, Copeptin: r_s=-0.04; p=0.66) (Supplementary Figure 3).

DISCUSSION

In this prospective-retrospective study, we found that early changes of circulating levels of three separate vasoactive peptides, MR-proADM, MR-proANP and Copeptin, are associated with an improved treatment outcome in mCRC patients receiving first line treatment with a bevacizumab-containing regimen. Substantial efforts have been directed at the identification of biomarkers predicting the efficacy of anti-angiogenic therapy, including tumour phenotype characteristics, circulating angiogenesis-related proteins, circulating tumour cells, and endothelial progenitor cells. Pharmacogenetic studies of polymorphisms in genes of

angiogenesis pathways, and new imaging guided criteria to predict treatment effects have also been explored [22]. Although these studies have resulted in an increased understanding of tumour angiogenesis, it currently remains uncertain whether any of these approaches will provide a useful baseline biomarker to predict the effect of anti-angiogenic treatment and change clinical praxis. In the present study, we have explored an alternative approach based on the hypothesis that dynamic effects of angiogenesis inhibition on the cardiovascular system of the host are correlated with effects on the tumour vasculature.

Hypertension is a common clinical manifestation of anti-angiogenic treatment, and some studies have linked this side effect to tumour response[4, 5, 6, 7, 8, 9]. Accordingly, systemic factors associated with blood pressure regulation should represent interesting candidates for response biomarkers of angiogenesis inhibition. We thus investigated early changes in circulating vasoactive peptides to monitor effects on cardiovascular pressure load and explore associations with treatment effects caused by angiogenesis inhibition. In a subgroup of patients who had increased levels for all three peptides, a prolongation of two months in TTP was demonstrated as compared to the group with an increase in only one or two of the peptide levels (Table 2). Although these results support the hypothesis of a link between treatment-induced effects on the systemic and tumour vasculature, the combined peptide analysis was based on small subgroups and the clinical impact of these findings should be interpreted with caution.

The biological actions of ADM and ANP are similar in their ability of inducing vasodilatation, diuresis and natriuresis, whereas vasopressin, as measured by Copeptin, is a vasoconstrictor. Interestingly, in addition to their effects on vascular tone and salt and water balance, vasoactive peptides may have direct effects on angiogenesis. ADM is a potent, pro-angiogenic factor, and genetic or pharmacological targeting of ADM resulted in reduced angiogenic and tumorigenic potential in CRC xenograft studies[23]. Our finding that an increased level of MR-proADM was associated with better clinical outcome thus appears paradoxical. However, ADM can act as a potent promoter of endothelial barrier stabilization, a process known as vascular normalization that supports improved tumour bioavailability of cytostatic agents[24, 25]. A strong vasoactive peptide response to the vascular rarefaction and increased cardiovascular pressure load induced by VEGF-inhibition may thus open a window of opportunity for better synergy with chemotherapy. This notion is supported by our finding of an association between increased peptide levels and ORR during the induction phase.

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In line with the comprehensive analysis by Hurwitz *et al.* [10], we found no significant correlation between the grade of hypertension during early induction treatment phase and treatment response in terms of TTP. Also, we found no significant association between changes in peptide concentrations and hypertension grade. Hypertension was graded retrospectively by using the CTCAE version 4.0, which besides variations in blood pressure also takes changes in antihypertensive medication into account, *i.e.* the risk of underestimating the grade of hypertension should be low. Notably, a single blood pressure measurement defined as hypertension grade 1 according to CTCAE 4.0 would not necessarily be considered as significant hypertension in clinical oncology. Moreover, grade 1 reflects the high normal/pre-hypertensive state, as defined by the European and American guidelines for blood pressure monitoring and antihypertensive treatment[18]. Consequently, we chose to analyse grade 0 and grade 1 hypertension as one group. The use of different classification systems, *e.g.* NCI-CTCAE 3.0 *vs.* CTCAE 4.0, diverging cut off values and frequencies of blood pressure monitoring may partly explain contradictions in the literature. Further, variations in the patient´s position, stress reaction in the treatment situation, compliance to antihypertensive drugs, and method of measurement introduce significant methodological bias, altogether pointing at blood pressure measurement as an unreliable surrogate biomarker of anti-angiogenic treatment response in clinical praxis.

The present study was based on a randomized controlled trial, which ascertains high quality clinical data, and excluded patients with significant cardiovascular disease that could have obscured peptide measurement data. The reason for using OR and TTP rather than overall survival as clinical endpoints was to avoid possible bias by second and third line treatment effects. Due to the design of the original clinical trial, however, direct links between vasoactive peptides and anti-angiogenesis could not be investigated, since all patients received bevacizumab. Also, different induction chemotherapy schedules were allowed, and in the maintenance phase patients were randomized to receive bevacizumab alone or in combination with erlotinib, or to single low-dose capecitabine. However, increased cardiovascular pressure load and hypertension are uncommon side-effects of chemotherapy. Therefore, it is conceivable that the early changes in vasoactive peptides were mainly caused by bevacizumab, although effects on the cardiovascular system and cardiotoxicity by *e.g.* fluoropyrimidines during induction treatment cannot be excluded. Another potential criticism of the design could be that the group treated with metronomic capecitabine as maintenance (n=21) only received bevacizumab in the induction phase. On the other hand, quantified peptide ratios and ORR in induction are independent of maintenance therapy, and results from

the original ACT2 trial showed no significant difference in PFS, OS or median duration of maintenance treatment when comparing bevacizumab alone with metronomic capecitabine as maintenance treatment[17]. The inclusion criteria of the present study were based on an intent to explore potential associations between vasoactive peptides and treatment outcome in patients with a well-defined progressive disease during the course of first line treatment, which limit the generalizability of the results. Clearly, the role of vasoactive peptides as possible biomarkers of the response to anti-angiogenic agents needs to be investigated in further studies using broader inclusion criteria as well as in patients with other tumour types including treatment with other anti-angiogenic agents.

In summary, we conclude that circulating, vasoactive peptides may reflect the patient's vascular response to anti-angiogenic therapy and could represent a novel class of early response biomarkers with potential to improve clinical benefit of angiogenesis inhibition.

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Conflicts of interest: None to declare.

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TABLES

Table 1. Baseline patient characteristics of the ACT2 biomarker cohort (n=97)

Characteristic	No. of patients	Percentage (%)
Age years, median (range)	64 (37-79)	
Gender, F/M	37/60	38/62
ECOG PFS 0/1	66/31	68/32
HT diagnosis at baseline, Yes/No	41/56	42/58
Induction regimen		
Bev + FOLFOX/XELOX	17/35	54
Bev + FOLFIRI/XELIRI	36/9	46
Best response to induction treatment		
PR	50	52
SD	39	40
PD	8	8
KRAS status		
wild type	46	47
mutant	49	51
unknown	2	2
Randomised, Yes/No	79/18	81/19
Maintenance regimen arms	(n=79)	
wt-BE	20	25
wt-B/mut-B	18/20	48
mut-C	21	27

ECOG PFS=Eastern Cooperative Oncology Group performance score; HT=Hypertension; Induction chemotherapy regimen (maximum 18 weeks): Bev=bevacizumab; FOLFOX/XELOX=oxaliplatin + 5FU/capecitabine, FOLFIRI/XELIRI=irinotecan + 5FU/capecitabine. PR=partial response; SD=stable disease; PD=progressive disease; KRAS=Kirsten rat sarcoma oncogene; Definitions of maintenance regimen arms: wt/mut=KRAS wild type/mutant; B=bevacizumab; E=erlotinib; C=metronomic capecitabine.

Table 2. Dichotomized peptide ratio score (n=97)

	n	Median TTP (days)	IQR (days)	p
All three peptide ratios ≤ 1 (levels equal or decreasing)	10	222	183-249	0.70[a]
One or two peptide ratios > 1 (1-2 out of the three peptide levels increasing)	59	225	60-436	
All three peptide ratios > 1 (levels increasing)	28	284	188-382	0.02[a]
				0.04[b]

[a]Mann-Whitney-rank-sum test comparing median TTP pairwise using patients with one or two of the three peptide B/A ratios > 1.00 as reference.
[b]Overall 2df-test of equal TTP in the three groups (Kruskal-Wallis test).

TTP=Time to tumour progression; IQR=Inter quartile range; Peptides: MR=Mid-regional; pro-ADM=Pro-adrenomedullin; pro-ANP=pro-atrial-natriuretic peptide; Copeptin=C-terminal-prepro-vasopressin; peptide ratio=vasoactive peptide concentration of sample B (at approximately 6 weeks) *vs.* A (at baseline).

FIGURE LEGENDS

Figure 1.

CONSORT diagram of the ACT2 biomarker cohort

PD=Progressive disease; EOT=End of Treatment in ACT2 study. See Supplementary Fig. 1 for further details.

Figure 2.

Peptide ratio (B/A) versus clinical outcome (best response)

A. MR-pro-Adrenomedullin**:** r_s= – 0.36; *P*=0.0003. **B.** MR-pro-atrial-natriuretic peptide: r_s= – 0.20; *P*=0.05. **C.** Copeptin: r_s= – 0.17; *P*=0.10. MR= Mid-regional. r_s = Spearman´s rank correlation coefficient. Peptide ratio = vasoactive peptide plasma concentrations ratio of sample B to A (at approximately 6 weeks/baseline) associate with best objective tumour response recorded in the induction phase. PR=partial response, SD=stable disease, PD=progressive disease.

Figure 3.

Clinical outcome (TTP) versus peptide ratio (B/A)

A. MR-pro-Adrenomedullin: r_s = 0.20; *P*=0.05. **B.** MR-pro-atrial-natriuretic peptide: r_s = 0.22; *P*=0.03. **C.** Copeptin: r_s= 0.23; *P*=0.02. MR= Mid-regional. r_s = Spearman´s rank correlation coefficient. Peptide ratio = vasoactive peptide plasma concentrations ratio of sample B to A (at approximately 6 weeks/baseline) associate with TTP = time to tumour progression.

Supplementary Figure 1.

Schematic presentation of trial design and evaluation for biomarker analyses. Induction chemotherapy regimen (maximum 18 weeks): FOLFOX/XELOX=oxaliplatin + 5FU/capecitabine, FOLFIRI/XELIRI=irinotecan + 5FU/capecitabine. KRAS=Kirsten rat sarcoma oncogene; R= Randomisation. Definitions of maintenance regimen arms: wt/mut=KRAS wild type/mutant; B=bevacizumab; E=erlotinib; C=metronomic capecitabine; CT=Computed Tomography scan of thorax and abdomen; PD=progressive disease; HT=Hypertension.

Supplementary Figure 2.

Clinical outcome (TTP) versus hypertension

S2A: Time to tumour progression (TTP) versus hypertension diagnosis at baseline (No/Yes). (r_s= – 0.05, *P*=0.64). **S2B**: TTP versus early hypertension grade 0-1<2<3 (according to common toxicity criteria for adverse events, CTCAE 4.0) at approximately 6 weeks (r_s= – 0.18, *P*=0.07). r_s = Spearman´s rank correlation coefficient.

Supplementary Figure 3.

Peptide ratio (B/A) versus early hypertension grade

S3A: r_s=0.005; *P*=0.96. **S3B**: r_s= – 0.03; *P*=0.74. **S3C**: r_s= – 0.04; *P*=0.66. r_s = Spearman´s rank correlation coefficient. Peptide ratio = vasoactive peptide plasma concentrations ratio of sample B to A (at approximately 6 weeks/baseline); hypertension grade 0-1<2<3 (according to common toxicity criteria for adverse events CTCAE 4.0) at approximately 6 weeks from baseline treatment start.
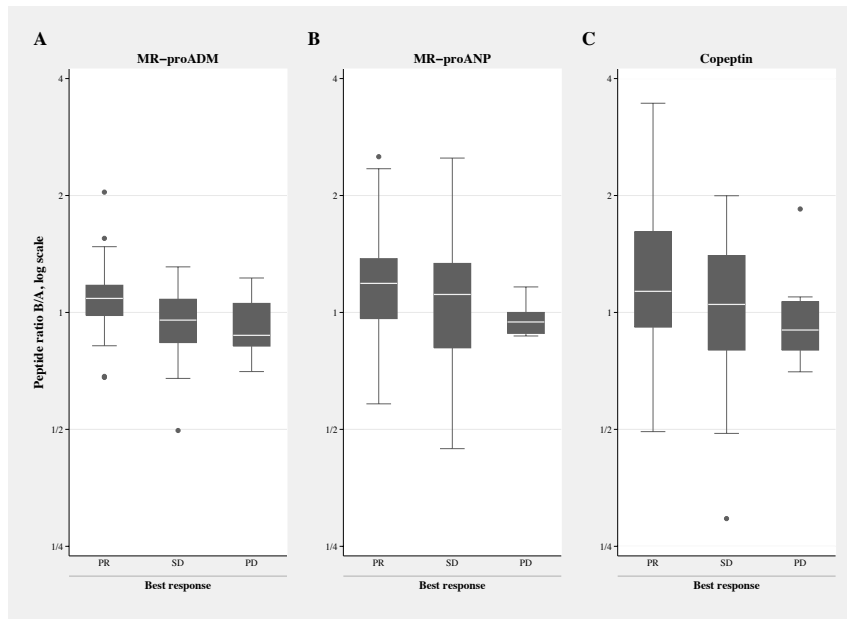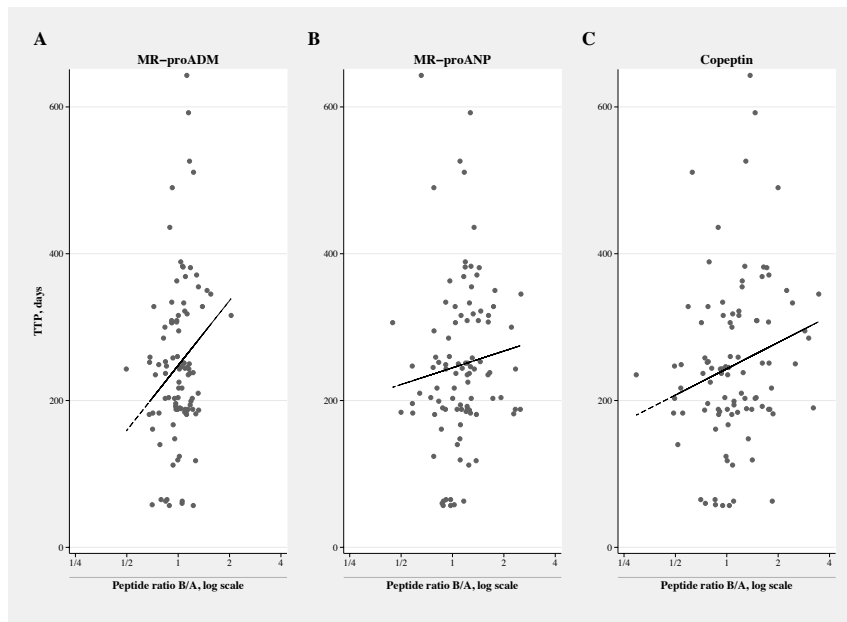
Figure 1.

Figure 2.

Figure 3.

A. MR−proADM   B. MR−proANP   C. Copeptin

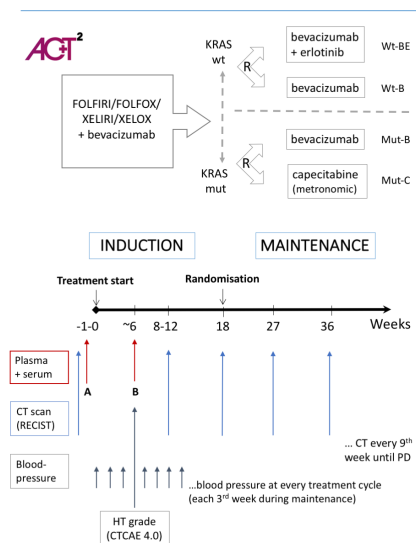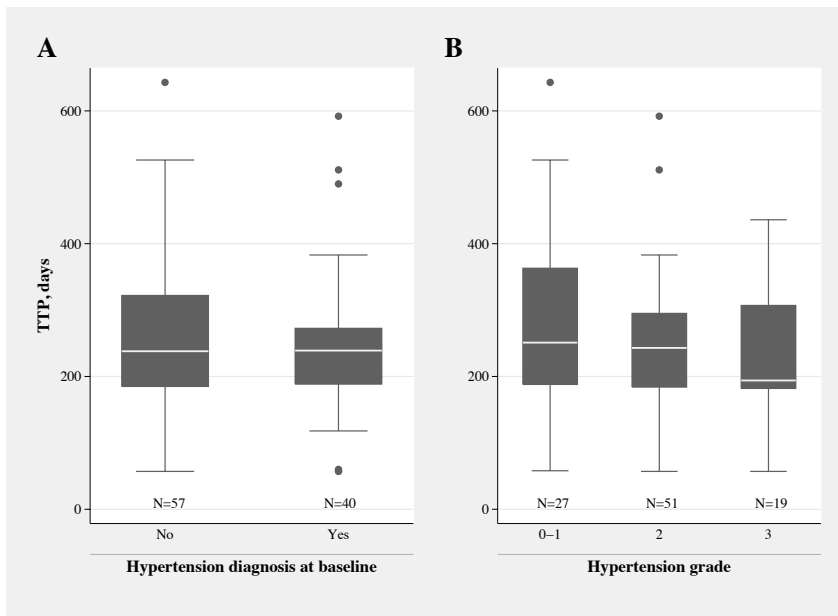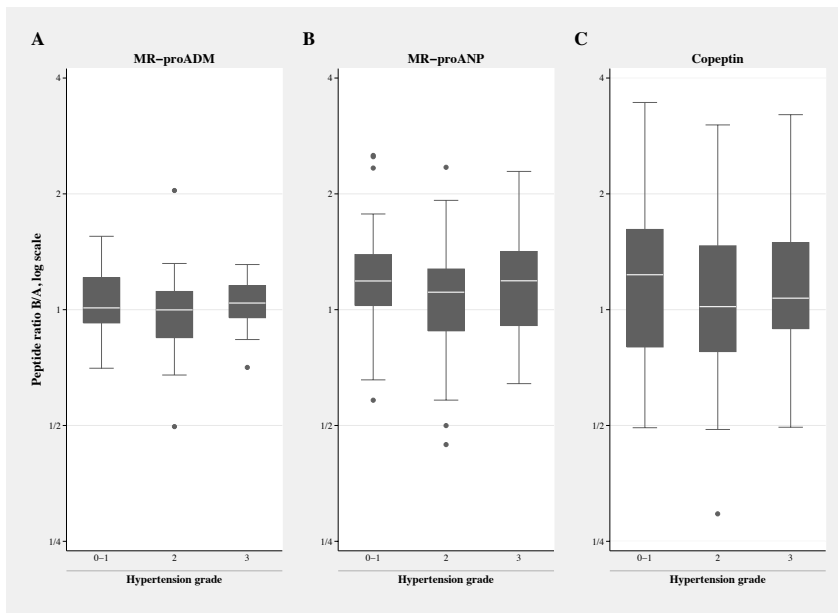(TTP, days vs Peptide ratio B/A, log scale)
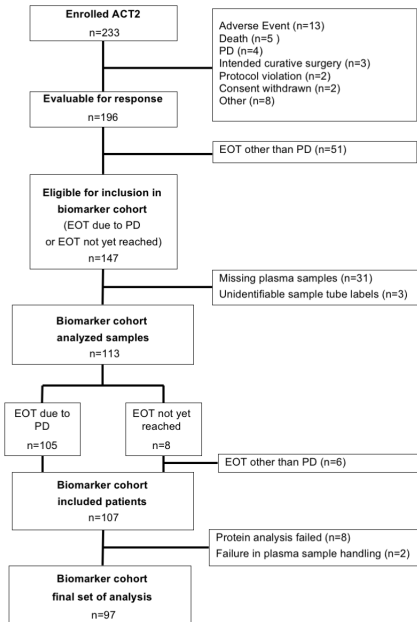
Suppl Fig 1.

Treatment design
and evaluation for biomarker analyses

Suppl Fig 2.

Suppl Fig 3.

Paper IV

Foto: Anna Hållams

I am a medical oncologist specialised in gastrointestinal cancers.
Scientific questions arise every day in my clinical practice, working
with patients with advanced colorectal cancer. An important aim of
oncological palliative care is to sustainably maintain the anti-tumoral
response to optimise survival and symptoms. This thesis looks at these
questions by investigating the role of maintenance treatment with
targeted therapies and explore associated response biomarkers.
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