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The quotation is paraphrasing John von Neumann.

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The simplest complete model of an organism is the organism itself.

ABSTRACT

Eva-Maria Birkman

Epidermal growth factor receptor and other tissue biomarkers in gastrointestinal cancers

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Personalised medicine plays an increasing role in the treatment of cancer. New therapeutic molecules are being developed, but their compatibility for each patient has to be tested before starting the treatment by examining the appropriate tissue biomarkers expressed in the tumour. These biomarkers can be utilised not only in treatment selection but also in predicting treatment efficacy and patient survival. They can also be used to classify tumours into specific molecular subtypes that have distinct characteristics related to tumour behaviour, response to cancer treatments and prognosis of the patients. In order to implement these classifications in clinical practice, instead of time-consuming sequencing-based techniques, the methods have to be simple enough and easy to interpret.

Gastrointestinal cancers are among the most prevalent malignancies and often lead to death. Monoclonal antibodies against the epidermal growth factor receptor (EGFR) can be used in the treatment of *RAS* wild-type metastatic colorectal cancer. It has been shown that in addition to *RAS* mutation testing, determining the *EGFR* gene copy number (GCN) of the tumours can aid in selecting the patients likely to benefit from the antibody treatment. In oesophagogastric cancer, *EGFR* GCN has not yet been shown to have a predictive role, although the overexpression of HER2, which belongs to the same receptor family as EGFR, is used as a biomarker to predict response to anti-HER2 antibody treatment. In this thesis, the prevalence of *EGFR* amplification was observed to be at a similar level with the prevalence of *HER2* amplification specifically among the intestinal-type oesophagogastric adenocarcinomas from 220 patients. This implies that it might be useful to examine whether *EGFR* GCN analysis could function as a biomarker predicting anti-EGFR treatment response in the intestinal-type tumours. In addition, in this thesis, tissue microarray was used to detect the different molecular subtypes of oesophagogastric cancers from 244 patients by staining methods applicable to clinical practice.

Comparative studies detecting *EGFR* GCN in primary colorectal tumours and their metastases are scarce. In this thesis, corresponding primary and metastatic tumours from 80 patients were examined. *EGFR* GCN was observed to decrease between the primary and metastatic tumours during anti-EGFR treatment but to remain stable or even increase among patients not treated with anti-EGFR antibodies. This *EGFR* GCN change may be relevant regarding the clinical response to anti-EGFR treatment.

Preoperative chemoradiotherapy can be used in the treatment of rectal cancer patients to enable a complete resection of the tumour or reduce the risk of local recurrence. However, treatment response among patients is variable. Thus, a suitable biomarker could be helpful in predicting response or stratifying patients into separate treatment groups according to their prognosis. In this thesis, CIP2A expression was examined in rectal cancer tissue samples from 210 patients. Low CIP2A expression level was observed to associate with a better response among patients treated with long-course chemoradiotherapy. Affirming results were obtained in cell culture studies, where the suppression of CIP2A expression was observed to render the cells more sensitive to irradiation than the cells with normal CIP2A expression.

Keywords: gastric cancer, colorectal cancer, EGFR, *in situ* hybridisation, molecular classification, tissue microarray

TIIVISTELMÄ

Eva-Maria Birkman

Epidermaalinen kasvutekijäreseptori ja muita kudosbiomarkkereita ruoansulatuskanavan syövissä

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieteen laitos, Patologia, Molekyylilääketieteen tohtoriohjelma

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Yksilöllistetty lääketiede on yhä merkittävämmässä osassa myös syöpätautien hoidossa. Syövän hoitoon kehitetään jatkuvasti uusia lääkemolekyylejä, joiden soveltuvuus kullekin potilaalle on ennen hoidon aloitusta selvitettävä kasvaimen ilmentämien molekulaaristen biomarkkereiden avulla. Biomarkkereita voidaan käyttää paitsi hoitojen valitsemisen apuna niin myös syövän käyttäytymisen ja potilaiden ennusteen arvioimiseen. Biomarkkereiden avulla tietty syöpätyyppi voidaan myös luokitella molekulaarisiin alatyyppeihin, joilla on toisistaan poikkeavia kasvaimen käyttäytymiseen, ennusteeseen ja hoitovasteisiin liittyviä ominaisuuksia. Molekulaaristen luokittelun käytännön soveltamiseen tarvitaan kuitenkin riittävän yksinkertaisia ja helposti tulkittavissa olevia menetelmiä aikaa vievien sekvennointitutkimusten sijaan.

Ruoansulatuskanavan syövät ovat yleisimpiä pahanlaatuisia kasvaimia ja johtavat usein kuolemaan. Levinneen suolistosyövän hoidossa voidaan käyttää epidermaaliseen kasvutekijäreseptoriin (EGFR) kohdistuvaa vasta-ainehoitoa, mikäli kasvaimessa ei ole osoitettavissa *Ras*-geenimutaatiota. Aikaisemmin on todettu, että *Ras*-geenitestin ohella *EGFR*:n geenikopiomäärän selvittämisen avulla voidaan valikoida pelkkää geenitestiä paremmin hoidosta todennäköisesti hyötyvät potilaat. Maha- ja ruokatorvisyövässä suurentuneen *EGFR*:n geenikopiomäärän ennusteellista merkitystä ei kuitenkaan ole vielä pystytty osoittamaan, vaikka samaan reseptoriperheeseen kuuluvan *HER2*:n geenimonistuman tiedetään ennustavan siihen kohdistuvan vasta-ainehoidon tehoa. Väitöstutkimuksessa *EGFR*:n geenimonistuman yleisyyden todettiin 220 potilaan aineistossa olevan nimenomaan intestinaalisen alatyypin maha- ja ruokatorvisyövissä *HER2*:n monistuman tasoa, joten sen selvittämisestä voisi olla hyötyä tutkittaessa EGFR-vasta-ainehoidon tehoa maha-ja ruokatorvisyövän hoidossa. Lisäksi maha- ja ruokatorvisyöpien molekulaaristen alatyyppien tunnistamista selvitettiin 244 potilaan näytteistä koostetun kudosmikrosirun avulla, ja tunnistamisen todettiin onnistuvan myös kliiniseen diagnostiikkaan soveltuvien menetelmien avulla.

EGFR:n geenikopiomäärää alkuperäisen paksusuolikasvaimen ja sen lähettämän etäpesäkkeen välillä vertailevia tutkimuksia on tehty niukasti. Väitöstutkimuksessa verrattiin 80 potilaan primaari- ja metastaattisia kasvaimia. *EGFR*:n kopiolukumäärän todettiin pienenevän vasta-ainehoitoa saaneilla potilailla mutta pysyvän samana tai jopa suurentuvan muuta hoitoa saaneiden potilaiden etäpesäkkeissä. Kopiolukumäärän muutoksella saattaisi olla merkitystä vasta-ainehoidon tehon kannalta.

Peräsuolipotilaiden hoidossa voidaan käyttää ennen leikkausta annettavaa kemosädehoitoa, jonka avulla pyritään mahdollistamaan kasvaimen täydellinen poisto. Osa potilaista jää kuitenkin ilman merkittävää hoitovastetta tai saa haitallisia sivuvaikutuksia, joten soveltuvasta biomarkkerista voisi olla hyötyä hoidon kohdentamisen parantamisessa. CIP2A:n ilmentymistä tutkittiin 210 potilaan peräsuolisyöpänäytteissä, ja matalan ilmenemistason todettiin olevan yhteydessä parempaan vasteeseen pitkän kemosädehoidon saaneilla potilailla. Samansuuntainen tulos saatiin syöpäsoluviljelmien sädetyskokeissa, joissa sädetyksen todettiin estävän enemmän CIP2A:ta ilmentämättömien kuin sitä normaalisti ilmentävien solujen kasvua.

Avainsanat: mahasyöpä, paksusuolisyöpä, EGFR, *in situ* hybridisaatio, molekulaarinen luokitus, kudosmikrosiru

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ABBREVIATIONS

ACI	average cytoplasmic staining index
ACRG	Asian Cancer Research Group
ADCC	antibody-dependent cell-mediated cytotoxicity
ADP	adenosine diphosphate
AKT	RAC-alpha serine/threonine-protein kinase
ALT	alternative lengthening of telomere
AP	alkaline phosphatase
APC	adenomatous polyposis coli
ARPP19	cAMP-regulated phosphoprotein 19
ATCC	American Type Culture Collection
ATM	serine-protein kinase ATM
AUC	area under the curve
BART	BamH1 A region rightward transcript
BOD1	biorientation of chromosomes in cell division protein 1
bp	base pair
BRAF	serine/threonine-protein kinase B-raf
BRCA1/2	breast cancer type 1/2 susceptibility protein
CA	California
$Ca2^{+}$	calcium ion
cAMP	cyclic adenosine monophosphate
CD274	gene for programmed cell death 1 ligand 1
CD4/8	T-cell surface glycoprotein CD4/8
CDH1	cadherin 1/E-cadherin, epithelial cadherin
CDKN2A	cyclin-dependent kinase inhibitor 2A
CHK1	serine/threonine-protein kinase CHK1 (*CHEK1*, checkpoint kinase 1)
Chr7	chromosome 7
CI	confidence interval
CIMP	CpG island methylator phenotype
CIMP-H	CIMP high
CIMP-L	CIMP low
CIN	chromosomal instability
CIN-B	CIN broad
CIN-F	CIN focal
CIP2A	cancerous inhibitor of protein phosphatase 2A
CMS	consensus molecular subtype
CpG	cytosine guanine dinucleotide

CRM	circumferential resection margin
CRT	chemoradiotherapy
CSS	cancer-specific survival
CT	computed tomography
CTC	circulating tumour cells
ctDNA	circulating tumour DNA
CTLA4	cytotoxic T-lymphocyte protein 4
CTNNB1	catenin beta-1
DAB	3,3'-diaminobenzidine
DFS	disease-free survival
DNA	deoxyribonucleic acid
DNMT	cytosine (DNA-5)-methyltransferase
Dnro	journal number
DSS	disease-specific survival
DTC	disseminated tumour cells
E2F1	transcription factor E2F1
EBER	EBV-encoded small RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ENSA	alpha-endosulphine
ETS1	protein C-ets-1
ERBB	receptor tyrosine-protein kinase ERBB
ERK	mitogen-activated protein kinase/ extracellular signal-regulated kinase
FAP	familial adenomatous polyposis
FBS	fetal bovine serum
FFPE	formalin-fixed paraffin-embedded
FISH	fluorescence *in situ* hybridisation
5-FU	5-fluorouracil
GADPH	glyceraldehyde-3-phosphate dehydrogenase
GAPPS	gastric adenocarcinoma and proximal polyposis of the stomach
GATA4/6	transcription factor GATA4/6
GCN	gene copy number
GIAC	gastrointestinal adenocarcinoma
GOJ	gastro-oesophageal junction
Gy	Gray

GS	genomic stability
H&E	hematoxylin-eosin
HBEGF	heparin-binding EGF
HER2	receptor tyrosine-protein kinase erbB-2/human epidermal growth factor receptor 2
HM-SNV	hypermutated tumours enriched with single-nucleotide variants
HNSCC	head and neck squamous cell carcinoma
HRAS	GTPase HRAS (*HRAS*, Harvey rat sarcoma viral oncogene homolog)
ICC	intraclass correlation coefficient
IFN-γ	interferon-γ
IHC	immunohistochemistry
JAK	tyrosine-protein kinase JAK (*Jak*, Janus kinase)
KRAS	GTPase KRAS (*KRAS*, Kirsten rat sarcoma viral oncogene homolog)
LGR5	leucine-rich repeat-containing G-protein coupled receptor 5
LOH	loss of heterozygosity
LOI	loss of imprinting
LQ	linear quadratic
MAPK	mitogen-activated protein kinase
MDM2	E3 ubiquitin-protein ligase MDM2
MGMT	6-O-methylguanine-DNA methyltransferase/methylated-DNA–protein-cysteine methyltransferase
MEK	dual specificity mitogen-activated protein kinase kinase
MET	hepatocyte growth factor receptor
MeV	megaelectronvolt
MICI	most intensive cytoplasmic staining index
µL	microliter
mL	millilitre
min	minute
MLH1	DNA mismatch repair protein MLH1
MMR	mismatch repair
MRI	magnetic resonance imaging
mRNA	messenger RNA
MSH2/3/6	DNA mismatch repair protein MSH2/3/6
MSI	microsatellite instability
MSI-H	MSI-high
MSI-L	MSI-low
MSS	microsatellite-stable
mTOR	serine/threonine-protein kinase mTOR (mechanistic target of rapamycin kinase)

mut	mutated
MYC	Myc proto-oncogene protein (*Myc*, v-myc avian myelocytomatosis viral oncogene homolog)
ND	not determined
ngTMA	next-generation tissue microarray
NOTCH1	neurogenic locus notch homolog protein 1
NRAS	GTPase NRAS (*NRAS*, neuroblastoma rat sarcoma viral oncogene homolog
NRG	neuregulin
NS	not significant
NSCLC	non-small cell lung cancer
NY	New York
Oct4	octamer-binding protein 4
OR	odds ratio
OS	overall survival
p14ARF	tumour suppressor ARF (alternative reading frame)
p16INK4A	alternative name for CDKN2A
PARP	poly(ADP-ribose)polymerase
PCR	polymerase chain reaction
PD-L1/2	programmed cell death 1 ligand 1/2
PFS	progress-free survival
PI3K	phosphatidyl-inositol-3-kinase
PIK3CA	phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
PME-1	protein phosphatase methylesterase 1
PMS2	mismatch repair endonuclease PMS2
POLD	DNA polymerase delta
POLE	DNA polymerase epsilon
PP1	serine/threonine-protein phosphatase 1
PP2A	protein phosphatase 2A
PPP2CA/B	serine/threonine-protein phosphatase 2A catalytic subunit alpha/beta isoform
PPP2R1A/B	serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha/beta isoform
PTB	phosphotyrosine-binding
R	residual tumour
RAC	Ras-related C3 botulinum toxin substrate
RAF	RAF proto-oncogene serine/threonine-protein kinase
RAS	rat sarcoma gene family

RECIST	Response Evaluation Criteria in Solid Tumors
RFS	recurrence-free survival
RHOA	transforming protein RhoA (ras homolog family member A)
RNA	ribonucleic acid
RNAi	RNA interference
RTK	receptor tyrosine kinase
S492R	serine substituted by arginine at amino acid position 492
SCNA	somatic copy number alteration
SET	protein SET
SF	surviving fraction
SH2	Src homology-2
siRNA	short interfering RNA
SISH	silver *in situ* hybridisation
SMAD4	mothers against decapentaplegic homolog 4
SNV	single nucleotide variant
SOX9	transcription factor SOX-9 (SRY-box 9)
SSA/P	sessile serated adenoma/polyp
STAT	signal transducer and activator of transcription
SV40	simian virus 40
TCGA	The Cancer Genome Atlas
TGF-α/β	transforming growth factor α/β
Th1	type 1 T helper cell
TKI	tyrosine kinase inhibitor
TMA	tissue microarray
TNM	TNM classification of malignant tumours (tumour, node, metastasis)
TP53	cellular tumour antigen p53
TSA	traditional serrated adenoma
TTR	time to recurrence
V600E	valine substituted by glutamic acid at amino acid position 600
VEGFA	vascular endothelial growth factor A
VEGFR2	vascular endothelial growth factor receptor 2
vs	versus
WA	Washington
WHO	World Health Organization
Wnt	protein Wnt
wt	wild-type

LIST OF ORIGINAL PUBLICATIONS

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III Birkman E-M, Elzagheid A, Jokilehto T, Avoranta T, Korkeila E, Kulmala J, Syrjänen K, Westermarck J, Sundström J. Protein phosphatase 2A (PP2A) inhibitor CIP2A indicates resistance to radiotherapy in rectal cancer. Cancer Medicine 2018;7(3):698–706.

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1 INTRODUCTION

Cancer cells have accumulated various genetic alterations ranging from single nucleotide mutations to whole-genome duplications during their evolution originating from a normal cell and leading to a malignant tumour. Recent cancer genome sequencing studies, particularly by The Cancer Genome Atlas (TCGA) project, have provided enormous amounts of information about the molecular characteristics of different types of cancers. (Chen *et al.* 2018).

This literature review discusses some general mechanisms leading to the development of cancer but mainly concentrates on certain molecular characteristic of gastric and colorectal adenocarcinomas, which have been studied in the original publications.

Gastrointestinal cancers, particularly gastric and colorectal cancer, belong to the most common malignancies and causes of cancer death worldwide (Bray *et al.* 2018; Ferlay *et al.* 2019). Adenocarcinomas form the large majority of gastrointestinal cancers. Over the last decades, the incidence of gastric cancer has gradually declined in Western Europe and North America even though this decline has recently been slowing down in some countries. Gastric cancer still remains very common in Eastern Europe, South America and Eastern Asia. (Smyth *et al.* 2016; Van Cutsem *et al.* 2016). In contrast, at the same time the incidence of adenocarcinoma of the oesophagus and gastro-oesophageal junction (GOJ) has increased in Western countries (Pohl *et al.* 2010). These proximal gastrointestinal adenocarcinomas are usually considered as separate entities from true gastric adenocarcinomas, but it has recently been shown that they share very similar molecular features with a subtype of gastric cancer characterised by chromosomal instability (CIN) (Liu *et al.* 2018).

Persistent *Helicobacter pylori* infection is one of the most important causes of sporadic gastric cancer. The chronic inflammation associated with the infection can lead to mucosal atrophy, intestinal metaplasia and in some patients further to dysplasia and adenocarcinoma. Other factors associated with an increased cancer risk include autoimmune atrophic gastritis, smoking, nutritional factors such as high salt intake and nitrates, and obesity. Chronic reflux disease and intestinal metaplasia in the distal oesophagus (Barrett's syndrome) increase the risk for GOJ adenocarcinoma. (Lordick *et al.* 2016; Smyth *et al.* 2016; Van Cutsem *et al.* 2016). Germline mutations, such as mutations in *CDH1* encoding E-cadherin, account for 1 – 3% of all gastric cancer (Smyth *et al.* 2016; Van Cutsem *et al.* 2016).

In contrast to gastric cancer, the incidence of colorectal cancer is highest in Western countries, while the lowest incidences are found in some Asian and African countries. Established risk factors include the so-called Western diet with high intake of red meat and low intake of plant-based and unrefined foods, smoking, alcohol consumption and obesity. Importantly, inflammatory bowel disease is an independent risk factor for colorectal cancer. Germline mutations leading to hereditary syndromes such as Lynch syndrome account for 3 – 5% of all colorectal cancer. (Brenner *et al.* 2014).

The purpose of different molecular biomarkers in the context of cancer management is mainly to function as diagnostic, prognostic or predictive factors that can help in specifying the type of disease and in selecting and optimising the treatments offered for the patients. A molecular biomarker can be, for example, a specific protein, mutation or gene amplification, the presence of which predicts response or lack of response to a specific treatment or indicates the likely behaviour of the disease. Alternatively, a biomarker can be used by proxy to classify a particular tumour into a molecular subtype associated with specific properties and behaviour, which are not necessarily directly linked with the biological functions of that biomarker.

One of the predictive biomarkers utilised in clinical pathology is HER2, the overexpression or gene amplification of which in breast and gastric cancer indicates favourable response to anti-HER2 antibody therapy (Bang *et al.* 2010). Another example is the detection of *RAS* mutations in patients with metastatic colorectal cancer, as anti-EGFR antibody treatment is beneficial only for patients with *RAS* wild-type (wt) tumours (Bokemeyer *et al.* 2009; Douillard *et al.* 2010). Furthermore, in retrospective studies, *EGFR* gene copy number (GCN) ≥ 4.0 in primary colorectal adenocarcinomas has been associated with a favourable anti-EGFR antibody treatment response in patients with *RAS* wt metastatic disease (Ålgars *et al.* 2011; Ålgars *et al.* 2014; Ålgars *et al.* 2017).

In contrast, no survival benefit has been observed in clinical trials for oesophagogastric cancer patients treated with an anti-EGFR antibody together with standard chemotherapy in comparison to only chemotherapy (Lordick *et al.* 2013; Waddell *et al.* 2013). However, these trials have not included any patient selection based on the histological subtype of the tumours or *EGFR* GCN analysis. As the clinical significance of HER2 overexpression or gene amplification has been demonstrated in the context of anti-HER2 therapy (Bang *et al.* 2010), it could be of interest to investigate if the presence of *EGFR* amplification might indicate those patients who could benefit from anti-EGFR antibodies. A prerequisite for the utilisation of *EGFR* GCN analysis in cancer treatment would be a high enough prevalence of *EGFR* amplification in oesophagogastric adenocarcinomas.

A large study has recently classified gastric adenocarcinomas into four distinct molecular subtypes based on their genomic alterations. These subtypes are characterised by either chromosomal instability, genomic stability (GS), microsatellite instability (MSI) or Epstein-Barr virus (EBV) infection. (TCGA 2014). Notably, even in the age of genome sequencing, the traditional Laurén classification (Laurén 1965) has remained a relevant part of cancer diagnostics. A modified version of the classification recognises three categories, proximal non-diffuse, distal non-diffuse and diffuse-type tumours, each with distinct epidemiology and gene expression patterns (Shah *et al.* 2011). The molecular characterisation studies have also demonstrated that the intestinal and diffuse histological subtypes originally described by Pekka Laurén are distinguishable to a large extent also at the molecular level (TCGA 2014; Cristescu *et al.* 2015). The CIN subtype is observed to be strongly associated with the intestinal histological subtype and the activation of the receptor tyrosine kinase (RTK)–RAS pathway, for example by RTK gene amplifications. In contrast, diffuse-type tumours are concentrated in the GS subtype. (TCGA 2014).

These studies have also provided information about the variability in biological properties among oesophagogastric adenocarcinomas. Instead of considering cancer of a specific organ as a single disease, it has become clear that when exploring new cancer therapies, future studies need to be conducted among defined sets of patients having tumours with specific genomic aberrations. However, genome-wide characterisation studies typically use complex and expensive methodologies that are not applicable for routine clinical diagnostics. Daily diagnostic work rather requires more straightforward and less costly methods that are still able to provide the relevant information needed for the subtype determination.

Preoperative (chemo)radiotherapy, (C)RT, is used in the treatment of rectal cancer patients. However, tumour response to (C)RT among patients is variable and currently no clinical biomarkers exist that could be used to predict response to this therapy or to stratify patients into different preoperative treatment groups according to their prognosis. High expression of cancerous inhibitor of protein phosphatase 2A (CIP2A) has been indicated to contribute to radioresistance in head and neck squamous cell carcinoma (Ventelä *et al.* 2015), but no studies so far have examined the role of CIP2A in radiation response in rectal cancer patients. If it were associated with the degree of tumour regression after preoperative (C)RT, CIP2A expression level might deserve further examination as a potential biomarker for radiosensitivity in rectal cancer patients.

It is a well-known phenomenon that cancer cells continue to acquire mutations and other genomic alterations during the metastatic process. At worst, these new aberrations might render the metastatic tumour unresponsive to treatment which was originally selected based on the properties of the primary tumour. Also the association between *EGFR* GCN ≥ 4.0 and a favourable response to anti-EGFR antibodies has been observed examining particularly primary *RAS* wt colorectal tumours (Ålgars *et al.* 2011; Ålgars *et al.* 2014; Ålgars *et al.* 2017). However, data comparing *EGFR* GCN between the primary tumours and the corresponding metastases are limited. In addition, few studies have examined the effect of anti-EGFR antibody treatment on the *EGFR* GCN in metastatic tumours. Possible GCN changes in the metastatic tumours might affect the response to antibody therapy during continuous treatment and thus could be one of the mechanisms responsible for acquired treatment resistance to anti-EGFR antibodies.

2 REVIEW OF LITERATURE

2.1 Development of the gastrointestinal tract

The epithelium of the gastrointestinal tract develops from the endoderm during embryogenesis, while the morphogenesis of the intestinal epithelial structures proceeds through the gestation and continues even after birth. Small intestine villi begin to form during embryogenesis, whereas the intestinal crypts form after birth. The formation of these structures requires constant signalling between the epithelial and mesenchymal cells mediated, for example, by the Wnt and Hedgehog pathways. (Barker *et al.* 2009; Brabletz *et al.* 2009). In addition, transforming growth factor β (TGF-β) is an essential regulator of signalling in the gastrointestinal epithelium starting from embryonic development and continuing thereafter throughout adulthood (Mishra *et al.* 2005; Liu *et al.* 2018).

The columnar epithelium of the stomach and intestines is continuously renewed by the division of stem cells located at the base of the gastric glands and intestinal crypts. These stem cells can be identified by leucine-rich repeat-containing G-protein coupled receptor (LGR5) expression. (Barker *et al.* 2007; Barker *et al.* 2009; Barker *et al.* 2010). Wnt signalling is the main pathway controlling the intestinal stem cells, and *LGR5* is one of the target genes of Wnt/β-catenin signalling (Clevers 2006; Brabletz *et al.* 2009). Also TGF-β signalling is involved in the regulation of intestinal stem cells (Mishra *et al.* 2005; Schepers & Clevers 2012). In the stomach, the LGR5 expressing stem cells are mainly located in the antrum (Zhao *et al.* 2015).

The common embryonic origin of the gastrointestinal tract is reflected in the development of gastrointestinal cancers in which the pathogenesis often involves the activation of developmental pathways, such as Wnt or TGF-β signalling. Normally, TGF-β mediates growth promoting signals during development but has a suppressive role in adult tissues. (Mishra *et al.* 2005; Schepers & Clevers 2012). Particularly, gastrointestinal adenocarcinomas in the lower gastrointestinal tract are enriched with active Wnt signalling (Schepers & Clevers 2012; Liu *et al.* 2018). According to the stem cell hypothesis, gastrointestinal cancer originates from intestinal cancer stem cells with dysregulated signalling pathways (Brabletz *et al.* 2009).

2.2 General mechanisms of cancer development

Cancer develops when cells acquire the ability to divide and grow uncontrollably, survive and invade. This development typically takes place over a long period of time, and it is due to the accumulation of genetic alterations. These alterations affect diverse signalling pathways that control a number of essential cellular functions. The result is a set of capabilities promoting carcinogenesis, and given their ubiquitous nature, they have been called the hallmarks of cancer. These hallmarks are enabled by genomic instability and supported by various molecules released by tumour-associated inflammatory cells. (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011). In

addition to somatic alterations, a notable proportion of cancers are associated with germ line variants of the signalling pathway genes that predispose to cancer (Huang *et al.* 2018).

2.2.1 Becoming a cancer cell

All cancers have their origin in normal cells. Every cell that is able to proliferate is also able to acquire changes in its genome during every cell division. Small changes in the DNA nucleotide sequence, such as mutations, can be fixed by DNA repair proteins present in the nucleus. However, if they remain unrepaired, the mutations will transfer into subsequent cell generations. Some of them may not have any effect on cell function, whereas others are harmful and lead to cell death, which cures the problem. Eventually, some changes remain and have a beneficial effect on the cell. That is, they give the cell a survival advantage among other cells that reside in the same environment and do not contain similar changes. As these beneficial effects transfer into new cell generations, additional genomic and posttranslational changes can accumulate and lead to progressive alterations. The cell also becomes able to escape the control mechanisms that normally keep it functioning properly. At some point, the output of this evolution can be considered a cancer cell. (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011; Lee *et al.* 2016).

In addition to mutations, the changes acquired during cell division can affect larger parts of the genome and lead to gain or loss of whole genes, chromosome arms or chromosomes. The susceptibility of a cell to accumulate these changes is related to genomic instability. The hallmarks that support the survival of cancer cells include the ability to resist cell death, to evade growth suppressors, to sustain proliferative signalling, to gain replicative immortality, to invade and metastasise, to induce angiogenesis, to reprogram energy metabolism and to evade immune destruction as well as to sustain tumour-promoting inflammation. In addition, cancer cells have the ability to interact with and recruit surrounding normal cells, such as stromal fibroblasts, to create a tumour microenvironment that supports the acquisition of other capabilities necessary for their survival. (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011).

2.2.2 Genome of a cancer cell

Across different cancer types, the functional changes driving tumorigenesis tend to be either somatic mutations or copy number alterations. This distinction is seen most clearly in tumours exhibiting high levels of genomic instability, which have been observed to contain either large numbers of mutations or copy number alterations but not both. *TP53* mutations are an exception because they are enriched in tumours typically containing also copy number alterations. (Ciriello *et al.* 2013).

This division between tumours characterised either by mutations or copy number alterations might be related to the variability in chromatin structure between patients caused by epigenetic changes

such as DNA methylation or histone modifications. The epigenetically affected chromatin state in a single progenitor cell could be inherited during cell division and lead to the accumulation of a particular set of aberrations over time. (Chen *et al.* 2018).

The chromatin structure can be either open or compact. Open chromatin, which is associated with active enhancers, tends to contain fewer mutations than closed chromatin because it can be accessed by the DNA repair proteins. However, open chromatin, being a much longer molecule than the closed form, is more likely to be involved in long-range DNA – DNA interactions. This could increase the likelihood of structural rearrangements such as somatic copy number alterations (SCNA). (Polak *et al.* 2014; Chen *et al.* 2018).

A limited number of alterations can suffice to transform many different cell types (Hanahan & Weinberg 2000; Hanahan & Weinberg 2011). Each tumour has a unique combination of genetic alterations but each specific type of alteration is almost always found across various types of tumours. By analysing large numbers and different types of tumours, patterns of co-occurring or mutually exclusive changes can be discerned among them. (Wood *et al.* 2007; Boland & Goel 2010; Sanchez-Vega *et al.* 2018).

The genetic alterations in a single tumour are typically several and affect several different pathways. Some of the observed carcinogenic alterations are mutually exclusive, which could indicate that they are functionally redundant and their simultaneous occurrence would not provide further selective advantage. Alternatively, their simultaneous occurrence could be disadvantageous to the cell and lead to apoptosis. In contrast, the co-occurrence of specific alterations could indicate that both of them are beneficial for the cell. (Mina *et al.* 2017; Sanchez-Vega *et al.* 2018). When analysing interactions between somatic driver genes, *TP53* is found to have most connections to other genes (Ding *et al.* 2018).

Driver gene mutations involved in the same pathways tend to show strong exclusivity. In colorectal cancer, oncogenic mutations in *KRAS*, *NRAS* and *BRAF* are usually mutually exclusive (TCGA 2012; Mina *et al.* 2017; Ding *et al.* 2018). *TP53* and *KRAS* mutations are mutually exclusive in colorectal and lung adenocarcinomas but often co-occur in pancreatic adenocarcinomas (Ding *et al.* 2018).

2.2.3 Genomic instability

Genomic instability can be detected in most cancers, but its degree and consequences are variable between different cancer types and even within the same cancer type. (Lee *et al.* 2016). Temporal and spatial differences in genomic instability may even contribute to intratumoural heterogeneity (Gerlinger *et al.* 2012; Bedard *et al.* 2013; de Bruin *et al.* 2014; Zhang *et al.* 2014). The major categories of genomic instability are chromosomal and nucleotide-level instability. The most

common manifestations of these are somatic copy number alterations (SCNA) and point mutations, respectively. (Lee *et al.* 2016; Chen *et al.* 2018).

2.2.3.1 Chromosomal instability

Chromosomal instability (CIN) refers to an increased rate of chromosomal change in comparison to normal cells and results in a wide range of different structural variations observed in cancer genomes. These include inversions, translocations, duplications, larger insertions/deletions (> 30 bp), chromothripsis and SCNAs. Large-scale SCNAs (comprising chromosome arms or whole chromosomes leading to aneuploidy) are usually described as gain or loss of copy number, while focal SCNAs are called amplifications or deletions. (Lee *et al.* 2016). Chromothripsis refers to the fragmentation of chromosomes or chromosomal regions within a restricted area followed by an incorrect rearrangement by DNA repair mechanisms. It is thought to occur early in cancer cell evolution and suggested to promote carcinogenesis by leading to oncogene amplifications and fusions or loss of tumour suppressor genes. (Forment *et al.* 2012).

CIN seems to be rather a driver than a passenger event in carcinogenesis even though this has been a topic of debate (Holland & Cleveland 2009; Gordon *et al.* 2012; Lee *et al.* 2016). Aneuploidy caused by CIN seems to both promote tumorigenesis (a low level of CIN) and cause tumour suppression (a high level of CIN) (Weaver *et al.* 2007; Weaver & Cleveland 2009; Gordon *et al.* 2012). As tumour cells with high levels of aneuploidy are still detected, it is thought that the harmful effects of aneuploidy could be compensated by the acquisition of other alterations (Weaver & Cleveland 2009; Lee *et al.* 2016) such as the inactivation of the TP53 tumour suppressor pathway (Thompson & Compton 2010). It has been suggested that a high level of CIN together with the loss of TP53 function is sufficient to promote carcinogenesis with fewer additional tumour suppressor mutations than is required in precursors without aneuploidy (Liu *et al.* 2018). Mechanisms leading to CIN include oncogene-induced replication stress, mitotic defects and telomere attrition (Lee *et al.* 2016).

2.2.3.1.1 Oncogene-induced replication stress, mitotic defects and telomere attrition

Oncogene-induced replication stress means the impairment of DNA replication as the cell is driven to excessive replication due to oncogene activation or tumour suppressor gene inactivation. Eventually, this can lead to DNA double-strand breakage. (Bartkova *et al.* 2006; Di Micco *et al.* 2006; Negrini *et al.* 2010; Lee *et al.* 2016). In the context of the stepwise carcinogenesis model described for colorectal cancer (Fearon 2011), mutations in oncogenes, tumour suppressor genes or DNA repair genes, such as mismatch repair (MMR) genes, are the early events maintaining cell proliferation but also leading to replication stress (Macheret & Halazonetis 2015). Replication stress

can lead to abnormalities in both the structure and number of chromosomes (Burrell *et al.* 2013; Lee *et al.* 2016).

Mitotic defects can result from, for example, whole-genome duplication, uneven distribution of chromosomes into daughter cells during earlier mitoses and mitotic checkpoint dysfunction. Whole-genome duplication is thought to be an early event in tumorigenesis. (Kops *et al.* 2005; Holland & Cleveland 2009; Holland & Cleveland 2012). Uneven distribution of chromosomes may be caused by defective sister-chromatid cohesion (Barber *et al.* 2008) or merotelic attachment, which means that a single kinetochore is attached to microtubules emanating from both spindle poles resulting in a lagging chromosome. Merotelic attachment is believed to be an important cause of CIN. (Kops *et al.* 2005; Thompson & Compton 2008; Holland & Cleveland 2012). Inactivating mutations in genes coding for mitotic checkpoint proteins are rarely observed in human cancers (Holland & Cleveland 2009).

Telomere attrition (shortening) is has been observed already in colorectal adenomas (Engelhardt *et al.* 1997; Roger *et al.* 2013; Lee *et al.* 2016). Telomere shortening leads to dysfunctional telomeres, activation of DNA repair mechanisms, genomic instability and apoptosis or senescence (Maser & DePinho 2002; Artandi & DePinho 2010; Lee *et al.* 2016). However, in more advanced tumours the cells have acquired mechanisms to regenerate telomeres either through the reactivation of telomerase or the alternative lengthening of telomere (ALT) pathway (Maser & DePinho 2002).

2.2.3.1.2 Somatic copy number alterations

SCNAs are responsible for a major part of somatic alterations in cancer cell genomes and can lead to both oncogene activation and tumour suppressor inactivation. Focal SCNAs are the most common SCNAs and typically have a higher amplitude (several copies) than larger SCNAs such as arm-level and chromosome-level alterations (usually single-copy changes). (Beroukhim *et al.* 2010; Zack *et al.* 2013; Lee *et al.* 2016). Similar types of functional SCNAs have been found across different cancer types implying positive selection (Beroukhim *et al.* 2010). However, decreased negative selection or increased SCNA formation may also be responsible for some recurring SCNAs. Some SCNAs may be passenger events and not affect tumorigenesis.

Recurrent gene amplifications and deletions tend to occur in specific regions across cancer cell genomes. Frequently amplified regions typically contain epigenetic regulators and genes such as *EGFR, ERBB2, MDM2* and *MYC*, which become activated by amplification and function as oncogenes. Frequently deleted regions have been observed to contain tumour suppressor genes such as *ATM*, *NOTCH1* and *PPP2R2A*. (Zack *et al.* 2013).

Whole-genome duplications and loss of heterozygosity (LOH) events (deletion of one gene allele and amplification of the other) can also be included in SCNAs (Zack *et al.* 2013). Whole-genome duplications have been observed in about one third of cancers. As an early event in tumorigenesis,

they could enable the acquirement of other chromosomal instabilities. (Zack *et al.* 2013; Dewhurst *et al.* 2014; Lee *et al.* 2016). In comparison to diploid cancer cells, cancer cells with whole-genome duplications tend to have higher rates of also other types of SCNAs (Zack *et al.* 2013).

2.2.3.2 Nucleotide-level instability

Nucleotide-level instability leads to single nucleotide variations (SNV) and small insertions or deletions (< 30 bp, indel mutations) (Lee *et al.* 2016). These nucleotide-level changes result in the distinct mutational signatures observed in various cancers that can be associated with, for example, defects in the DNA repair mechanisms or specific carcinogens (Alexandrov *et al.* 2013).

Nucleotide-level instability is typically found in tumours with high levels of somatic mutations. It is often the consequence of defective DNA repair mechanisms like the MMR system or the proofreading function of DNA polymerases (Preston *et al.* 2010; Kim *et al.* 2013a; Lee *et al.* 2016). As in the case of CIN, it has been debated whether a hypermutated phenotype is advantageous to cancer cells (Fox *et al.* 2013). Nevertheless, it has been observed that cancers characterised by somatic mutations tend not to require additional SCNAs to increase genomic instability and drive tumorigenesis (TCGA 2012; Ciriello *et al.* 2013).

Among the hypermutated colorectal adenocarcinomas, the most commonly mutated genes include *BRAF, MSH3* and *MSH6*, while *APC* and *TP53* mutations are more common in non-hypermutated tumours together with mutations in *KRAS, NRAS, PIK3CA* and *SMAD4*. This supports the hypothesis that hypermutated and non-hypermutated tumours progress through different genetic pathways. (TCGA 2012).

2.2.3.2.1 Mismatch repair system defects

Defects in the MMR system lead to the accumulation of small insertions and deletions in DNA microsatellite regions, which results in a phenotype called microsatellite instability (MSI). Its role in cancer was first described in colorectal cancer patients. (Aaltonen *et al.* 1993; Ionov *et al.* 1993; Thibodeau *et al.* 1993; Lynch *et al.* 2015). Normally, MMR proteins recognise mismatched nucleotides during DNA replication and recruit other proteins to remove them in order to enable the insertion of the correct nucleotide. The MMR proteins function as dimers (MSH2 together with MSH6 or MSH3, MLH1 together with PMS2). Inactivation of both of the MMR alleles results in defective MMR and in the MSI phenotype. This inactivation can be caused either by mutation or promoter hypermethylation. (Lynch & de la Chapelle 2003; de la Chapelle & Hampel 2010).

Microsatellites are usually located in non-coding regions of the genome, but they are also found in coding regions of genes involved in cell proliferation or apoptosis (Ionov *et al.* 1993; Kinzler & Vogelstein 1996). MSI events most often occur in euchromatin regions (in contrast to

heterochromatin) and in early-replicating (in contrast to late-replicating) DNA segments (Kim *et al.* 2013a). The MMR system has been suggested to suppress mutations especially in the early-replicating euchromatin regions, which typically contain active genes essential for cell functions (Supek & Lehner 2015). Approximately 17% of cancers with amino-acid altering somatic mutations in MMR genes have been observed to have high MSI (MSI-H) status. Among cancers with germline MMR mutations, MSI-H has been observed in about 30% of them, most of which contain both pathogenic germline and somatic MMR mutations. (Ding *et al.* 2018).

2.2.3.2.2 DNA polymerase mutations

The proofreading function of DNA polymerases depends on their 3' to 5' exonuclease activity. Mutations in *POLE* and *POLD1*, coding for polymerase epsilon and delta, have been detected in both sporadic and hereditary colorectal cancers. (Flohr *et al.* 1999; TCGA 2012; Palles *et al.* 2013; Heitzer & Tomlinson 2014). Tumours with *POLE* mutations are characterised by even a greater number of mutations than MSI-H tumours (TCGA 2012). The presence of mutated *POLE* may be sufficient to drive tumorigenesis, as these tumours usually have microsatellite-stable (MSS) phenotype even in the presence of MMR mutations (Kim *et al.* 2013a) and do not contain SCNAs (Shlien *et al.* 2015). The functional role of *POLD1* mutations in cancer is uncertain (Lee *et al.* 2016).

2.2.3.3 Epigenetic changes

Epigenetic changes are heritable modifications of DNA or chromatin that do not alter the DNA sequence. Epigenetic modifications are required for normal cellular functions, but they are also commonly detected in cancer cells. Epigenetic changes are suggested to be functionally similar to mutations in being able to cause oncogene activation or tumour suppressor inactivation. Epigenetic modifications include events such as DNA methylation, genomic imprinting as well as histone and other modifications of chromatin. (Iacobuzio-Donahue 2009). Early epigenetic alterations affecting the stem cell population in the intestinal crypts and causing changes in their gene expression have been suggested to promote the accumulation of subsequent mutations and the development of cancer stem cells (Pardal *et al.* 2003; Feinberg *et al.* 2006).

DNA methylation refers to the covalent modification of DNA by a family of cytosine (DNA-5)-methyltransferases (DNMTs). In this process, a methyl group is transferred from S-adenosylmethionine to a cytosine located 5' to a guanosine (CpG dinucleotides). Short regions of DNA enriched with CpG dinucleotides are called CpG islands. Most CpG islands are found in areas such as microsatellites, centromeres and in promoter regions of approximately half of the genes in normal cells. (Jones & Baylin 2002; Issa 2004; Klose & Bird 2006). The methylation of the promoters prevents the transcription factors from binding to their binding sites, which results in gene silencing (Noffsinger 2009).

In normal cells, hypermethylation is suggested to contribute to genomic stability by repressing repetitive, often retroviral, elements. In contrast, cancer cells are often characterised by global hypomethylation, which may lead to the expression of normally silent genes, such as those which are normally expressed only during embryogenesis. (Iacobuzio-Donahue 2009). Global hypomethylation may also promote overall genomic instability and thus tumorigenesis (Eden *et al.* 2003).

However, promoter hypermethylation leading to loss of gene function is also commonly observed in cancer cells (Esteller 2007). It is one of the mechanisms leading to tumour suppressor inactivation as observed for example in patients with sporadic MSI-H colorectal cancer. In these patients, *MLH1* promoter hypermethylation has been detected in both the tumour cells and surrounding normal intestinal mucosa, which suggests that hypermethylation is an early event of tumorigenesis. (Ricciardiello *et al.* 2003; Kawakami *et al.* 2006; Iacobuzio-Donahue 2009). Promoter hypermethylation results in permanent gene silencing that is preserved in offspring cells (Kondo & Issa 2004). Increased methylation has been associated with older age and chronic inflammation such as in inflammatory bowel disease. Oxidative stress associated with inflammation may reduce DNA repair and thus lead to MSI. (Boland & Goel 2010).

Genomic imprinting refers to a mechanism where only one gene allele is transcriptionally active and the other becomes inactivated by methylation. The active allele is determined by the parent of origin. (Iacobuzio-Donahue 2009; Noffsinger 2009). Loss of imprinting (LOI) leading to abnormal gene expression has been observed in different cancers. LOI can be obtained by activation of the normally inactive allele or by inactivation of the remaining active allele. (Iacobuzio-Donahue 2009).

2.2.4 *Phosphorylating enzymes and phosphatases*

In addition to translational regulation, the various proteins involved in different cell signalling pathways are regulated post-translationally by the addition or removal of different types of chemical groups. This regulation is performed by a vast number of different enzymes, each of which is specialised to a certain reaction type. One of the post-translational regulatory mechanisms is reversible phosphorylation involving the addition or removal of phosphate groups. (Hunter 1995; Khanna & Pimanda 2016).

Phosphorylating enzymes are called protein kinases and they usually activate their target proteins. In contrast, protein phosphatases are dephosphorylating enzymes and usually deactivate their target proteins. Disturbances in the balance between kinase and phosphatase expression or activity alter intracellular signalling and thus the regulation of cellular functions. (Hunter 1995; Khanna & Pimanda 2016). Some of these alterations may promote cell survival, carcinogenesis and tumour growth. Cancer cells typically contain mutations leading to constitutively active protein kinases. (Hanahan & Weinberg 2011). In contrast, protein phosphatases tend to act as tumour suppressors and therefore become inactivated in cancer cells (Khanna & Pimanda 2016). In cancer cells, this

regulation by phosphorylation and dephosphorylation can function together with activating mutations of *KRAS, NRAS* and *HRAS* (Prior *et al.* 2012). For example, dephosphorylation of signalling proteins by protein phosphatase 2A (PP2A) can counter the oncogenic effects of constantly active RAS (Sablina *et al.* 2010; Naetar *et al.* 2014).

2.2.4.1 Protein phosphatase 2A

The PP2A family of heterotrimeric phosphatases contains together with the protein phosphatase 1 (PP1) family the predominant serine-threonine phosphatases in eukaryotic cells. PP2A is involved both in normal cell functions and malignant transformation. (Sablina & Hahn 2008; Westermarck & Hahn 2008; Eichhorn *et al.* 2009). The three-part structure of the core enzyme consists of a scaffolding A subunit (PR65α, encoded by *PPP2R1A,* or PR65β, encoded by *PPP2R1B*), a catalytic C subunit (PP2ACα, encoded by *PPP2CA,* or PP2ACβ, encoded by *PPP2CB*) and one of the several isoforms of a regulatory B subunit binding the other subunits. (Janssens & Goris 2001; Westermarck & Hahn 2008; Eichhorn *et al.* 2009; O'Connor *et al.* 2018).

The B subunit defines both the localisation and substrate specificity of the enzyme complex. The variable combinations of these subunits give rise to different PP2A complexes with diverse substrate specificities. (Janssens & Goris 2001; Westermarck & Hahn 2008; Eichhorn *et al.* 2009; O'Connor *et al.* 2018). Normally, PP2A inhibits the oncogenic signalling by dephosphorylation, but only some of the B subunits direct the PP2A complex to function as a tumour suppressor (Westermarck & Hahn 2008; Eichhorn *et al.* 2009; Sablina *et al.* 2010).

PP2A plays a role in carcinogenesis through its inactivation, which mainly occurs by non-genomic mechanisms. These include the overexpression of endogenous inhibitor proteins and post-translational modifications of the catalytic subunit. (Chen *et al.* 1992; Westermarck & Hahn 2008; Kauko & Westermarck 2018). Non-genomic mechanisms allow a more selective and transient regulation of PP2A activity than can be obtained through the inactivating mutations (Kauko & Westermarck 2018). Mutations in PP2A subunits seem to have a relatively minor role in malignant transformation (Sablina & Hahn 2008; Zack *et al.* 2013, Kauko & Westermarck 2018).

2.2.4.1.1 Inhibition of protein phosphatase 2A

Several proteins have been recognised to function as endogenous PP2A inhibitors. For example, cancerous inhibitor of PP2A (CIP2A), protein phosphatase methylesterase 1 (PME-1) and protein SET (SET) are involved in the regulation of cell proliferation, while cAMP-regulated phosphoprotein 19 (ARPP19), biorientation of chromosomes in cell division protein 1 (BOD1) and alpha-endosulphine (ENSA) are inhibiting PP2A during mitosis. (Westermarck & Hahn 2008; Puustinen *et al.* 2009; Ventelä *et al.* 2012; Laine *et al.* 2013; Kauko & Westermarck 2018).

The post-translational mechanisms of PP2A inhibition include the phosphorylation or demethylation of PP2A subunits at specific sites (Kauko & Westermarck 2018). PME-1 can inhibit PP2A both by demethylation and binding directly to the catalytic site of PP2A (Kaur & Westermarck 2016). Viral proteins such as the simian virus 40 (SV40) small t antigen have also been suggested to act as PP2A inhibitors (Westermarck & Hahn 2008). However, the role of SV40 or other viruses in promoting human cancers by PP2A inhibition is uncertain (Kauko & Westermarck 2018).

Mutations of PP2A are most frequently observed in *PPP2R1A* and *PPP2R2A* (Zack *et al.* 2013). *PPP2R2A* encodes one of the regulatory subunits of PP2A (Eichhorn *et al.* 2009). In addition, genomic inhibition of PP2A can be obtained by larger alterations such as arm-level deletions. For example, loss of the 8p chromosome arm, containing *PPP2R2A*, is frequently observed among different cancer types. Deletions of *PPP2R2A* and mutations in *PPP2R1A* have been found to be associated with whole-genome duplication. (Zack *et al.* 2013).

2.2.5 *From primary to metastatic tumours*

When acquiring metastatic properties, cancer cells typically change their shape as well as their attachments to other cells and the extracellular matrix. The purpose of these changes is to enable cancer cells to leave the primary tumour, move through the surrounding tissues into blood or lymphatic vessels, exit the vessel and form a new tumour at a distant site. These metastatic properties are achieved through modifications in gene expression and intracellular signalling leading to alterations in the cytoskeletal structures and adhesion proteins on cell membranes. (Hanahan & Weinberg 2011).

Normally, the molecular mechanisms of the epithelial-to-mesenchymal transition (EMT) are involved in embryogenesis and wound healing. In cancer cells, EMT is likely an essential process for acquiring the properties needed for invasion, apoptosis resistance and metastasis. The central mechanism of EMT is the suppression of E-cadherin and upregulation of N-cadherin expression through the action of a number of transcription factors. E-cadherin is normally involved in adherence junctions between epithelial cells, while N-cadherin is expressed in migrating mesenchymal cells and neurons during embryonic development. (Hanahan & Weinberg 2011).

Metachronous metastases, occurring after the resection of the primary tumour, are thought to arise from disseminated tumour cells (DTC) that have travelled to distant sites already before the primary surgery. However, it cannot yet be predicted which molecular characteristics are required from those DTCs that will eventually form metastatic tumours. (Klein 2009).

2.2.5.1 Linear and parallel progression

The two principal models of metastasis are the linear progression model and the parallel progression model. Stepwise linear progression is thought to occur within the primary tumour where the tumour cells are continuously acquiring and selected for growth promoting mutations and epigenetic alterations. The surviving clones are able to proliferate and seed the distant metastases. Therefore, according to this model, the DTCs would share many of the molecular characteristics present in the primary tumour. (Nowell 2002; Klein 2009). The expansion of the surviving clones has been associated with tumour size, and larger tumours are thought to have been able to acquire more significant mutations than smaller tumours. Larger tumour size has also been associated with a higher frequency of metastases. (Klein 2009).

However, the presence of similar changes in primary and metastatic tumour does not provide an ultimate proof that the metastatic cells have clonally descended from the primary tumour. As it is known that certain mutations or other aberrations are selected over others, it is feasible that similar alterations may occur independently both in the primary and metastatic tumour as well as in different cells within the same tumour. Genetic differences between primary and metastatic tumours would indicate, however, that some selection has occurred and led to the survival of divergent clones. (Klein 2009).

According to the parallel progression model, tumour cells leave the primary tumour while still evolving towards a fully malignant phenotype, and metastases could start to develop even before the occurrence of the first symptoms from the primary tumour (Klein 2009). In this case, the genomic aberrations needed for metastasising should already be present in the primary tumours and show intratumoural heterogeneity (Hühns *et al.* 2018). After reaching a suitable distant site, tumour cells would acquire further genomic alterations and undergo clonal selection. This mechanism would enable the adaptation of different tumour cell populations to different microenvironments even after dissemination. According to the parallel model, the primary tumour would not be the optimal reference when selecting systemic therapy for the patient, as the primary and metastatic tumour cells could be genetically different. Thus, the characterisation of DTCs would be required for predicting therapy responses. (Klein 2009).

In support of the parallel model, it has been observed that notable heterogeneity of genomic aberrations may already be present in colorectal adenomas and early primary adenocarcinomas rather than only in later occurring metastatic tumours (Hühns *et al.* 2018). In this case, genomic heterogeneity observed in metastatic tumours could develop later due to genomic instability but might not be required for growth advantage (Sottoriva *et al.* 2015).

It has not been definitely proven that one of these models would apply for all metastatic processes. They need not to be mutually exclusive. In some tumours, the required mutations necessary for metastasising might be acquired in the beginning of carcinogenesis, whereas in others the mutations could occur in a step-wise fashion over a longer time period. (Hühns *et al.* 2018). However, at the

moment, the parallel progression model seems to be favoured over the linear progression model (Klein 2009; Hühns *et al.* 2018).

2.3 Mechanisms of molecular pathogenesis in gastrointestinal cancer

2.3.1 Adenoma – carcinoma sequence

The classical model for cancer development has been derived from the adenoma-carcinoma sequence originally proposed for the development of colorectal adenocarcinomas (Vogelstein *et al.* 1988; Boland & Goel 2010; Fearon 2011). According to this model, the conventional low grade adenomas progress into high grade adenomas and finally into invasive tumours due to a stepwise accumulation of genetic changes. At the beginning of this pathway, the inactivation of *APC* leads to the development of an adenoma, which then gains the ability to grow due to subsequent *KRAS* mutations and deletions on chromosome 18q. Finally, biallelic loss or inactivation of *TP53* leads to the transition from adenoma to carcinoma. (Fearon 2011).

This pathway applies predominantly to sporadic colorectal cancers, but it is also detected in familial adenomatous polyposis (FAP). It is sometimes also called the chromosomal instability pathway because tumours arising by this pathway are characterised by chromosomal abnormalities including deletions, insertions and LOH. (Noffsinger 2009). Later, it has been noticed that only a few colorectal cancers actually evolve along this pathway (Wood *et al.* 2007) and alternative routes have been suggested.

Aberrant Wnt signalling, due to *APC* or β-catenin mutations, or other alterations leading to stabilisation and nuclear accumulation of β-catenin, is detected already in colorectal adenomas, and it remains involved in tumour progression during invasion and metastasis (Brabletz *et al.* 2009). Alterations in the Wnt pathway are observed in over 90% of colorectal adenocarcinomas. The majority of these is due to biallelic inactivation of *APC* or activating mutations in *CTNNB1* (encoding β-catenin). Defective Wnt signalling is common both in hypermutated and non-hypermutated colorectal tumours. *APC* mutated tumours often contain also other mutations along the Wnt pathway, which could provide selective advantage. (TCGA 2012). In sporadic colorectal cancer, the loss of both *APC* alleles is required for the loss of APC function (Kinzler & Vogelstein 1996; Clevers & Nusse 2012). Later in tumorigenesis, *KRAS* and *TP53* mutations occur together with CIN (Lengauer *et al.* 1997; Smith *et al.* 2002; Fearon 2011) and LOH (Fearon 2011).

The development of focal CIN (CIN-F), characterised by fragmented genomes with focal, short and high-amplitude SCNAs, is associated with *TP53* mutations particularly in the upper gastrointestinal tract. In the lower gastrointestinal tract, loss of APC is often an earlier event than loss of TP53. APC loss may lead the carcinogenic process to another direction than TP53 loss, which may explain the lower prevalence of CIN-F in colorectal adenocarcinomas. *APC* mutant

cells in the lower gastrointestinal tract might be able to undergo malignant transformation without the need for *TP53* mutation or aneuploidy. Upper gastrointestinal tumours with broad and low-amplitude SCNAs (CIN-B) often contain mutations in tumour suppressors such as *APC*, *CDKN2A* and *SMAD4* instead of *TP53* mutations. This implies that genomic instability leading to aneuploidy and CIN-F does not occur very easily in the absence of *TP53* aberrations. In general, early *APC* loss and activating mutations in oncogenes like *KRAS* are typical of colorectal cancer, while extensive aneuploidy and resulting oncogene amplification are more characteristic of upper gastrointestinal tract adenocarcinomas. (Liu *et al.* 2018).

Wnt signalling is the central activator of EMT through which cancer cells acquire their invasive properties. Nuclear accumulation of β-catenin, which is a sign of Wnt activation, is typically observed at the invasive front of the tumours where cancer cells can also have a dedifferentiated phenotype. (Brabletz *et al.* 2001). In addition, due to the activation of Wnt signalling, cancer cells might be able to undergo EMT and gain stem cell properties, both of which could contribute to their metastatic ability (Brabletz *et al.* 2001; Fodde & Brabletz 2007; Brabletz *et al.* 2009; Vermeulen *et al.* 2010). In contrast, at the metastatic sites cancer cells can undergo mesenchymal-epithelial transition (MET) and regain a more differentiated phenotype showing E-cadherin and membranous β-catenin expression (Brabletz *et al.* 2009).

It has been hypothesised that only a small subset of tumour cells, cancer stem cells, has tumorigenic properties, while the rest of the tumour cells have lost their tumorigenic capacity due to differentiation. Maintaining active Wnt signalling may require interaction between the cancer stem cells and surrounding myofibroblasts. Myofibroblasts may also be able to induce stem cell properties in more differentiated tumour cells by promoting activation of the Wnt pathway. The concentration of nuclear β-catenin into the invasive tumour front could indicate Wnt activation by the surrounding myofibroblasts. (Vermeulen *et al.* 2010).

2.3.2 *CpG island methylator phenotype*

Cancers showing elevated frequencies of aberrant CpG island methylation are described as having a CpG island methylator phenotype (CIMP) (Issa 2004). Colorectal cancers with CIMP form a distinct subgroup characterised by methylation of tumour suppressor genes such as *CDKN2A* (encoding p16 and p14ARF) and DNA repair genes such as *MLH1* and *MGMT* (encoding methylguanine methyltransferase) (Issa 2004; Ogino *et al.* 2009; Boland & Goel 2010).

CIMP can be observed in 30 – 50% of colorectal cancers (Kambara *et al.* 2004; O'Brien *et al.* 2006). The inactivation of *MLH1* and *MGMT* lead to high-level MSI (MSI-H) and low-level MSI (MSI-L), respectively. *MLH1* and *MGMT* methylation may also coexist in MSI-H tumours. (Noffsinger 2009). Colon tumours with CIMP tend to occur in elderly patients, and they are typically right-sided, mucinous, poorly differentiated, MSI-H and *BRAF* mutated (Kambara *et al.* 2004; Noffsinger 2009).

A small subset of colorectal cancer does not show CIN or MSI-H but are still characterised by CIMP. These tumours are associated with serrated morphology and poor prognosis of the patients. (De Sousa E Melo *et al.* 2013). In a large analysis, MSI-H due to epigenetic silencing of *MLH1* was found to be lacking in about 40% of the high-level CIMP (CIMP-H) gastrointestinal adenocarcinomas. Most commonly, these were CIN tumours located in the oesophagus or proximal stomach, descending colon or rectum. Conversely, some MSI-H gastrointestinal adenocarcinomas without *MLH1* methylation or CIMP were observed to contain somatic mutations in *MLH1* or *MSH2*, which suggests an alternative way to the loss of DNA MMR function. In these tumours, *KRAS* mutations are more common than *BRAF* mutations. In a small number of MSI-H gastrointestinal adenocarcinomas, the hypermutated status is explained neither by MMR promoter methylation or MMR mutations. (Liu *et al.* 2018).

2.3.3 Mismatch repair deficiency and microsatellite instability

MSI was first described in tumours arising in patients with Lynch syndrome. Among them, mutations in DNA MMR genes (usually *MLH1* and *MSH2*, rarely *MSH6* and *PMS2*) typically lead to MSI-H tumours. (Aaltonen *et al.* 1993; Peltomäki *et al.* 1993; Thibodeau *et al.* 1993). *MGMT* methylation is rare in Lynch syndrome. In sporadic colon cancers, loss of MMR function and MSI-H result from promoter methylation, whereas in Lynch syndrome they usually result from germline MMR mutations (Deng *et al.* 2004). In addition to point mutations, MMR genes may become inactivated by insertions, deletions or rearrangements. Instead of germ line mutations, epigenetic MMR inactivation by promoter hypermethylation can be observed in some Lynch syndrome patients. (Boland & Goel 2010).

MMR defects have been detected in about 12 – 15% of sporadic colorectal adenocarcinomas (Noffsinger 2009; Lynch *et al.* 2015). *MLH1* promoter methylation has been observed to be responsible for almost 90% of sporadic MSI-H colorectal cancers, while methylation of other MMR gene promoters is rare (Jass 2005). The carcinogenic DNA mismatch repair pathway (Aaltonen *et al.* 1993; Thibodeau *et al.* 1993) is characterised by CIMP and gives rise to sporadic MSI-H colorectal cancer (Toyota *et al.* 1999; Goel *et al.* 2007; Ogino *et al.* 2009). Conversely, most of the sporadic colorectal cancers with MSI-H arise from a CIMP background (Weisenberger *et al.* 2006; Nagasaka *et al.* 2008).

APC mutations, β-catenin activation, *KRAS* mutations and LOH are unusual in sporadic MSI-H colorectal cancer (Salahshor *et al.* 1999; Jass *et al.* 2003; Kambara *et al.* 2004; Boland & Goel 2010). Instead of the *APC* mutation pathway, sporadic MSI-H tumours are thought to develop via an alternative pathway initiated by an epigenetic mechanism causing CIMP-H. In this context, epigenetic silencing of *MLH1* would give rise to CIMP-H MSI-H tumours. If *MLH1* remains unaffected, the tumour would develop into CIMP-H MSS. (Liu *et al.* 2018).

In addition, sporadic MSI-H colorectal cancers seem to develop along a different pathway than MSI-H tumours associated with Lynch syndrome. Sporadic adenomas rarely demonstrate MSI-H, while adenomas associated with Lynch syndrome are often characterised by MSI-H. It has been suggested that MSI-H occurs late in adenomas developing to sporadic colon cancers and early in adenomas of patients with Lynch syndrome. (Noffsinger 2009).

Most sporadic MSI-H colorectal cancers occur in elderly patients and contain *BRAF* mutations (V600E) (Ribic *et al.* 2003; Popat *et al.* 2005). The association between the *BRAF* V600E mutation and MSI-H is observed particularly in proximal colon tumours (Liu *et al.* 2018). Patients with MSI-H colorectal tumours do not benefit from fluorouracil-based adjuvant therapy (Ribic *et al.* 2003; Popat *et al.* 2005). MSI-H tumours are also associated with a reduced mortality (Ogino *et al.* 2009; Boland & Goel 2010), which may be related to the typically abundant presence of tumour-infiltrating lymphocytes (Brenner *et al.* 2014).

CIMP-H MSI-H tumours are most prevalent in distal stomach and proximal colon (Budinska *et al.* 2013; Liu *et al.* 2018). The reason for this may be that these areas have a higher epithelial cell turnover and DNA replication rate, and thus are more sensitive to *MLH1* silencing than other areas of the gastrointestinal tract. Gastrointestinal adenocarcinomas developing due to germline MMR mutations are also more often observed in areas with highly proliferative epithelia. (Lynch *et al.* 2015; Liu *et al.* 2018).

2.3.4 Sessile serrated adenomas/polyps, traditional serrated adenomas and serrated adenocarcinomas

Sessile serrated adenomas/polyps (SSA/P) have been associated with sporadic MSI-H colorectal cancer. The exact risk of malignant transformation in SSA/Ps without dysplasia is still unknown but it is likely at least equivalent to that of conventional adenomas. SSAs located in the proximal colon are typically characterised by CIMP and also contain activating *BRAF* mutations. Sporadic adenocarcinomas developing from these serrated precursor lesions usually show MSI-H caused by epigenetic *MLH1* promoter methylation. (Aaltonen *et al.* 1993; Thibodeau *et al.* 1993; Noffsinger 2009; Bettington *et al.* 2013; Brenner *et al.* 2014).

Traditional serrated adenomas (TSA) may also show CIMP-H but they contain more often *KRAS* than *BRAF* mutations. TSAs may progress to left-sided serrated colorectal adenocarcinomas characterised by either MSI-L or MSS. (Noffsinger 2009). Loss of MLH1 expression does not usually occur in TSAs (Sawyer *et al.* 2002; Goldstein *et al.* 2003).

Serrated adenocarcinomas comprise about 7.5% of colorectal cancers. They are predominantly located in the right-sided colon but also occur in the rectum (Mäkinen *et al.* 2001; Tuppurainen *et al.* 2005). The left-sided tumours are typically MSS or MSI-L (Dong *et al.* 2005; Tuppurainen *et al.* 2005) and are associated with *KRAS* mutations (Jass *et al.* 2002). Between 15% and 20% of serrated

adenocarcinomas arise in proximally located SSA/Ps (Goldstein *et al.* 2003; Tuppurainen *et al.* 2005). These serrated adenocarcinomas frequently show MSI-H and CIMP (Jass *et al.* 2002; Noffsinger 2009). *APC* and *TP53* mutations as well as LOH are rare in serrated tumours (Jass *et al.* 2002; Sawyer *et al.* 2002; Yamamoto *et al.* 2003; Noffsinger 2009). *MGMT* promoter methylation is characteristic of serrated adenomas and can be observed in about 50% of serrated adenocarcinomas (Dong *et al.* 2005; Mäkinen 2007).

The most reliable method for MSI detection is based on PCR. Tumours in which > 30% of the microsatellite PCR markers are mutated are defined as MSI-H. Tumours in which at least one but < 30% of the markers are mutated are defined as MSI-L, and they typically resemble MSS tumours in their clinical behaviour. (Boland *et al.* 1998; Boland & Goel 2010). However, immunohistochemistry is typically used as a MSI screening method in clinical practice (de la Chapelle & Hampel 2010). The majority of MSI-H colorectal tumours have lost the expression of both MLH1 and PMS2, while a smaller percentage of MSI-H tumours are negative for both MSH2 and MSH6. It has been estimated that MSI IHC identifies MMR-deficient colorectal tumours with approximately 93% sensitivity (most insensitivity is caused by *MSH6* mutations), and that MLH1 and MSH2 IHC can identify MMR defects with 92% sensitivity and 100% specificity (Boland & Goel 2010).

2.3.5 Epstein-Barr virus-related gastric cancer

Epstein-Barr virus (EBV) positivity has been detected in approximately 9 – 10 % of gastric adenocarcinomas. It is more common in males than females and in tumours located in gastric cardia or corpus than in antrum. (Murphy *et al.* 2009; Sanchez-Vega *et al.* 2018). Persistent EBV infection leads to the expression of latent viral genes encoding latent membrane proteins (LMP), EBV nuclear antigens (EBNA), EBV-encoded small RNAs (EBER) and BamH1 A region rightward transcripts (BART). These products are thought to promote tumorigenesis by affecting different tumour suppressors and signalling pathways. (Akiba *et al.* 2008; Murphy *et al.* 2009; Chen *et al.* 2012; Shinozaki-Ushiku *et al.* 2015).

Phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MEK) alterations are observed to co-occur in EBV positive gastric cancer and thus the combination of PI3K and MEK inhibitors has been suggested as an treatment option (Sanchez-Vega *et al.* 2018). The immune signatures related to CD8 or IFN-γ signalling and the presence of PD-L1/2 overexpression observed in EBV positive gastric tumours could be indicators for a possible therapeutic effect from immune checkpoint inhibitors (TCGA 2014).

2.4 Clinipathological aspects of gastric cancer

The treatment of gastric cancer with curative intent includes surgery. In addition, adjuvant chemotherapy is used for locally advanced disease, which refers to tumours invading muscularis

propria or beyond. Preoperative chemoradiotherapy (CRT) or perioperative chemotherapy for oesophageal adenocarcinomas as well as perioperative chemotherapy for gastric cancer can also be used for certain patients. Patients with metastatic disease can be treated with chemotherapy and targeted therapies such as trastuzumab or ramucirumab. Patients with metastatic gastric cancer do not generally benefit from metastasectomy. Trastuzumab is a monoclonal antibody against human epidermal growth factor receptor 2 (HER2) and can be used in the treatment of patients with HER2 overexpressing tumours. Ramucirumab is a monoclonal antibody against vascular endothelial growth factor receptor 2 (VEGFR2). (Brenner *et al.* 2014; Smyth *et al.* 2016; Van Cutsem *et al.* 2016).

2.4.1 Histopathological classification of gastric cancer

The histological Laurén classification was first proposed by Pekka Laurén in 1965. It uses morphological characteristics of gastric adenocarcinomas to divide them into two histologically distinct subtypes, intestinal and diffuse, which were also demonstrated to have different clinical characteristics. A small proportion of tumours does not fit into these categories and can be classified as mixed- or indeterminate-type. Intestinal-type tumours are usually well- or moderately differentiated and composed of glandular structures. In contrast, diffuse-type tumours are poorly differentiated, composed of poorly cohesive cells and can include a signet-ring cell component. (Laurén 1965; Bosman *et al.* 2010). An alternative histology-based classification method is the WHO classification system dividing gastric adenocarcinomas into tubular, papillary, mucinous, poorly cohesive and rare variants (Bosman *et al.* 2010).

Based on anatomical location, gastric cancers can be divided into true gastric cancers and GOJ cancers located in cardiac area. The Siewert classification (Siewert & Stein 1998) has been developed to determine if the tumour should be classified as a distal oesophageal carcinoma, true cardiac carcinoma or subcardial carcinoma according to the location of the tumour epicentre in relation to the GOJ. The TNM classification uses a simplified categorisation and divides the tumours into either carcinomas of the oesophagus and oesophagogastric junction or gastric carcinomas (Brierley *et al.* 2017).

According to the TNM classification, intraepithelial gastric tumours are classified as Tis and invasive tumours as T1 – T4 according to the level of invasion. Tumours invading lamina propria or submucosa are T1, tumours invading muscularis propria are T2, tumours invading subserosal connective tissue are T3, and tumours perforating visceral peritoneum or invading adjacent structures are classified as T4. If no lymph node metastases are found (N0), the tumour belongs to stage 0, IA – B, IIA – B or IIIA according to T. If lymph node metastases are found (N1 – 3), the tumour is stage IB, IIA – B or IIIA – C according to T and the number of metastatic regional lymph nodes (N1, ≤ 2 metastases; N2, 3 – 6 metastases; N3, ≥ 7 metastases). In the presence of distant metastases (M1), the stage is IV regardless of T or N. (Brierley *et al.* 2017).

The most essential prognostic factors in gastric cancer are the TNM category, HER2 status and the presence of residual disease (R0, R1 or R2). Additional factors include the location of the tumour, histological type, the presence of lymphovascular invasion and patient age. (Brierley *et al.* 2017).

2.4.2 Hereditary gastric cancer

Germline mutations associated with cancer susceptibility account for 1 – 3% of all gastric cancer. These include conditions such as hereditary diffuse gastric cancer (due to *CDH1* mutation) (Richards *et al.* 1999) and gastric adenocarcinoma and proximal polyposis of the stomach (GAPPS) as well as syndromes more often associated with colorectal cancer such as FAP and Lynch syndrome (Keller *et al.* 1996). Also patients with Peutz-Jeghers syndrome have an increased risk for gastric cancer as well as for tumours in many other organs (Smyth *et al.* 2016; Van Cutsem *et al.* 2016).

2.5 Clinicopathological aspects of colorectal cancer

The treatment of colorectal cancer with curative intent includes surgery. In addition, adjuvant chemotherapy is used for stage III/IV or high-risk stage II colorectal cancer. Preoperative (chemo)radiotherapy, (C)RT, can be used for rectal cancer patients. Patients with metastatic disease can be treated with chemotherapy and targeted therapies such as cetuximab, panitumumab, bevacizumab, aflibercept or regorafenib. Liver or lung metastases from colorectal cancer are sometimes suitable for surgical resection. Cetuximab and panitumumab are monoclonal antibodies against epidermal growth factor receptor (EGFR) and can be used in the treatment of patients with *RAS* wt tumours. Bevacizumab is a monoclonal antibody against vascular endothelial growth factor A (VEGF-A) and aflibercept is a recombinant protein binding to circulating VEGF. Regorafenib is a molecule inhibiting several receptor tyrosine kinases. (Brenner *et al.* 2014; Smyth *et al.* 2016; Van Cutsem *et al.* 2016).

2.5.1 Histopathological classification of colorectal adenocarcinoma

Histological grading of colorectal adenocarcinomas is based on the proportion of glandular structures. Well-differentiated tumours (grade I) are composed of > 95% glandular structures, moderately differentiated (grade II) tumours have 50 – 95% and poorly differentiated (grade III) tumours have < 50% glandular structures. Over 90% of colorectal cancers can be classified as adenocarcinomas, while the remaining tumours include rare variants such as mucinous adenocarcinoma, serrated adenocarcinoma, signet ring cell carcinoma, micropapillary adenocarcinoma and undifferentiated carcinoma. (Bosman *et al.* 2010).

According to the TNM classification, intramucosal tumours are classified as T0, tumours invading submucosa as T1, tumours invading muscularis propria as T2, tumours invading subserosa as T3, and tumours either perforating visceral peritoneum or invading other organs or structures are classified as T4. If no lymph node metastases are found (N0), the tumour belongs to stage 0, I or IIA – C according to T. If lymph node metastases are found (N1 – 2), the tumour is stage IIIA – C according to T and the number of metastatic regional lymph nodes (N1, ≤ 3 metastases; N2, > 4 metastases). In the presence of distant metastases, the stage is IV regardless of T or N. (Brierley *et al.* 2017).

Histopathological staging is still the most important prognostic factor for colon cancer (Bijlsma 2017). The essential prognostic factors include also patient age, participation in a screening programme and the circumferential resection margin (CRM) for rectal cancer. Additional factors include the presence of lymphovascular and perineural invasion, histological differentiation grade, tumour budding and bowel perforation as well as the presence of MSI and *KRAS* or *BRAF* mutations. (Brierley *et al.* 2017). High-risk features that indicate adjuvant treatment in stage II colon cancer include large tumour size, bowel obstruction or perforation, the presence of lymphovascular invasion and poorly differentiated histology (Bijlsma *et al.* 2017).

2.5.2 Hereditary colorectal cancer

Lynch syndrome is the most common of hereditary colorectal cancer syndromes. Patients with Lynch syndrome have a germline mutation in one of their MMR gene alleles, and the inactivation of the other allele by mutation or epigenetic silencing can lead to malignant transformation. (Boland & Goel 2010). Colorectal tumours associated with Lynch syndrome may contain *KRAS* mutations but practically never *BRAF* mutations (Bettstetter *et al.* 2007; Brenner *et al.* 2014). In clinical diagnostics, detection of *BRAF* mutations is used to distinguish patients with sporadic cancer from those with Lynch syndrome (Brenner *et al.* 2014). Patients with Lynch syndrome develop tumours at an early age and often at multiple sites. In addition to colorectal adenocarcinomas, they are prone to have tumours in the endometrium, stomach, ovaries, urinary tract and small intestine. (Boland & Goel 2010). The life-time risk of colorectal cancer in Lynch syndrome patients has been estimated to be as high as 78% (Aarnio *et al.* 1995).

Another hereditary form of colorectal cancer is familial adenomatous polyposis (FAP), which is associated with germline mutations in one of the alleles for the *APC* tumour suppressor gene. Both Lynch syndrome and FAP have autosomal dominant inheritance. The estimated allele frequencies are 1:350 – 1:1700 for Lynch syndrome and 1:10 000 for FAP. (Brenner *et al.* 2014).

2.6 Molecular classification of gastric adenocarcinoma

The molecular classification of gastric adenocarcinoma has identified four tumour subgroups, which are characterised by EBV positivity, MSI, CIN or genomic stability (GS). Regarding the Laurén classification, MSI tumours typically have an intestinal phenotype, whereas diffuse tumours are concentrated in the GS subtype. Both EBV positive and MSI tumours are characterised by hypermethylation, which in the MSI tumours often affects the *MLH1* promoter. MSI tumours typically have a hypermutated genome. The remaining tumours are divided according to the presence of SCNAs into either CIN tumours enriched with SCNAs or into GS tumours without hypermutation or SCNAs. In addition to chromosomal-level structural changes and aneuploidy, CIN tumours usually have intestinal-type histology and mutations in proto-oncogenes and tumour suppressor genes. Approximately 36 – 50% of gastric adenocarcinomas are characterised by CIN. (TCGA 2014).

The Asian Cancer Research Group (ACRG) has divided gastric adenocarcinomas into four subgroups, which include MSI tumours, MSS tumours showing EMT (MSS/EMT), MSS tumours with intact TP53 activity (MSS/TP53+) and MSS tumours with functional loss of TP53 (MSS/TP53-) (Cristescu *et al.* 2015). The latter is somewhat comparable with the CIN subtype.

Several other studies have applied these results and proposed slightly variable classification systems for gastric cancer. Some of these proposals are mainly based on gene-expression patterns (Tan *et al.* 2011; Lei *et al.* 2013; Kim *et al.* 2017; Min *et al.* 2017; Oh *et al.* 2018), while others have concentrated on immunohistochemical and *in situ* hybridisation methods (Kim *et al.* 2016; Park *et al.* 2016; Setia *et al.* 2016; Díaz Del Arco *et al.* 2018). A few of these studies have also included histopathological criteria for subtype specification (Park *et al.* 2016; Min *et al.* 2017). The proportions of different subtypes as reported by some of these studies have been summarised in Table 1.

Table 1. The distribution of different molecular subtypes of oesophagogastric cancer in relevant studies together with the typical genomic alterations according to the TCGA studies (Cerami *et al.* 2012; Gao *et al.* 2013; TCGA 2014, 2017; Liu *et al.* 2018).

	TCGA 2014[a]	**Cristescu et *al.* 2015**[b]	**Kim et *al.* 2016**[c]	**Park et *al.* 2016**[d]	**Setia et *al.* 2016**[e]	**Ahn et *al.* 2017**	**Díaz del Arco et *al.* 2018**[f]
Number of patients (%)	295	300	438	993	146	349	206
Intestinal	196 (66)	150 (50)	98 (22)	518 (52)	ND	199 (57)	111 (54)
Diffuse	69 (23)	142 (47)	130 (30)	475 (48)		147 (42)	71 (34)
Mixed	19 (6.4)	8 (2.7)	17 (3.9)			3 (0.9)	24 (12)
EBV pos	26 (8.8)	18 (6.0)	14 (3.2)	61 (6.1)	7 (4.8)	26 (7.4)	ND
EBV neg	269 (91)	257 (86)	424 (97)	910 (92)		323 (93)	
MSI	64 (22)	68 (23)	21 (5.0)	114 (11)	24 (16)	24 (6.9)	48 (24)
MSS	205 (69)	232 (77)	403 (95)	876 (88)		299 (86)	158 (77)
GS	58 (20)			253 (25)	30 (21)		
CIN	147 (50)			565 (57)	75 (51)		
MSS/EMT		46 (15)					12 (6.0)
MSS/TP53+		79 (26)			[10 (6.8)]		110 (54)
MSS/TP53-		107 (36)					35 (17)
TCGA subtype	**EBV**	**MSI**[g]	**GS**	**CIN**	**Oeso-phageal adeno-carci-noma**	**Oesophagogastric adenocarcinoma** CIN-F	CIN-B
Clinicopatho-logical features	Males, gastric corpus	Females, elderly patients, distal stomach	Younger patients, diffuse-type	Proximal stomach			
Mutations	*PIK3CA* (80%) *ARID1A* (55%) *PTEN* (15%)	*ARID1A* (84%) *EGFR* (19%) *ERBB2* (11%) *ERBB3* (33%) *PIK3CA* (42%) *TP53* (39%) *BRAF* (28%) *KRAS* (23%)	*CDH1* (34%) *RHOA* (14%) *ARID1A* (16%) *TP53* (14%)	*TP53* (71%) *ARID1A* (9%)	*ERBB2* (15%) *ARID1A* (13%) *CDKN2A* (15%) *SMAD4* (10%) *TP53* (81%)	*TP53* (76%)	*TP53* (54%) *ERBB2* *KRAS APC* *CDKN2A* *SMAD4*
Amplifications[h]	*JAK2* (15%) *CD274*[i] (15%) *CD273*[i] (12%) *ERBB2* (12%)	*PIK3CA* (3%)	*MYC* (4%)	*EGFR* (9%) *ERBB2* (22%) *GATA4* (10%) *GATA6* (14%) *KRAS* (14%) *MYC* (19%) *VEGFA* (13%)	*ERBB2* (29%) *EGFR* (8%) *GATA4* (15%) *GATA6* (20%) *KRAS* (9%) *VEGFA* (12%)	*EGFR* *ERBB2* *ERBB3* *KRAS NRAS* *BRAF* *PIK3CA* *CDKN2A*	
Deletions	*PTEN* (8%)	*PTEN* (3%)	*CDKN2A* (10%)	*ARID1A* (3%) *CDKN2A* (14%) *PTEN* (5%) *SMAD4* (10%)	*CDKN2A* (13%) *SMAD4* (13%)		
Characteristic protein expression	JAK2, PD-L1/L2, IFN-γ signalling	IFN-γ signalling					

[a]Histological subtype was not determined for eleven tumours.
[b]EBV information was available for 275 tumours.
[c]Laurén classification was determined for 245 tumours.
[d]Intestinal- and mixed-type tumours were combined. EBV was analysed in 971 and MMR information in 990 tumours.
[e]Ten intestinal-type tumours with TP53 wt, EBV neg and MSS were identified.
[f]One tumour with an isolated loss of MSH6 expression was excluded from the analyses.
[g]MSI-H, CIMP-H tumours with epigenetic silencing of *MLH1* and defective MMR.
[h]Upper gastrointestinal CIN-F tumours have more intense amplifications than lower CIN-F tumours.
[i]CD274 encodes PD-L1; *CD273*, also known as *PDCD1LG2*, encodes PD-L2.

CIN, chromosomal instability; EBV, Epstein-Barr virus; GS, genomic stability; IFN-γ, interferon-γ; MSI, microsatellite instability; MSS, microsatellite-stable; ND, not determined; TP53-, functional loss of TP53; TP53+, functional TP53.

2.6.1 Characteristics of the EBV, MSI, GS and CIN subtypes of gastric adenocarcinomas

Among all cancers, EBV is mainly detected in gastric adenocarcinomas (Liu *et al.* 2018, Thorsson *et al.* 2018). CIMP-H is often observed among the EBV positive tumours (Matsusaka *et al.* 2011; Wang *et al.* 2014), but these contain different mutations and have different gene expression profiles than the CIMP-H MSI tumours. For example, hypermethylation of the *MLH1* promoter is not observed among the EBV positive tumours, while *CDKN2A* (p16INK4A) promoter hypermethylation is common. *TP53* mutations are rare. (TCGA 2014). The presence of EBV associates with high *CTLA4* and *CD274* (encodes PD-L1) expression levels (Thorsson *et al.* 2018). EBV positive tumours also contain high levels of CD8+ T cells, M1-macrophages and interferon-γ signatures (Derks *et al.* 2016; Koh *et al.* 2017).

In contrast to sporadic MSI colorectal cancers (TCGA 2012), *BRAF* (V600E) mutations are not typical of upper gastrointestinal MSI adenocarcinomas (TCGA 2014; Liu *et al.* 2018). While the TCGA classification could not demonstrate survival differences between the different molecular subtypes, the ACRG classification system showed that patients with MSI tumours have the best prognosis and patients with MSS/EMT tumours have the shortest survival. The MSS/EMT subtype shares some properties with the GS subtype. (Cristescu *et al.* 2015). Alterations in the RTK–RAS signalling pathways, RTK amplifications and *TP53* aberrations are often detected in CIN tumours. In addition to *EGFR* amplification, elevated levels of phosphorylated EGFR are observed in CIN tumours reflecting the functional significance of the amplification. (TCGA 2014). Especially gastro-oesophageal adenocarcinomas with CIN-F are enriched with focal, short and high-amplitude SCNAs. (Liu *et al.* 2018).

RHOA and somatic *CDH1* mutations are enriched in the GS subtype. RHOA is involved in controlling cell motility and contractility and thus mutations in both *RHOA* and *CDH1* may contribute to the low cohesion growth pattern typical of diffuse-type tumours (Ridley *et al.* 2003; Hanahan & Weinberg 2011; TCGA 2014).

2.7 Molecular classification of oesophageal adenocarcinoma

Oesophageal adenocarcinomas have been observed to share their molecular profile with the CIN gastric adenocarcinomas to the extent that they cannot be consistently separated from each other at the molecular level. In contrast, oesophageal squamous cell carcinomas contain distinctly different mutations. EBV positive or MSI tumours have not been observed within the oesophagus, but adenocarcinomas arising around the GOJ include some EBV positive and MSI tumours. GS subtype is very rare in this area. The only differential feature among oesophageal and CIN gastric adenocarcinomas is seen in the extent of DNA hypermethylation, which is enriched in the oesophageal and most proximal CIN gastric tumours. (TCGA 2017). The characteristic features of oesophageal adenocarcinomas are summarised in Table 1.

Similar to CIN gastric adenocarcinomas, RTK alterations are typical of oesophageal adenocarcinomas (Secrier *et al.* 2017, TCGA 2017). The most common oncogenic alterations are found in *ERBB2*, which is either amplified or mutated in 32% of oesophageal adenocarcinomas. *EGFR* alterations can be found in 15% of oesophageal adenocarcinomas. (TCGA 2017).

Barrett's oesophagus predisposes to the development of oesophageal adenocarcinomas and thus these tumours have not been thought to be of gastric origin. However, it has been suggested that Barrett's oesophagus could have its origin in the proximal stomach or GOJ (Wang *et al.* 2011), which could also explain the similar molecular features observed in oesophageal and gastric adenocarcinomas. (TCGA 2017).

2.8 Molecular classification of colorectal adenocarcinoma

The different molecular classification systems suggested for colorectal cancer have recognised three to six distinct subtypes (Jass 2007; Perez-Villamil *et al.* 2012; Schlicker *et al.* 2012; Budinska *et al.* 2013; De Sousa E Melo *et al.* 2013; Marisa *et al.* 2013; Sadanandam *et al.* 2013; Roepman *et al.* 2014). The most stable subtypes appear to be those characterised by MSI-H and frequently associated with CIMP, and those enriched with mesenchymal gene expression due to EMT (Guinney *et al.* 2015; Bijlsma *et al.* 2017). About 75% of the hypermutated colon tumours have MSI-H, usually due to *MLH1* promoter hypermethylation. The rest of the hypermutated tumours have somatic mutations in *POLE* or in genes encoding MMR proteins. (TCGA 2012).

Integrating the available mutation, copy number, methylation, microRNA, proteome and survival data, four distinct consensus molecular subtypes (CMS) have been suggested for colorectal adenocarcinomas (Guinney *et al.* 2015; Dienstmann *et al.* 2017). These four subtypes comprise 86% of the tumours included in the analyses. The remaining 14% show mixed features, which could be due to intratumoural heterogeneity, or they could represent so-called transition phenotypes. *RAS* mutations are detected in all CMS subtypes of colorectal cancer, but specific biological differences in each of these subtypes may modify the response from anti-EGFR therapy even in *RAS* wt tumours. What those differences might be is not yet known. (Guinney *et al.* 2015). The consensus subtypes have been summarised in Table 2.

CMS1 tumours typically contain dense immune cell infiltrates, which indicate immunological activation. This is also reflected in the increased expression of genes associated with the activation of type 1 T helper cells (Th1) and cytotoxic T cells. (Guinney *et al.* 2015). In comparison with the TCGA subtypes, the CMS1 group contains both MSI-H tumours and hypermutated tumours enriched with single-nucleotide variants (HM-SNV). The CMS system does not clearly discriminate between CIN and GS tumours (Liu *et al.* 2018).

Table 2. The consensus subtypes of colorectal adenocarcinoma and their characteristics together with a summary of the TCGA molecular subtypes (TCGA 2012; Guinney *et al.* 2015; Dienstmann *et al.* 2017; Liu *et al.* 2018).

	CMS1	**CMS2**	**CMS3**	**CMS4**	
	Immune subtype (14%)	**Canonical subtype (37%)**	**Metabolic subtype (13%)**	**Mesenchymal subtype (23%)**	
Clinicopathological characteristics	Right > left, females > males	Left > right		Advanced stage, worst prognosis	
Histological characteristic	Poor differentiation, dense lymphocytic infiltrates	Well-differentiated			
Overall genomic characteristics	MSI-H, hypermethylation, hypermutation, active RTK and MAPK pathways	Active Wnt and Myc pathways	Hypermutation, active RTK and MAPK pathways	Active TGF-β signalling	
Characteristic mutations	*BRAF*		*KRAS*		
Characteristic amplifications	Fewest SCNAs	*ERBB2* (4%)			
TCGA subtypes	**MSI**[a]	**HM-SNV**	**GS**		**CIN**
				CIN-F	CIN-B
Clinicopathological characteristics	Proximal colon, rare in descending colon and rectum		Right > left		Distal colon, rectum
Characteristic mutations	*BRAF* (56%, mainly proximal colon) *APC* (41%) *KRAS* (26%) *PIK3CA* (30%)	*POLE*	*APC* (81%) *KRAS NRAS BRAF PIK3CA SOX9* TGF-β pathway genes; *TP53* (16%)		*TP53* (80%) *APC* (85%) *KRAS NRAS BRAF PIK3CA*
Characteristic amplifications				*CDX2* *ERBB2*	
Other features	Hypermutation, epigenetic *MLH1* silencing, *CDKN2A* methylation, low Wnt signalling, IFN-γ signalling				

[a]MSI-H, CIMP-H tumours with epigenetic silencing of *MLH1* and defective MMR.

CIN, chromosomal instability; CIN-B, CIN broad; CIN-F, CIN focal; CMS, consensus molecular subtype; GS, genomic stability; HM-SNV, hypermutated tumours enriched with single-nucleotide variants; IFN-γ, interferon-γ; MAPK, mitogen-activated protein kinase; MSI-H, high level of microsatellite instability; RTK, receptor tyrosine kinase; SCNA, somatic copy number alteration; TCGA, The Cancer Genome Atlas.

CMS2 tumours have retained their epithelial differentiation and are enriched with SCNAs. Activating alterations in the Wnt and Myc signalling pathways are typical (Guinney *et al.* 2015) but not exclusive to the CMS2 tumours (TCGA 2012). CMS3 tumours are characterised by deregulation of metabolic pathways responsible for processing different sugars, aminoacids and lipids (Dienstmann *et al.* 2017). About a third of these tumours are hypermutated and thus CMS3 overlaps to some extent with the CMS1 subtype (Guinney *et al.* 2015). CMS3 tumours also share some molecular features with the GS subtype (TCGA 2012, TCGA 2014, Liu *et al.* 2018).

CMS4 tumours are characterised by the activation of TGF-β-signalling and other pathways involved in EMT, invasion, angiogenesis and complement-mediated immune response. However, alterations in the TGF-β pathway components are also typical of the hypermutated tumours. (Guinney *et al.* 2015).

2.9 Molecular classification of gastrointestinal adenocarcinomas

A collective study on adenocarcinomas of the oesophagus, stomach, colon and rectum has revealed that all gastrointestinal adenocarcinomas share some molecular characteristics at the genomic, epigenomic, mRNA, microRNA and protein level, which is consistent with their common developmental origin (Liu *et al.* 2018). Based on their molecular characteristics, gastrointestinal adenocarcinomas can be identified as a distinct group of tumours among all different cancers (Hoadley *et al.* 2018).

In comparison with other cancer types, gastrointestinal adenocarcinomas are observed to contain on average more somatic mutations, some of which are specific for the gastrointestinal tract. Also some genes associated with EGFR signalling pathways or gastrointestinal stem cells have higher expression levels in gastrointestinal adenocarcinomas than in other cancers. SCNAs typical of gastrointestinal adenocarcinomas include amplifications in *EGFR, FGFR1, GATA4, GATA6* and *IGF2* as well as deletions in *APC* and *SOX9*. Arm-level gain of the region (Chr13q), containing the tumour suppressor gene *RB1* and transcription factor gene *CDX2*, is also common in gastrointestinal adenocarcinomas. (Liu *et al.* 2018).

Although the classification systems have been able to define distinct tumour subtypes and recognise similarities between subtypes in different gastrointestinal organs, it is still uncertain whether or not these subtypes share a common origin. It has been suggested that they could arise from a so-called ground state, or canonical subtype, which diverges into different subtypes under the influence of various events. Alternatively, each subtype could be determined at the earliest stages of tumorigenesis, and the tumour would progress along that line throughout its development. (Bijlsma *et al.* 2017). The percentages of the five molecular subtypes for upper and lower gastrointestinal adenocarcinomas are summarised in Table 3.

Table 3. The distribution of the molecular subtypes among upper and lower gastrointestinal adenocarcinomas (n = 921) (Liu *et al.* 2018).

	EBV (n = 30)	MSI (n = 138)	HM-SNV (n = 19)	GS (n = 625)	CIN (n = 109)	
Upper GIACs	100%	54%	47%	47%	48%	
					CIN-F (74%)	CIN-B (26%)
Lower GIACs	0%	46%	53%	53%	52%	
					CIN-F (22%)	CIN-B (78%)

CIN, chromosomal instability; CIN-B, CIN broad; CIN-F, CIN focal; EBV, Epstein-Barr virus; GIAC, gastrointestinal adenocarcinoma; GS, genomic stability; HM-SNV, hypermutated tumours enriched with single-nucleotide variants; MSI, microsatellite instability.

Hypermutated tumours form a distinct group among all gastrointestinal adenocarcinomas and can be divided into two subgroups: MSI tumours and *POLE* mutated tumours (HM-SNV) (TCGA 2012; Palles *et al.* 2013; Liu *et al.* 2018).

MSI tumours form the most consistent subtype among all gastrointestinal adenocarcinomas. In contrast, the EBV positive or GS tumours of the upper gastrointestinal tract contain alterations that

are not observed in the other subtypes. The co-occurrence of CIMP-H and MSI is predominantly observed in tumours located in distal stomach and proximal colon, while CIMP-H or MSI is rare in tumours of descending colon and rectum. The majority of CIMP-H MSI tumours display methylation of the tumour suppressor *CDKN2A* (encodes p16). Among all gastrointestinal adenocarcinomas, promoter hypermethylation is observed to silence particularly genes encoding DNA binding proteins such as transcription factors (Liu *et al.* 2018).

The GS subtype differs most between the upper and lower gastrointestinal adenocarcinomas (Bijlsma *et al.* 2017). The upper gastrointestinal GS tumours comprise a more distinct subgroup than GS colorectal carcinomas, which display overlapping features with the CIN subtype. For example, loss of *APC* is equally common in both GS and CIN colorectal carcinomas. However, GS colorectal carcinomas are more frequently right- than left-sided, while the reverse is true for CIN tumours. (Liu *et al.* 2018).

CIN is the most common molecular subtype among all gastrointestinal adenocarcinomas (Dulak *et al.* 2012; Liu *et al.* 2018). However, SCNAs are less common and less intense in the lower than in the upper gastrointestinal tumours. In colorectal CIN tumours, oncogene activation is more often obtained by mutation than amplification. With regard to SCNAs and somatic mutations, CIN-F and CIN-B subgroups are more similar to each other among colorectal than oesophagogastric tumours. Mutation patterns in colorectal CIN tumours also resemble more the oesophagogastric CIN-B than CIN-F tumours. Nevertheless, CIN-F, but not CIN-B, colorectal tumours are associated with decreased survival. (Liu *et al.* 2018).

2.10 Biomarkers and signalling pathways

2.10.1 Receptor tyrosine kinases

Receptor tyrosine kinases are evolutionarily conserved cell membrane receptors, of which 58 are known to be expressed in human cells (Lemmon & Schlessinger 2010; Yarden & Pines 2012). All RTKs have an extracellular ligand-binding region, a single helical transmembrane region, and a domain with tyrosine kinase activity together with regulatory regions on the cytoplasmic side. The ligand is typically a growth factor. RTKs transmit information from the mesenchyme to the inside of the cell, and they are involved in the regulation of several cellular processes such as cell proliferation, cell migration, cell cycle and angiogenesis the aberrations in which are often beneficial for cancer cell survival. In human cancers, altered RTK activation is obtained by increased autocrine signalling, chromosomal translocations, receptor overexpression or by gain-of-function mutations. (Lemmon & Schlessinger 2010).

In general, the active form of RTKs is a dimer, the formation of which can be induced by ligand binding. Alternatively, some RTKs form dimers on the cell surface even in the absence of the

ligand, and the ligand is only needed for inducing structural changes that lead to receptor activation. (Lemmon & Schlessinger 2010).

The activation of the kinase is followed by the recruitment of several cytoplasmic signalling molecules containing Src homology-2 (SH2) and phosphotyrosine-binding (PTB) domains. They interact with the RTKs either binding directly to phosphorylated tyrosines in the receptor or indirectly via docking proteins. A single RTK can interact with several different proteins leading to a complex intracellular signalling network. (Lemmon & Schlessinger 2010). The networks associated with different RTKs often have overlapping components but can nevertheless produce distinct effects. The behaviour of a specific signalling route is to a great extent determined by the expression levels of the different components. (Jones *et al.* 2006). The variability in cellular responses to RTK signalling is partly related to changes in positive and negative feedback mechanisms. The inputs from the several RTKs are thought to converge on a relatively small number of conserved processes such as mitogen-activated protein kinase (MAPK), PI3K and $Ca2^{+}$ signalling. From there, the signalling diverges again to produce the various effects on different cellular functions. (Lemmon & Schlessinger 2010).

2.10.2 EGFR/ERBB family

The EGFR/ERBB family includes four members: EGFR, ERBB2 (HER2), ERBB3 and ERBB4 (Yarden & Sliwkowski 2001; Hynes & MacDonald 2009; Lemmon & Schlessinger 2010; Arteaga & Engelman 2014). The ERBB signalling has effects on cell proliferation and migration, adhesion, differentiation and apoptosis. ERBB receptor heterodimers have been shown to be more mitogenic and transforming than homodimers, and heterodimers containing ERBB2 are the most potent complexes. (Yarden & Sliwkowski 2001, Hynes & MacDonald 2009).

The extracellular part of an ERBB receptor monomer consists of four domains (I – IV) among which domains I and III bind the activating ligand (Lemmon & Schlessinger 2010). ERBBs can bind to several ligands, which are stored in the mesenchyme (Yarden & Sliwkowski 2001). In the absence of a ligand, the intracellular tyrosine kinase domains interact in an autoinhibitory fashion, which is reversed by ligand binding. When a ligand binds to a monomeric receptor tyrosine kinase, the receptor monomers dimerise, and one of them phosphorylates the other at the kinase domain, which activates the cytoplasmic catalytic function. (Hynes & MacDonald 2009; Lemmon & Schlessinger 2010; Arteaga & Engelman 2014).

All ERBB ligands have an EGF-like domain, which is a motif of about 50 amino acids (Jones *et al.* 1999; Yarden & Sliwkowski 2001). Eleven different ligands are known to bind the different ERBB receptors, and in human malignancies they mainly exert their activating effect through autocrine secretion (Yarden & Pines 2012). EGFR binds not only EGF but also amphiregulin, epiregulin, betacellulin, TGF-α, epigen and heparin-binding EGF-like growth factor (HBEGF). HER2 has no known ligand. ERBB3 binds neuregulins (NRG) 1 and 2 but has no or little kinase activity. ERBB4

binds NRG1 – 4 in addition to epiregulin, betacellulin and HBEGF. (Jones *et al.* 1999; Yarden & Sliwkowski 2001; Hynes & MacDonald 2009; Yarden & Pines 2012). The ERBB receptors may also respond to other signals including hormones, neurotransmitters and stress stimuli such as ultraviolet light (Carpenter 1999).

The ERBB family members generate potent signals needed for the initiation and maintenance of several types of cancer (Yarden & Sliwkowski 2001; Arteaga & Engelman 2014). In different cancer types, the ERBB pathway may become hyperactivated by various mechanisms such as overproduction of ligands or receptors, or by constant activation of receptors due to mutations (Yarden & Sliwkowski 2001). In colorectal cancer, mutations or amplifications in ERBB genes have been detected in 13% of the non-hypermutated and 53% of the hypermutated tumours (TCGA 2012).

2.10.3 Epidermal growth factor receptor

The association between ERBBs and cancer was first recognised in the 1980s. The product of a viral oncogene, analogous to EGFR, was observed to be tumorigenic in birds affected by the avian erythroblastosis virus. (Yarden & Sliwkowski 2001; Yarden & Pines 2012; Arteaga & Engelman 2014).

EGFR is has an essential role in embryonal signalling pathways regulating the development of many organs such as brain, kidney, liver and gastrointestinal tract as well as pathways involved in tooth growth and eye opening (Miettinen *et al.* 1995; Threadgill *et al.* 1995; Sibilia *et al.* 1998). It is also involved in promoting epithelial proliferation and differentiation in, for example, the skin, lung and pancreas. (Yarden & Sliwkowksi 2001).

EGFR can function as a homodimer or it can form heterodimers with ERBB2. Ligand binding induces receptor dimerization, which is both necessary and sufficient for the kinase activity in wild-type receptors. (Yarden & Pines 2012). However, the exact structural changes required for EGFR activation are still unclear. EGFR can also be activated without ligand binding if the autoinhibitory mechanism is disrupted by mutation. (Lemmon & Schlessinger 2010).

Both EGFR overexpression and mutations are observed in several human malignancies (Yarden & Sliwkowski 2001; Hynes & MacDonald 2009; Yarden & Pines 2012). EGFR can be overexpressed in head and neck, breast, bladder, prostate, kidney and non-small-cell lung cancer as well as in gliomas. The overexpression often occurs due to *EGFR* amplification, which is most common in glioblastomas where it is observed in as many as 50% of patients. EGFR overexpression has also been associated with reduced survival in, for example, breast cancer and gliomas. (Yarden & Sliwkowski 2001; Yarden & Pines 2012). *EGFR* mutations are observed especially in gliomas and lung, ovarian and breast cancer (Yarden & Sliwkowski 2001; Lemmon & Schlessinger 2010; Yarden & Pines 2012; Arteaga & Engelman 2014).

EGFR expression has been reported in about 10 – 80% of gastric (Zhang *et al.* 2017) and 50 – 80% of colorectal cancers (Cunningham *et al.* 2004; Scartozzi *et al.* 2007) depending on the evaluation criteria. *EGFR* amplification has been detected in 5.3% of gastric adenocarcinomas but only 0.4% of colorectal adenocarcinomas (Cerami *et al.* 2012; Gao *et al.* 2013). Instead of true amplification, increased *EGFR* GCN in colorectal cancer is more often observed to be related to Chr7 polysomy (Ålgars *et al.* 2011). It is thought that EGFR overexpression can lead to malignant transformation only in the presence of a ligand. Accordingly, EGF-like ligands are often overexpressed together with EGFR in malignant tumours. (Yarden & Sliwkowski 2001).

Anti-EGFR antibodies cetuximab and panitumumab are indicated for the treatment of *RAS* wt metastatic colorectal cancer (Cunningham *et al.* 2004; Benvenuti *et al.* 2007; Di Fiore *et al.* 2007). Mutated *RAS* can directly activate downstream signalling without input from the EGFR, which could explain the lack of therapeutic response from these antibodies in patients with *RAS* mutated tumours (Arteaga & Engelman 2014). However, only about 60 % of the *RAS* wt patients are responsive to anti-EGFR treatment, which implies that some additional mechanisms affect the antibody response (Misale *et al.* 2014). This has led to proposals of new predictive methods such as *EGFR* GCN assessment. Indeed, it has been shown that *RAS, BRAF* and *PIK3CA* wt colorectal cancer patients with *EGFR* GCN ≥ 4.0 tumours have a better treatment response and increased progression-free survival (PFS) than patients with *EGFR* GCN < 4.0 tumours regardless of the *RAS*, *BRAF* or *PIK3CA* mutation status (Ålgars *et al.* 2017).

2.10.4 HER2/ERBB2

ERBB2 was first identified as a mutated ortholog (Neu) in carcinogen-induced brain tumours of rats (Yarden & Pines 2012; Arteaga & Engelman 2014). Overexpression of HER2 has been observed in breast, lung, pancreatic, colon, oesophageal, endometrial and cervical cancer. The overexpression is most often due to gene amplification, which is detected in up to 30% of breast cancers (Yarden & Sliwkowski 2001) and 13% of gastric cancers (Cerami *et al.* 2012; Gao *et al.* 2013). *HER2* amplification has been associated with reduced survival in, for example, breast (Yarden & Sliwkowski 2001; Yarden & Pines 2012; Arteaga & Engelman 2014) and gastric cancer (Tanner *et al.* 2005, Gravalos & Jimeno 2008; Begnami *et al.* 2011).

With regard to anti-HER2 antibody treatment, overexpression of HER2 is determined by IHC and *in situ* hybridisation (ISH). Tumours with either strong protein expression (3+) or moderate protein expression (2+) together with gene clusters in ISH are defined as showing HER2 overexpression. *HER2* mutations are also observed in several cancers but almost solely in tumours without *HER2* amplification (Arteaga & Engelman 2014).

ERBB2 has not been observed to have any specific ligand. (Klapper 1999 *et al.*; Yarden & Pines 2012; Arteaga & Engleman 2014). However, the conformation of ERBB2 favors dimerisation (Arteaga & Engelman 2014), and the formation of ERBB2-containing heterodimers are preferred

over other combinations (Tzahar *et al.* 1996; Graus-Porta *et al.* 1997; Olayioye *et al.* 1998). Especially ERBB2 – ERBB3 heterodimers are potent activators of both MEK – ERK and PI3K – AKT pathways (Yarden & Pines 2012). *ERBB2* amplification has been detected to promote the formation of both ERBB2 homo- and heterodimers (Olayioye *et al.* 1998). ERBB2 homodimer formation can also be induced by point mutations in the transmembrane region of the receptor or by antibody binding (Olayioye *et al.* 1998; Klapper *et al.* 1999).

2.10.5 EGFR/ERBB signalling

The main determinants of the specificity and potency of intracellular EGFR/ERBB signalling are the intracellular proteins that bind to the phosphorylated tyrosines after receptor dimerisation. The type of these proteins, and thus the output, is determined by the ligand and by the structure of the intracellular part of the receptor monomer. Thus the ability to form heterodimers results in more diverse intracellular signalling. In addition, the amount of receptor monomers, especially ERBB2, adjusts the function of the network. (Olayioye *et al.* 1998; Yarden & Pines 2012).

The central signalling pathways connected to the ERBB receptors are the RAS-RAF-MEK-ERK pathway and the PI3K-AKT-mTOR pathway. Co-occurring alterations in the RAS and PI3K signalling cascades can be observed in about one-third of colorectal adenocarcinomas. (TCGA 2012). Although sharing some intracellular secondary messenger pathways, each ERBB receptor is coupled with a distinct set of signalling proteins. The positive feedback mechanisms of EGFR signalling include the autocrine production of EGFR ligands, while negative feedback is provided by receptor endocytosis and ubiquitination-mediated protein degradation. (Yarden & Sliwkowski 2001; Yarden & Pines 2012; Arteaga & Engelman 2014). Other pathways associated with ERBB receptors include JAK/STAT, Wnt and Src kinase pathways (Yarden & Sliwkowski 2001). A simplified depiction of ERBB signalling pathways is presented in Figure 1.

EGFR and ERBB2 use the same signalling pathways in both cancer cells and normal cells. However, constitutively active signalling, together with impaired feedback regulation, contributes to the abnormal properties of cancer cells. (Hynes & MacDonald 2009). This state of aberrant signalling can be obtained by, for example, the overexpression of ERBB ligands (Arteaga & Engelman 2014) or by mutations. *KRAS*, *NRAS* and *BRAF* mutations result in continuous activation of their signalling pathways and thus promote cell proliferation (Weisenberger *et al.* 2006). *KRAS* mutations are present in about 40%, *NRAS* mutations in 9% and *BRAF* mutations in 9 – 14% of colorectal cancers (Weisenberger *et al.* 2006; Cerami *et al.* 2012; Gao *et al.* 2013).

The main mechanism to turn off EGFR signalling is ligand-mediated receptor endocytosis. After ligand binding, EGFR molecules concentrate on clathrin-coated regions of the plasma membrane, which then invaginate to form endocytic vesicles. In these vesicles, the receptor parts are degraded by hydrolytic enzymes. In contrast, the other three ERBB proteins are usually recycled back to the cell surface instead of degradation. (Yarden & Sliwkowski 2001). Sorting to degradation is

determined by the composition of the dimer: phosphorylated EGFR homodimers are directed primarily to lysosomal degradation from the endosome by ubiquitinylation, while heterodimerisation with ERBB2 decreases the rate of endocytosis and increases the rate of recycling back to the cell membrane. (Levkowitz *et al.* 1998; Waterman *et al.* 1998; Yarden & Sliwkowski 2001).

The therapeutic effect of anti-EGFR antibodies is thought to occur through the downregulation of the receptor from the cell membrane. Consequently, signalling via the affected pathway ceases and this may inhibit cell proliferation and induce apoptosis. Trastuzumab may be able not only to inhibit intracellular signalling by uncoupling HER2-containing dimers (Arteaga & Engelman 2014) but also to recruit natural killer cells by its constant region. This can induce antibody-dependent cell-mediated cytotoxicity (ADCC), which could contribute to the therapeutic effect. (Yarden & Pines 2012; Arteaga & Engelman 2014). In contrast, ADCC may not be central to the effect of cetuximab and panitumumab (Arteaga & Engelman 2014).

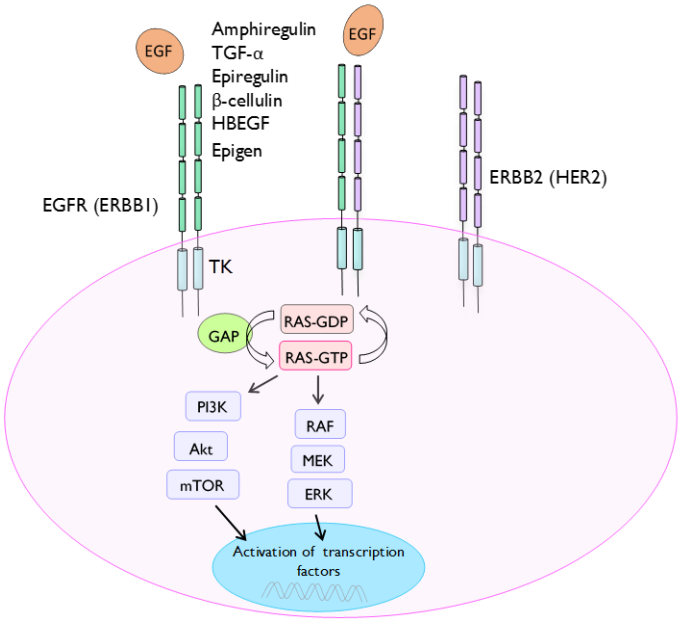

Figure 1. The main EGFR signalling pathways associated with transcriptional regulation and typically activated in cancer cells. Modified from Yarden & Sliwkowski 2001; Hynes & Lane 2005; Ciardiello & Tortora 2008.

2.10.6 Resistance to ERBB-targeting therapies

Several different ERBB-targeting antibodies and tyrosine kinase inhibitors (TKIs) have been developed for the treatment of various cancers. Among TKIs, gefitinib and erlotinib are used for patients with non-small cell lung cancer (NSCLC) containing activating EGFR mutations. (Yarden & Pines 2012). TKIs predominantly bind to mutated EGFR, but they are not as effective as anti-EGFR antibodies against tumours that depend on ligand-mediated receptor activation (Arteaga & Engelman 2014). Lapatinib is a dual inhibitor of EGFR and ERBB2, and it is approved for patients with HER2-positive breast cancer. However, the clinical efficacy of ERBB-targeting therapies is known to vary between patients (primary or intrinsic resistance), and a significant number of patients become resistant to these therapies (secondary or acquired resistance). (Yarden & Pines 2012). Both types of resistance may involve the same molecular mechanisms (Arteaga & Engelman 2014).

A well-known example of primary resistance to anti-EGFR antibodies is the presence of *RAS* mutations in colorectal carcinomas. In fact, the antibody treatment may even be harmful for patients with *RAS* mutated tumours. (Amado *et al.* 2008; Karapetis *et al.* 2008; Bokemeyer *et al.* 2009; Van Cutsem *et al.* 2009; Peeters *et al.* 2010; Bokemeyer *et al.* 2011; Van Cutsem *et al.* 2011; Douillard *et al.* 2013; Bokemeyer *et al.* 2015; Van Cutsem *et al.* 2015; Peeters *et al.* 2015; Van Cutsem *et al.* 2016; Stintzing *et al.* 2017). The *KRAS* and *NRAS* codons that should be tested for mutations include codons 12 and 13 in exon 2, codons 59 and 61 in exon 3 and codons 117 and 146 in exon 4 (Allegra *et al.* 2016).

However, about 40% of patients with *RAS* wt colorectal cancer do not respond to anti-EGFR therapy (Misale *et al.* 2014). Among them, alterations such as *BRAF*, *PI3K* and *PTEN* mutations have been implicated in causing primary resistance to anti-EGFR therapies (De Roock *et al.* 2011; Misale *et al.* 2014). Some evidence suggests that patients with *RAS* wt/*BRAF* wt tumours could benefit more from anti-EGFR therapy than patients with *RAS* wt/*BRAF* mutated tumours (Pietrantonio *et al.* 2015; Rowland *et al.* 2015). High levels of amphiregulin and epiregulin have also been reported to predict favourable response to cetuximab (Khambata-Ford *et al.* 2007).

Acquired resistance to ERBB-targeting treatments can develop either through changes in the target receptor or by the utilisation of an alternative signalling pathway that circumvents the inhibitory effect of the antibody (Yarden & Pines 2012; Arteaga & Engelman 2014). For example, resistance to cetuximab, but not to panitumumab, has been observed in patients with an acquired *EGFR* mutation (S492R) preventing antibody binding (Montagut *et al.* 2012; Bertotti *et al.* 2015). Aberrant HER2 signalling, either due to *ERBB2* amplification or increased NRG1 levels, may also contribute to cetuximab resistance (Yonesaka *et al.* 2011; Bertotti *et al.* 2015). In addition, the emergence of activating mutations in downstream signalling pathways can promote resistance. It is also possible that intrinsically resistant subclones are present in the tumour due to tumour heterogeneity even before starting the ERBB-targeting therapy and subsequently expand under the selective pressure from the treatment. (Diaz *et al.* 2012; Misale *et al.* 2012; Arteaga & Engelman 2014; Misale *et al.*

2014). Treatment resistance related to the usage of single agents might be overcome by the development of combination therapies targeting more than one component of the ERBB network. Alternatively, a key regulator could be targeted by two or more drugs with different mechanisms of action. (Yarden & Pines 2012).

2.10.7 Cancerous inhibitor of PP2A

One of the endogenous inhibitory proteins of PP2A is the cancerous inhibitor of PP2A (CIP2A) (Junttila *et al.* 2007; Westermarck & Hahn 2008). The specific structure of CIP2A is unknown, and it has not been assigned to any enzyme gene family. Currently, among the PP2A complexes, two forms with specific B subunits are known to be involved in CIP2A-mediated inhibition. (Khanna *et al.* 2013b). CIP2A is expressed at very low levels in normal cells (Junttila *et al.* 2007), but it is overexpressed in several cancers such as head and neck squamous cell carcinoma (Junttila *et al.* 2007), gastric adenocarcinoma (Khanna *et al.* 2009), breast carcinoma (Côme *et al.* 2009; Laine *et al.* 2013), serous ovarian carcinoma (Böckelman *et al.* 2011) and colorectal adenocarcinoma (Böckelman *et al.* 2012).

One explanation for the widespread overexpression of CIP2A in different malignancies might be related to DNA damage and the activation of DNA damage response proteins in cancer cells. One of these proteins, checkpoint kinase 1 (CHK1) has been observed to promote CIP2A transcription and c-Myc activity. (Khanna *et al.* 2013a).

By inhibiting PP2A from dephosphorylating Myc proto-oncogene protein (c-Myc), CIP2A stabilises c-Myc and promotes malignant transformation. CIP2A can bind directly to c-Myc that is associated with the PP2A protein complex. The stabilisation, and inhibition of proteolytic degradation, is attained by selectively keeping the serine 62 of c-Myc in phosphorylated form, which enables its function as a transcription factor. Inversely, inhibition of CIP2A leads to increased PP2A dephosphorylation activity. (Junttila *et al.* 2007; Westermarck & Hahn 2008). Among the different B subunit isoforms, PPP2R5A is mediating the regulation of serine 62 phosphorylation in c-Myc. (Kauko & Westermarck 2018). c-Myc can also positively regulate CIP2A, and thus c-Myc activation by, for example, gene amplification could contribute to the inhibition of PP2A. (Khanna *et al.* 2009; Myant 2015 *et al.*; Kauko & Westermarck 2018). CIP2A has also been shown to function independently of c-Myc at least in promoting cell migration (Niemelä *et al.* 2012).

PP2A can also act as a tumour suppressor by inhibiting the Wnt/β-catenin signalling pathway (Westermarck & Hahn 2008; Perrotti & Neviani 2013), and has a role in the regulation of senescence and TP53 mediated tumour suppression (Laine *et al.* 2013). TP53 activity can inhibit the expression of E2F1 transcription factor, which leads to the suppression of CIP2A expression. Further, CIP2A suppression can inhibit E2F1 expression by PP2A-mediated dephosphorylation resulting in cancer cell senescence. Conversely, the positive feedback loop between E2F1 and

CIP2A can contribute to senescense inhibition and tumour growth if TP53 activity is lost. (Laine *et al.* 2013; Laine & Westermarck 2014).

In addition to malignant transformation, CIP2A has a role in promoting anchorage-independent growth, cancer cell viability and in protecting the cancer cells from apoptosis or senescence related to cancer therapies (Côme *et al.* 2009; Khanna *et al.* 2009; Böckelman *et al.* 2011; Dong *et al.* 2011; Lucas *et al.* 2011; Niemelä *et al.* 2012; Laine *et al.* 2013). CIP2A is also involved in the regulation of mitosis and cell cycle (Kim *et al.* 2013b).

High *CIP2A* mRNA or protein levels have been associated with poor prognosis in, for example, gastric (Khanna *et al.* 2009), breast (Laine *et al.* 2013) and colorectal (Wiegering *et al.* 2013) cancer as well as with poor histological differentiation in breast (Côme *et al.* 2009) and colorectal (Böckelman *et al.* 2011) cancer. CIP2A overexpression has also been associated with EGFR overexpression and *EGFR* amplification in serous ovarian carcinoma (Böckelman *et al.* 2011). It has been found that EGFR signalling upregulates CIP2A expression through the activation of the EGFR-MEK1/2-ETS1 pathway (Khanna *et al.* 2011; Khanna & Pimanda 2016).

It has been noticed *in vitro* that in order to undergo malignant transformation, the cells require both the constitutive activity of mutated *RAS* and the inhibition of PP2A (Westermarck & Hahn 2008). CIP2A-mediated inhibition of PP2A can function synergistically with RAS activity to drive the cell towards a more malignant phenotype. (Junttila *et al.* 2007; Mathiasen *et al.* 2012). In addition, both CIP2A- and RAS-mediated signalling have been shown to share several common phosphorylation target proteins (Kauko *et al.* 2015). They may also have overlapping regulatory functions and be co-operating during cancer progression (Hahn *et al.* 2002). In survival analyses, patients with both high CIP2A expression and KRAS or NRAS expression, or *KRAS* mutations, have been observed to have worse survival than patients with low CIP2A and KRAS/NRAS expression (Kauko *et al.* 2015).

Molecules activating PP2A or inhibiting the endogenous PP2A inhibitors might have potential as cancer treatment (Kauko & Westermarck 2018; O'Connor *et al.* 2018). As many existing cancer therapies are protein kinase inhibitors (Eifert & Powers 2012), additional therapeutic benefits might be obtained by combining kinase inhibitors with PP2A reactivating agents (Westermarck & Hahn 2008; Perrotti *et al.* 2013; Kauko & Westermarck 2018; O'Connor *et al.* 2018).

2.11 Tumour infiltrating lymphocytes and immunological therapies

The prominent lymphocytic infiltration observed in some tumours has been associated with the presence of neoantigens that have triggered a cell-mediated immune response against cancer cells (Sæterdal *et al.* 2001; Segal *et al.* 2008). The production of these neoantigens can result from a hypermutated phenotype as observed for example in MSI-H tumours (Sæterdal *et al.* 2001; Llosa *et*

al. 2015). The most frequently predicted neoantigens in cancer include *KRAS*/*NRAS*/*HRAS* and *BRAF* V600 mutations (Ding *et al.* 2018; Thorsson *et al.* 2018).

Lymphocytic infiltrations associated with MSI-H contain mainly cytotoxic T cells (CD8+) and type 1 helper T cells (Th1, CD4+) (Phillips *et al.* 2004; Llosa *et al.* 2015), and the overall number of frameshift mutations in MSI-H colorectal cancer tumours has been associated with the density of tumour infiltrating lymphocytes (Tougeron *et al.* 2009). *BRAF* mutated tumours have been observed to contain a higher proportion of CD8+ T cells than *NRAS* mutated tumours, and the predominance of CD8+ T cells has been associated with a better outcome (Ding *et al.* 2018; Thorsson *et al.* 2018). Overall, the presence of a prominent lymphocytic infiltration has been associated with an improved survival regardless of clinical stage (Galon *et al.* 2006; Ogino *et al.* 2009; Mlecnik *et al.* 2011). In contrast, low levels of T cell infiltration associate with poor prognosis even in patients with stage I colorectal tumours (Galon *et al.* 2006).

The immune response triggered by the neoantigens can be suppressed by immune checkpoint molecules such as PD-1 and PD-L1. These proteins are highly expressed in the infiltrating immune cells and tumour cells in MSI-H tumours. (Llosa *et al.* 2015). Monoclonal antibodies against PD-1 and PD-L1/2 have been implicated to provide clinical benefit also in the treatment of metastatic or recurrent PD-L1-positive gastric cancer (Muro *et al.* 2016) and metastatic MSI-H colorectal cancer (Le *et al.* 2015; Le *et al.* 2017).

3 AIMS OF THE STUDY

The specific aims of this study were:

1. To study the prevalence, clinicopathological associations and prognostic role of EGFR and HER2 protein expression and gene amplification in intestinal-type adenocarcinomas of the stomach, gastro-oesophageal junction and distal oesophagus.
2. To study the prevalence of EBV positivity and MSI together with aberrant E-cadherin and TP53 expression in intestinal- and diffuse-type oesophagogastric adenocarcinomas using next-generation tissue microarray. This information was combined with the Laurén classification and *EGFR* and *HER2* amplification data to identify subgroups with distinct molecular and clinicopathological characteristics.
3. To study the association between CIP2A expression and clinical response to long-course (chemo)radiotherapy in rectal cancer patients. To support the finding, the effect of *CIP2A* suppression by siRNA on the viability of colorectal cancer cells after irradiation was examined *in vitro*.
4. To analyse the *EGFR* GCN change between the primary and recurrent tumours from colorectal cancer patients. The *EGFR* GCN change among patients treated with anti-EGFR therapy after primary surgery was compared with the GCN change among patients not exposed to anti-EGFR antibodies.

4 PATIENTS AND METHODS

4.1 Patients and tumours (I – IV)

All of the studies were retrospective in nature. The characteristics of the patients and tumours included in the studies I – IV are presented in Table 4. The materials and methods are presented in more detail in the original publications I – IV.

Table 4. The clinicopathological characteristics of the patients and the gastric and colorectal tumours included in studies I – IV.

	Gastric cancer			Colorectal cancer		
	Study I	**Study II**		**Study III**		**Study IV**
Number of patients	220	244		210		80
Median age in years (range)	74 (33–93)	72 (33–91)		70 (34–92)		66 (34–87)
Median follow-up time in years	10.5	10.4		6.2		8.1
Patient sex						
Female	79 (35.9)	101 (41.4)		89 (42.4)		38 (47.5)
Male	141 (64.1)	143 (58.6)		121 (57.6)		42 (52.5)
Histological type						
Intestinal	220	190 (77.9)				
Diffuse		54 (22.1)				
Grade[a]						
I	30 (13.6)	17 (7.0)		32 (15.2)		9 (11.3)
II	103 (46.8)	93 (38.1)		135 (64.3)		55 (68.8)
III	87 (39.5)	134 (54.9)		36 (17.1)		14 (17.5)
KRAS status						
wild-type						47 (58.8)
mutated						33 (41.3)
Location of tumour						
Distal oesophagus	20 (9.1)	19 (7.8)	Colon			40 (50.0)
GOJ/cardia	63 (28.6)	60 (24.6)				
Corpus	65 (29.5)	106 (43.4)	Rectum	210 (100.0)		40 (50.0)
Antrum/pylorus	72 (32.7)	59 (24.2)				
Postoperative stage[b]						
I	59 (26.8)	46 (18.9)		56 (26.7)		7 (8.8)
II	83 (37.7)	102 (41.8)		70 (33.3)		27 (33.8)
III	64 (29.0)	83 (34.0)		82 (39.0)		42 (52.5)
IV	14 (6.4)	13 (5.3)				4 (5.0)

	Gastric cancer		Colorectal cancer			
	Study I	**Study II**		**Study III**		**Study IV**
Residual tumour[c]			CRM (mm)			
R0	167 (75.9)	180 (73.8)	0	15 (7.1)		
R1	24 (10.9)	34 (13.9)	0 – 2	24 (11.4)	< 2	13 (16.3)
R2	17 (7.7)	20 (8.2)	> 2	117 (55.7)	≥ 2	28 (35.0)
Rx	12 (5.5)	10 (4.1)	ND	54 (25.7)		38 (48.8)
Preoperative therapy						
Chemotherapy	18 (8.2)	29 (11.9)	Short-course RT[d]	89 (42.4)		10 (12.5)
			Long-course (C)RT[d]	51 (24.3)		7 (8.8)
No treatment	202 (91.8)	215 (88.1)	No RT	70 (33.3)		63 (78.8)
Disease recurrence (≥ 6 months after dg)[e]			Disease recurrence (all recurrences)			
Yes	58 (29.8)	73 (29.9)		67 (31.9)		
No	137 (70.3)	134 (54.9)		143 (68.1)		
Recurrence site (≥ 6 months after dg)[f]			Recurrence site (all recurrences)			
Local	21 (9.5)	24 (9.8)		18 (8.6)		21 (26.3)
Distant	37 (16.8)	49 (20.1)		49 (23.3)		59 (73.8)
Tumour regression[g]						
			Poor	26 (51.0)		
			Moderate	15 (29.4)		
			Excellent	10 (19.6)		
Antibody therapy[h]						
Anti-EGFR						24 (30.0)
Anti-HER2	5 (2.3)	5 (2.0)				
Patient status at the end of follow-up						
Alive	55 (25.0)	49 (20.1)		114 (54.3)		22 (27.5)
Dead	165 (75.0)	195 (79.9)		96 (45.7)		58 (72.5)

[a]Grade could not be determined for seven tumours in study III and two tumours in study IV.
[b]According to the WHO Classification manual (2010) for studies I – II. The TNM classification applicable at the time of surgery was used for study III (Sobin & Wittekind 2002). In study III, no vital tumour was observed in two patients.
[c]Determined only for gastric and oesophageal tumours.
[d]Short-course RT consisted of a total dose of 25 Gy delivered over 5 days in 5 Gy fractions and long-course RT was given in 1.8 Gy fractions to a total dose of 50.4 Gy over 6 weeks with or without chemotherapy.
[e]In study I, 14 patients had metastatic disease at the time of primary diagnosis and 11 patients < 6 months after diagnosis. In study II, 13 patients had metastatic disease at the time of primary diagnosis and 22 patients < 6 months after diagnosis. Disease recurrence was not known for two patients. In study III, follow-up information was available for 206 patients.
[f]In study I, disease recurrences < 6 months after diagnosis included two local recurrences and nine distant metastases. In study II, disease recurrences < 6 months after diagnosis included six local recurrences and sixteen distant metastases.
[g]Tumour regression was determined only after long-course (chemo)radiotherapy.
[h]Anti-EGFR therapy included either cetuximab or panitumumab, with or without irinotecan. Anti-HER2 therapy was trastuzumab administered together with chemotherapy.

CRM, circumferential resection margin; (C)RT, (chemo)radiotherapy; dg, diagnosis; GOJ, gastro-oesophageal junction; ND, not determined.

4.1.1 Gastric cancer patients (I – II)

The study population in the original publications I – II consisted of patients diagnosed with adenocarcinoma of the stomach, gastro-oesophageal junction or distal oesophagus at the Turku University Hospital in 1993–2012. Intestinal-type tumours from thirty patients included in study I were excluded from study II due to insufficient sample material for next-generation tissue microarray (ngTMA).

Primarily, tissue samples from surgical specimens were used in study I. Representative biopsies were analysed in case of 22 patients because four patients were not operated due to stage IV disease at the time of diagnosis and 18 patients had received perioperative chemoradiotherapy resulting in insufficient surgical material for immunohistochemistry and *in situ* hybridisation.

4.1.2 Rectal cancer patients (III)

The study population in the original publication III consisted of 210 rectal cancer patients with tumours located in either middle or distal rectum. They were operated at Turku University Hospital in 2000–2009. Patients with superficial tumours operated by local excision and patients with distant metastases at the time of diagnosis were excluded from the study. The patients received either short-course preoperative RT, long-course preoperative (C)RT or no treatment before surgery. Long-course RT was given with (n = 43) or without (n = 8) chemotherapy. Chemotherapy included either 5-fluorouracil (5-FU, n = 5) or capecitabine (n = 38). The type of treatment was chosen based on preoperative tumour staging. Patients with established high-risk features were treated with adjuvant chemotherapy.

Tumour regression after long-course (C)RT was determined according to a simplified classification based on Dworak and Rödel scales (Dworak *et al.* 1997; Rödel *et al.* 2005; Korkeila *et al.* 2009; Avoranta *et al.* 2012). The response to RT was divided into three categories: poor (only minimal or no tumour regression), moderate (some detectable vital tumour cells or cell groups), or excellent response (very few or no detectable tumour cells).

4.1.3 Colorectal cancer patients (IV)

The study population in the original publication IV consisted of 80 patients treated for colorectal cancer at the Turku University Hospital and Central Finland Central Hospital in 2000–2015. Three of the Turku patients had their liver metastasis resection performed at the Helsinki University Hospital.

Altogether 24 patients were treated with anti-EGFR therapy. Of those, the relationship between *EGFR* GCN change and anti-EGFR antibody treatment was analysed in 14 *KRAS* wt patients

whose primary tumour samples were obtained prior to and recurrent tumour samples after the administration of anti-EGFR therapy. The *EGFR* GCN of their tumour samples was compared to the samples of patients having received adjuvant chemotherapy or no adjuvant therapy after primary surgery. In addition, the clinical response to anti-EGFR antibody treatment was evaluated in 13 patients receiving antibodies before the sample was obtained from the recurrent tumour. The evaluation was performed by computed tomography (CT) or magnetic resonance imaging (MRI) according to the Response Evaluation Criteria in Solid Tumors (RECIST) (Eisenhauer *et al.* 2009).

4.2 Immunohistochemistry, *in situ* hybridisation, Western blot and *KRAS* mutation analysis

4.2.1 Antibodies, in situ *hybridisation probes and staining procedures (I – IV)*

The same EGFR and HER2 IHC and SISH samples were included in studies I – II.

The antibodies and staining procedures used in studies I – IV are described in more detail in Table 5 together with specifics of the *KRAS* mutation analysis. The IHC and *in situ* hybridisation scoring principles are described in Table 6.

Table 5. The antibodies and *in situ* hybridisation probes used in studies I – IV together with specifics of *KRAS* mutation analysis used in study IV.

IHC antibody (clone)	Dilution	Tissue sections	Reagents, signal detection, procedures	Antibody/ probe incubation
EGFR monoclonal (5B7)[a] Ventana/Roche	ready-to-use	3 µm	ultraView Universal DAB Detection Kit, BenchMark XT (Ventana/Roche)	
HER2 monoclonal (4B5) Ventana/Roche	ready-to-use	”	”	
MLH1 (G168-15) BD Pharmingen	1:5	4 µm	” & amplification kit	36 min
MSH2 (G219-1129) BD Pharmingen	1:200	”	”	28 min
MSH6 (EP49) Epitomoc	1.200	”	”	32 min
PMS2 (EPR3947) Ventana/Roche	ready-to-use	”	OptiView Universal DAB Detection Kit & amplification kit (Ventana/Roche)	44 min
TP53 (Bp53-11) Ventana/Roche	ready-to-use	”	ultraView universal DAB Detection Kit (Ventana/Roche)	28 min
E-cadherin (NHC-38) Agilent Technologies	1:100	”	” & amplification kit	32 min
CIP2A polyclonal (Soo Hoo *et al.* 2002)	1:4000		Antibody Diluent Buffer (Dako Denmark A/S), Dual Link System–HRP and DAB Chromogen System (Dako Denmark A/S), Lab Vision Autostainer	60 min

ISH probe				
EGFR DNA Probe Ventana/Roche		5µm	ultraVIEW SISH Detection Kit, BenchMark XT (Ventana/Roche)	
HER2 DNA Probe and INFORM Chromosome 17 Probe Ventana/Roche		„	” & ultraView Alkaline Phosphatase Red ISH Detection Kit, BenchMark XT (Ventana/Roche)	
EBER (Epstein-Barr virus –encoded small RNA) Ventana/Roche		”	ISH iVIEW Blue Detection Kit, BenchMark XT (Ventana/Roche)	60 min
Western blot				
CIP2A (2G10-3B5) monoclonal Santa Cruz Biotechnology anti-GAPDH monoclonal (mAbcam 9484) Abcam			cell lysis in RIPA buffer, HRP-conjugated anti-GAPDH was used as a loading control	
***KRAS* mutation analysis**[b]				
60 patients (pyrosequencing)		10 µm	QIAamp DNA FFPE tissue kit, Qiagen TheraScreen KRAS Pyro kit, PyroMark Q24 analysis program (Qiagen)	
20 patients (real-time PCR)		”	QIAamp DNA FFPE tissue kit (Qiagen), DxS K-RAS Mutation kit (DxS Ltd)	

[a]Targeted against the internal domain of EGFR.
[b]Included the analysis of codons 12, 13 and 61.

DAB, 3,3'-diaminobenzidine; FFPE, formalin-fixed paraffin-embedded; DAB, GADPH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; SISH, silver *in situ* hybridisation.

4.2.2 CIP2A staining indices (III)

In study III, the most intense cytoplasmic staining index (MICI) and the average cytoplasmic staining index (ACI) were used to classify the samples into two subgroups according to the index value being either below or above median level. The indices were calculated with the following formula: $I = 0 \times f0 + 1 \times f1 + 2 \times f2 + 3 \times f3$, where I is the staining index and f0 – f3 the fraction of cells (from 0 to 1) showing a defined level of staining (from 0 to 3). To obtain MICI, the area containing the most intense staining of cancer cells was chosen from each sample, and the fraction of cancer cells (percentage/100) belonging to each staining intensity category was estimated from that area. ACI was calculated as an average of three randomly selected areas from which the fraction of cancer cells belonging to each staining intensity category was estimated. (Lipponen & Collan 1992).

Table 6. The scoring principles of immunohistochemical stainings and *in situ* hybridisations used in studies I – IV.

Immunohisto-chemistry	Negative (0)	Weak (1+)	Moderate (2+)	Strong (3+)
EGFR	No staining (membranous or membranous + cytoplasmic)	Detected only with 10x objective magnification	Clearly identified with 5x objective magnification	Intense reaction with 5x objective magnification
HER2[b]	No reactivity or membranous reactivity in < 10% of tumour cells	Faint membranous reactivity in ≥ 10% of tumour cells; only partial membranous reaction	Weak to moderate complete, basolateral or lateral membranous reactivity in ≥ 10% of tumour cells	Moderate to strong complete, basolateral or lateral membranous reactivity in ≥ 10% of tumour cells
	MSI[c]		**MSS**	
MLH1 **MSH2** **MSH6** **PMS2**	Complete loss of nuclear reactivity in tumour cells together with positive reaction in normal epithelium, lymphocytes, stromal and smooth muscle cells		Positive nuclear reaction in tumour cells together with positive reaction in normal epithelium, lymphocytes, stromal and smooth muscle cells	
	Aberrant		**Wild-type**	
TP53	Complete loss of or strong diffuse nuclear positivity in tumour cells		Moderate or weak nuclear reaction in tumour cells	
E-cadherin	Loss of membranous reactivity or only weak cytoplasmic reaction in tumour cells		Moderate or strong membranous reaction in tumour cells	
	Negative (0)	**Weak (1+)**	**Moderate (2+)**	**Strong (3+)**
CIP2A	No detectable cytoplasmic staining with 10x objective magnification	Cytoplasmic staining still distinguishable from the background with 10x objective magnification	Cytoplasmic staining intermediate between weak and strong with 10x objective magnification	Cytoplasmic staining corresponding to the positive control (normal testis) with 10x objective magnification

***In situ* hybridisation**	**Positive**	**Negative**		
EBER	Positive nuclear reaction	No detectable reaction		
	Gene copy number		**Amplification**	**No amplification**
***EGFR*[a]**	A mean value from forty tumour cells was calculated from the areas of highest IHC reactivity[d]		Surgical specimens: detectable clusters[e] in forty tumour cells Biopsies: a group of ≥ 5 tumour cells containing clusters	No detectable clusters[e]
***HER2*[a, b]**	*HER2*/Chr17 GCN ratio was calculated as a mean value from forty tumour cells from the areas of highest IHC reactivity		Surgical specimens: detectable clusters in ≥ 10% of tumour cells Biopsies: a group of ≥ 5 tumour cells containing clusters[e]	No detectable clusters[e]

[a]Samples with EGFR or HER2 IHC 2+ or 3+ in ≥ 10% of tumour cells in surgical specimens or in ≥ 5 clustered tumour cells in biopsies were further analysed with SISH.
[b]Hofmann 2008, Bang 2010.
[c]A tumour was classified as MSI if at least one of the markers (MLH1, MSH2, MSH6 and PMS2) showed a complete loss of nuclear reactivity together with positive reaction in benign colorectal epithelium, lymphocytes, stromal and smooth muscle cells. Tumours showing negative nuclear reactivity with negative background were not used for classification (inconclusive staining).
[d]Ålgars *et al.* 2011; Ålgars *et al.* 2014.
[e]A cluster contains numerous overlapping SISH signals. One *EGFR* cluster was approximated to contain ≥ 10 gene copies and one *HER2* cluster was approximated to contain ≥ 6 gene copies. In practice, HER2/Chr17 ratio was always ≥ 2.0 when clusters were detected and < 2.0 when no clusters were detected in tumour cells.

Chr7, chromosome 7; EBER, Epstein-Barr virus encoded small RNA; GCN, gene copy number; IHC, immunohistochemistry; MSI, microsatellite instability; MSS, microsatellite-stable.

4.3 Next generation tissue microarray (II)

The next generation tissue microarray (ngTMA) was created by using representative paraffin blocks containing invasive carcinoma from each tumour. The blocks were selected by evaluating the original hematoxylin-eosin (H&E) stained sections. New H&E slides were produced, scanned (Pannoramic P250, 3DHistech) and uploaded into the university digital microscopy web portal (casecenter.utu.fi) for annotation. The digital slides were viewed using Pannoramic Viewer software (3DHistech). From each tumour, two areas were selected in the centre and two areas in the periphery or invasive front by using the 1.0 mm annotation tool. The annotated digital slide was overlaid with the corresponding tissue specimen, and the corresponding tissue cores were transferred into the TMA blocks by using an automated TMA instrument (TMA Grandmaster, 3DHistech). (Zlobec *et al.* 2014). One core containing benign tissue was selected from each tumour to act as a control. The constructed TMA blocks were sectioned, stained, scanned and uploaded into the web portal.

4.4 *In vitro* experiments (III)

4.4.1 *Cell culture and* CIP2A *siRNA transfection*

The RKO human colorectal cancer cell line (ATCC® CRL-2577™) was purchased from ATCC (Manassas, VA, USA) and its validity was affirmed by sequencing (Eurofins Genomics, Ebersberg, Germany). Cells were grown in Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine and 1% penicillin/streptomycin.

Cells in the logarithmic growth phase were transfected with *CIP2A* or scrambled double-stranded small interfering RNAs (siRNA) using Oligofectamine (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). The siRNA sequences have been previously published (Côme *et al.* 2009). The CIP2A protein levels were analysed by Western blotting using a monoclonal CIP2A antibody (Table 3).

4.4.2 *Cell irradiation experiment and cell survival assay*

Radiation experiments were performed on RKO cells transfected with either *CIP2A* siRNA or scrambled siRNA (control). Forty-eight hours after the transfections, the cells were harvested into single-cell suspensions. The cells were irradiated at room temperature in separate tubes containing 25 000 cells/treatment in 6 mL culture medium. The irradiation was performed at the radiotherapy department using a linear accelerator (Clinac 2100; Varian CA) with 6 MeV photon irradiation at a dose rate of 2 Gy/min. After irradiation, the cells were further diluted into 50 mL culture medium in appropriate concentration and 200 µL of cell suspension/well was pipetted in duplicate into 96-well plates (Pekkola-Heino *et al.* 1989). The cell plating is described in Figure 2.

The surviving fractions (SF) were calculated with the formula:

$$SF = \frac{\text{no. of positive wells/no. of plated cells in control}}{\text{no. of positive wells in control}}$$

The survival curves of cancer cells were fitted using the linear quadratic (LQ) model ($SF = \exp[-(\alpha D + \beta D2)]$; D, radiation dose). The area under the curve (AUC) values were calculated with a numerical integration algorithm. The results were calculated from three experiments for each treatment with duplicate plates for each radiation dose.

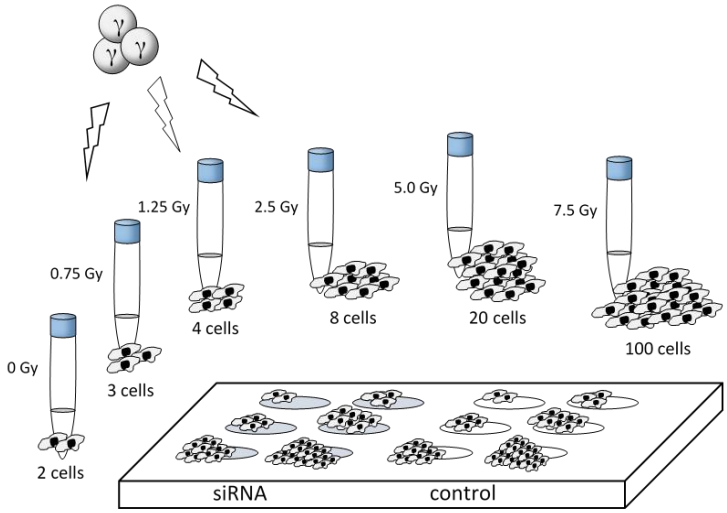

Figure 2. The number of cells per well was adjusted according to the expected cell death. The plates were incubated in the cell culture incubator until visible colonies were formed. The plates were examined using an inverted phase contrast microscope. Wells containing colonies of at least 32 cells were considered positive.

4.5 Statistical analyses

Statistical analyses were performed with IBM SPSS Statistics for Windows (IBM Corporation, Armonk, NY), version 21.0 (studies I – III) and version 24.0 (study IV). Frequency table data were analysed using the Pearson's $\chi 2$ test or Fisher's exact test for categorical variables. 2 x 2 tables were used to calculate odds ratios (OR) and 95% confidence intervals (CI) using the exact method. In Study IV, to compare the mean GCN in relation to categorical variables, non-parametric Mann-Whitney and Kruskal-Wallis tests were used as the *EGFR* GCN was not normally distributed. Pairwise concordance of *EGFR* GCN between primary and metastatic tumours were analysed using a non-parametric paired-samples test (McNemar and Wilcoxon signed rank test).

In study III, interobserver reproducibility of the IHC assessments was tested with weighted kappa, which was calculated with the intraclass correlation coefficient (ICC) test in parallel mode with a two-way random model using consistency assumption and the average measures option. The interobserver reproducibility was very good for MICI (weighted kappa 0.83, 95% CI: 0.67–0.91) and moderate for ACI (weighted kappa 0.56, 95% CI: 0.16–0.77). For the irradiation experiments, calculations were performed with Microsoft Excel 2007 (Microsoft Corporation, Redmond, WA) and paired t-test was used to compare the mean AUC values.

Kaplan-Meier method and log-rank test as well as Cox's proportional hazards regression model were used for univariate survival analysis. Multivariate survival analysis was performed using Cox's

proportional hazards regression model. In multivariate analyses, all covariates were entered simultaneously in studies I – II and in a stepwise backward manner in study III. The multivariate analyses included variables with a p value under 0.2 in univariate analysis in study I, variables with a p value under 0.05 in univariate analysis in study II and variables considered clinically relevant in study III. The different clinical survival endpoints are described in Table 7. All statistical tests were two-sided and p-values under 0.05 were considered statistically significant.

Table 7. The definition of different clinical endpoints used in survival analyses in studies I – III. All variables were calculated from the time of diagnosis. (Punt *et al.* 2007; Birgisson *et al.* 2011).

	Original publication	Recurrent disease (local or distant)	Second primary cancer	Death from primary cancer	Death from other cancer	Non-cancer related death	Loss to follow-up
RFS[a]	II	E	I	E	E	E	C
DFS[a]	III - IV	E	E	E	E	E	C
TTR[a]	I	E	I	E	C	C	C
CSS	I	I	I	E	C	C	C
DSS	III	I	E	E	C	C	C
OS	I – IV	I	I	E	E	E	C

[a]In studies I – II and IV, only recurrences occurring ≥ 6 months after diagnosis were considered relevant. Earlier detection of a local or distant recurrence was considered likely to present an initially advanced disease. Patients treated with surgery or surgery and adjuvant therapy without disease recurrence ≥ 6 months after diagnosis were considered curatively treated.

C, censored; E, event; I, ignored. CSS, cancer-specific survival; DFS, disease-free survival; DSS, disease-specific survival; OS, overall survival; RFS, recurrence-free survival; TTR, time to recurrence.

In study I, five patients (2.3%) who had received trastuzumab treatment for recurrent cancer were excluded from the cancer-specific survival (CSS) and overall survival (OS) analyses and additionally 14 patients with stage IV disease (6.4%) from the time to recurrence (TTR) analysis. In study II, five patients (2.0%) who had received trastuzumab treatment for recurrent cancer were excluded from the OS analysis and additionally 13 patients with stage IV disease (5.3%) from the RFS analysis. In study III, exact follow-up information for survival analyses was unavailable for four patients.

4.6 Ethical issues

The studies were conducted in accordance with the Declaration of Helsinki and the Finnish legislation for the use of archived tissue specimens and associated clinical information. The clinical data were retrieved and the histological samples collected and analysed with the endorsement of the National Authority for Medico-Legal Affairs and the Institutional Review Board of the Hospital District of Southwest Finland and, in studies I – II, also with the permission of Auria Biobank hosting the specimen archive. For study I, information about the cause of death of the patients was obtained from Statistics Finland (Dnro TK-53-1286-14). In study III, the clinical data were

retrieved and the histological samples were collected and analysed with the endorsement of the National Supervisory Authority for Welfare and Health, Finland (Dnro 1709/32/300/02, 13.5.2002). In study IV, oral or written informed consent was not obtained due to the fact that the majority of the patients included in this study had died of their disease. The need for informed consent from participants was waived by the National Authority for Medico-Legal Affairs (Dnro 4423/32/300/02, 15.10.2002).

5 RESULTS

5.1 Gastric cancer (I – II)

5.1.1 EGFR and HER2 IHC and SISH (I)

In study I, all 220 intestinal-type adenocarcinomas were analysed with EGFR and HER2 IHC. *EGFR* or *HER2* SISH was performed on all tumours with high EGFR or HER2 IHC staining intensity (2+/3+), respectively. The summary of EGFR and HER2 IHC and SISH results is presented in Figure 3. In study I, in order to validate the method of including only tumours with high EGFR IHC intensity for *EGFR* SISH, *EGFR* GCN was also assessed in fifteen randomly selected tumours in which EGFR IHC was scored as negative/weak. No *EGFR* amplification was found in these tumours (GCN 2.1–3.3).

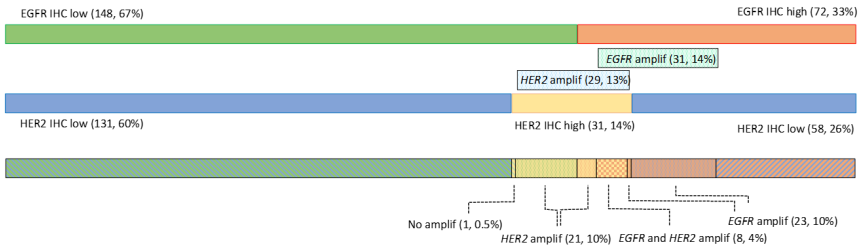

Figure 3. The distribution of EGFR and HER2 high (2+/3+) and low (0/1+) staining intensity in relation to *EGFR* and *HER2* gene amplification. The number of tumours (%) in each subgroup is presented in parentheses.

5.1.2 EGFR, HER2, EBV, MSI, TP53 and E-cadherin in relation to clinicopathological variables (I – II)

The distribution of EBV positivity, MSI, E-cadherin aberrations and TP53 aberrations together with the occurrence of *EGFR* and *HER2* amplifications among the intestinal- and diffuse type oesophagogastric adenocarcinomas is presented in Figure 4.

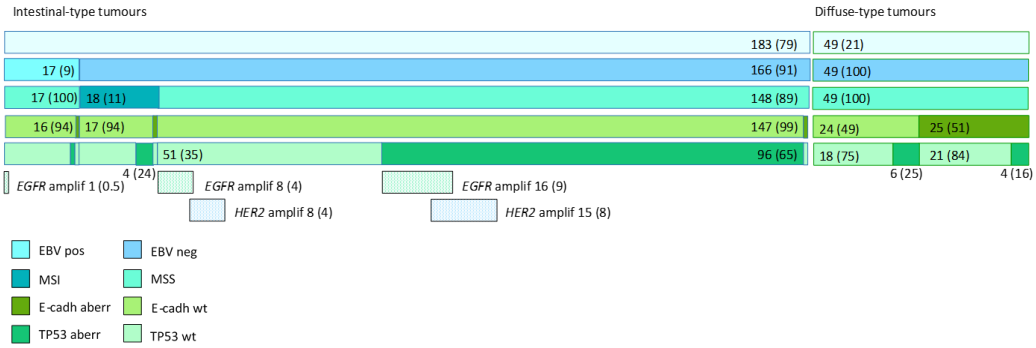

Figure 4. The distribution of EBV positivity, MSI, E-cadherin and TP53 aberrations among intestinal- and diffuse-type adenocarcinomas together with *EGFR* and *HER2* amplifications. The figures show the number of tumours (%). In study II, 183 out of the 190 intestinal-type adenocarcinomas included in the TMA had been evaluated for EGFR and HER2 protein expression levels in study I. EBV, MSI and TP53 were analysed in 238 tumours and E-cadherin in 232 tumours. In remaining tumours, the markers could not be evaluated due to insufficient tissue material.

EBV RNA ($p = 0.028$) and MSI ($p = 0.017$) were detected only in the intestinal-type tumours. Aberrant TP53 expression was also observed to be more common among intestinal-type than diffuse-type tumours ($p < 0.0001$). The intestinal-type tumours with aberrant TP53 were typically EBV negative ($p < 0.0001$) or MSS ($p = 0.003$). The majority of the *EGFR* (17/98, 17%) and *HER2* (15/98, 15%) amplifications as well as co-amplifications (5/98, 5.1%) were also found in tumours characterised by EBV negativity, MSS and aberrant TP53. The EBV negative, MSS and TP53 wt intestinal-type tumours were the second most common subgroup for *EGFR* and *HER2* amplifications (8/52, 15% for both genes). Among the diffuse-type tumours, aberrant E-cadherin expression could be detected in 25/49 (51%) tumours, whereas only 3/183 (1.6%) of the intestinal-type tumours had aberrant E-cadherin expression.

The combination of EBV negativity, MSS and TP53 wt was found in 52/186 (28%) of the intestinal-type and 42/52 (81%) of the diffuse-type tumours ($p <0.0001$). Among these, 21/39 (54%) of the diffuse-type tumours but none of the intestinal-type tumours ($n = 51$) had aberrant E-cadherin expression.

The association between *EGFR* amplification, EBV positivity, MSI and TP53 aberration and selected clinicopathological variables among the intestinal-type tumours is shown in Table 8. In study I, the presence of *HER2* amplification or diffuse-type histology was not associated with the examined variables. No significant associations were observed between the presence of *EGFR* or *HER2* amplification and EBV, MSI or TP53 status.

Table 8. The association between selected clinicopathological variables and some of the molecular markers from studies I – II in intestinal-type oesophagogastric tumours. The figures show the number of patients (%) together with p values.

	***EGFR* amplification**	**EBV positivity**	**MSI**	**TP53 aberration**
Patient sex	NS	**0.035**	**0.042**	NS
Female	8 (25.8)	2 (11.8)	11 (57.9)	34 (33.0)
Male	23 (74.2)	15 (88.2)	8 (42.1)	69 (67.0)
Location	**0.016**	NS	**0.003**	**0.002**
Distal oesophagus/GOJ/cardia	18 (58.1)	7 (41.2)	2 (10.5)	54 (52.4)
Corpus/antrum/pylorus	13 (41.9)	10 (58.8)	17 (89.5)	49 (47.6)
Location	**0.013**	**0.011**	**0.002**	**0.010**
Distal oesophagus	5 (16.1)	0 (0)	0 (0)	15 (14.6)
GOJ/cardia	13 (41.9)	7 (41.2)	2 (10.5)	39 (37.9)
Corpus	2 (6.5)	9 (52.9)	4 (21.1)	23 (22.3)
Antrum/pylorus	11 (35.5)	1 (5.9)	13 (68.4)	26 (25.2)
Grade	NS	**< 0.0001**	NS	NS
I	2 (6.5)	0 (0)	1 (5.3)	10 (9.7)
II	17 (54.8)	2 (11.8)	8 (42.1)	54 (52.4)
III	12 (38.7)	15 (88.2)	10 (52.6)	39 (37.9)
T	**0.020**	NS	NS	NS
T1 – T2	4 (13.3)	5 (29.4)	3 (15.8)	28 (27.2)
T3 – T4	26 (86.7)	12 (70.6)	16 (84.2)	75 (72.8)
Stage	**0.024**	NS	NS	NS
I – II	14 (45.2)	11 (64.7)	13 (68.4)	65 (63.1)
III – IV	17 (54.8)	6 (35.3)	6 (31.6)	38 (36.9)

EBV, Epstein-Barr virus; GOJ, gastro-oesophageal junction; MSI, microsatellite instability; NS, not significant.

EGFR gene amplification was more common in tumours with deep invasion ($p = 0.020$) and overall in more advanced tumours ($p = 0.024$). It was also most commonly detected in proximally located tumours ($p = 0.016$). Among the intestinal-type tumours, aberrant TP53 expression was more frequent in proximal than distal tumours ($p = 0.002$). Additionally, the co-localisation of aberrant TP53 expression and either *EGFR* or *HER2* gene amplification was detected more often in the proximal (distal oesophagus/GOJ/cardia) than distal (corpus/antrum/pylorus) intestinal-type tumours ($p = 0.019$, data not shown). In contrast, tumours with MSI were most frequent in distal location ($p = 0.002$).

EBV positivity was least common in the most proximal and distal tumours ($p = 0.011$), and it was associated with poor histological differentiation ($p < 0.0001$). EBV positivity was less often detected in female than male patients ($p = 0.035$), while MSI tumours were more common among female than male patients ($p = 0.042$).

5.1.3 EGFR and HER2 gene amplification and MSI in relation to survival (I – II)

Patients with intestinal-type tumours containing *EGFR* amplification had shorter TTR ($p = 0.026$) and CSS ($p = 0.033$) in univariate survival analysis than other patients. In addition, increasing depth of tumour invasion ($p < 0.0001$), and accordingly, increasing tumours stage, were associated with

decreased TTR ($p = 0.005$) and CSS ($p < 0.0001$) of the patients. Additional factors associated with shorter CSS were tumour differentiation grade (grade II, $p = 0.020$; grade III, $p = 0.029$) and older age of the patients at the time of diagnosis ($p = 0.048$). In multivariate analysis, only tumour stage remained as a predictive factor. In study II, the presence of MSI was predictive for longer OS both in univariate ($p = 0.040$) and multivariate ($p = 0.015$) analysis together with increasing tumour stage and patient age above median at the time of diagnosis. Increasing depth of tumour invasion, increasing tumour stage and older age were associated with shorter RFS in univariate, but not in multivariate, analysis.

In study I, EGFR or HER2 protein expression level or *HER2* amplification status was not associated with survival. In study II, no significant associations were observed between EBV, TP53 or E-cadherin status and survival. The association of *EGFR* amplification, MMR status and selected clinicopathological variables with survival endpoints in the intestinal-type tumours is presented in Tables 9 – 10.

In study I, the multivariate model for TTR included *EGFR* amplification status, postoperative tumour stage, histological differentiation grade and anatomical location of the tumour (proximal *vs* distal). The multivariate analysis for CSS included *EGFR* gene amplification status, postoperative tumour stage, histological differentiation grade and patient age at the time of diagnosis. Tumour stage remained as a single predictive factor for shorter TTR in patients with stage III tumours ($p = 0.014$) and for shorter CSS in patients with stage III ($p = 0.023$) or stage IV tumours ($p < 0.0001$).

In study II, the multivariate model for OS included patient age at diagnosis, postoperative T, postoperative tumour stage and MMR status of the tumour. MSI status was found to be predictive for longer OS ($p = 0.015$) while patient age above median ($p = 0.009$) and tumour stage III – IV ($p = 0.036$) were predictive for shorter OS among patients with intestinal-type tumours. Age above median remained as a single predictive factor for shorter OS ($p = 0.030$) among patients with diffuse-type tumours.

Table 9. Univariate survival analysis with selected clinicopathological variables for intestinal-type oesophagogastric tumours in studies I–II.

	TTR				CSS			
Study I	p value log-rank	p value Cox test	HR	95% CI	p value log-rank	p value Cox test	HR	95% CI
EGFR amplif	0.026	0.028	1.73	1.06–2.83	0.033	0.035	1.67	1.04–2.69
Age (cont)		NS				0.048	1.02	1.00–1.04
T	< 0.0001	< 0.0001	1.46	1.19–1.80	< 0.0001	< 0.0001	1.60	1.30–1.96
T1		ref				ref		
T2		NS				NS		
T3		NS				NS		
T4		0.002	2.59	1.44–4.67		0.001	2.94	1.58–5.47
Stage[a]	0.005	0.001	1.52	1.18–1.96	< 0.0001	< 0.0001	1.94	1.53–2.45
I		ref				ref		
II		NS				NS		
III		0.001	2.33	1.38–3.92		0.002	2.36	1.37–4.08
IV						< 0.0001	14.2	6.86–29.3
Grade	NS				NS			
I		ref				ref		
II		0.043	1.95	1.02–3.74		0.020	2.22	1.13–4.36
III		NS				0.029	2.15	1.08–4.27

	RFS				OS			
Study II	p value log-rank	p value Cox test	HR	95% CI	p value log-rank	p value Cox test	HR	95% CI
MSS		ref				ref		
MSI	NS	NS			0.040	0.043	0.54	0.30–0.98
T1 – T2		ref				ref		
T3 – T4	0.045	0.046	1.53	1.01–2.32	0.030	0.031	1.54	1.04–2.28
Stage I–II		ref				ref		
Stage III–IV[a]	0.019	0.020	1.56	1.07–2.26	< 0.0001	< 0.0001	1.84	1.32–2.57
Age < median		ref				ref		
Age ≥ median	0.006	0.006	1.67	1.16–2.42	0.026	0.027	1.46	1.04–2.03

[a]Stage IV excluded from TTR and RFS.

amplif, amplification; CI, confidence interval; cont, continuous variable; CSS, cancer-specific survival; HR, hazard ratio; ref, reference; TTR, time to recurrence

Table 10. Multivariate survival analysis with selected clinicopathological variables for intestinal-type tumours in studies I–II.

	TTR			CSS			OS			
Study I	p	HR	95% CI	p	HR	95% CI	**Study II**	p	HR	95% CI
Stage[a]										
I	ref			ref			MSS	ref		
II	NS			NS			MSI	0.015	0.46	0.25–0.86
III	0.014	2.05	1.16–3.63	0.023	1.99	1.10–3.61	Stage I–II	ref		
IV				< 0.0001	11.4	5.34–24.4	Stage III–IV	0.036	1.50	1.03–2.18
							Age < median	ref		
							Age ≥ median	0.009	1.57	1.12–2.21

[a]Stage IV excluded from TTR.

CI, confidence interval; CSS, cancer-specific survival; HR, hazard ratio; MSS, microsatellite-stable; MSI, microsatellite instability; NS, not significant; OS, overall survival; ref, reference; TTR, time to recurrence.

5.2 Colorectal cancer (III - IV)

5.2.1 CIP2A expression in relation to selected clinicopathological variables, tumour regression grade and survival

The association between CIP2A expression and selected clinicopathological variables is presented in Table 11. Moderate or excellent response to long-course (C)RT was associated with both low CIP2A MICI ($p = 0.006$) and ACI ($p = 0.007$). Low CIP2A MICI was also more common in younger patients ($p = 0.023$) and in the most invasive tumours ($p = 0.022$). Low CIP2A ACI tended to be more common in the well-differentiated tumours ($p = 0.050$). In addition, patients with low CIP2A MICI were more likely to be alive 36 months after diagnosis than patients with high CIP2A MICI ($p = 0.014$). No association was found between CIP2A expression level and patient sex, postoperative histological differentiation grade, lymph node status, postoperative stage, CRM or the presence of lymphovascular invasion.

In the univariate analysis, CIP2A MICI was not associated with patient survival. The multivariate analysis for DSS included CIP2A MICI, RT treatment group, patient sex, patient age at diagnosis, postoperative lymph node status, the presence of lymphovascular invasion, CRM and disease recurrence status. In the multivariate analysis, high CIP2A MICI ($p = 0.014$), patient age above median ($p = 0.002$), long-course (C)RT ($p = 0.040$) and disease recurrence ($p < 0.0001$) were found to predict reduced DSS. CIP2A MICI did not remain as an independent predictive factor for DFS.

5.2.2 The effect of CIP2A knockdown on radiosensitivity

The RKO cells with reduced *CIP2A* expression obtained by *CIP2A* siRNA transfection were observed to be more sensitive to irradiation than the control cells with intact *CIP2A* expression level ($p = 0.015$). The cell survival curves from the irradiation experiments and corresponding Western blot results are presented in more detail in Figure 2 in the respective publication (III).

Table 11. The association between the most intensive or average CIP2A expression level and selected clinicopathological variables in rectal adenocarcinomas.

	Most intensive cytoplasmic staining index (MICI, n = 204)[a]			**Average cytoplasmic staining index (ACI, n = 198)[a]**		
	Below median	Above median	p value	Below median	Above median	p value
Tumour regression[b]						
Poor	15 (42.9)	11 (91.7)	0.006	16 (50.0)	10 (100.0)	0.007
Moderate/excellent	20 (57.1)	1 (8.3)		16 (50.0)	0 (0)	
Age at dg						
≤ median	67 (57.8)	36 (40.9)	0.023			NS
> median	49 (42.2)	52 (59.1)				
T[c]						
T1 – T2	36 (31.1)	37 (42.0)	0.022			NS
T3	63 (54.8)	48 (54.5)				
T4	16 (13.9)	3 (3.4)				
Grade[d]						
I			NS	21 (21.2)	9 (9.5)	0.050
II				59 (59.6)	70 (73.7)	
III				19 (19.2)	16 (16.8)	
DSS[e]						
≥ 36 months	95 (90.5)	60 (76.9)	0.014			NS
< 36 months	10 (9.5)	18 (23.1)				

[a]Six tumours (MICI) and 12 tumours (ACI) could not be evaluated with IHC due to limited amount of tumour cells.
[b]Tumour regression was assessed only after long-course (C)RT (n = 47).
[c]Postoperative T could not be determined for one tumour.
[d]Four tumours could not be graded.
[e]Disease-specific survival, alive *vs* death of disease.

ACI, average cytoplasmic staining index; (C)RT, (chemo)radiotherapy; dg, diagnosis; DSS, disease-specific survival; MICI, most intensive cytoplasmic staining index; NS, not significant.

5.3 Primary and recurrent colorectal tumours (IV)

5.3.1 EGFR *gene copy number in the primary and recurrent colorectal tumours*

The *EGFR* GCN of the primary and recurrent tumours and the GCN change during disease progression are presented in Figures 5 and 6 both for the whole study population and with regard to the type of therapy after primary surgery (patients treated with anti-EGFR antibodies *vs* patients not receiving anti-EGFR therapy).

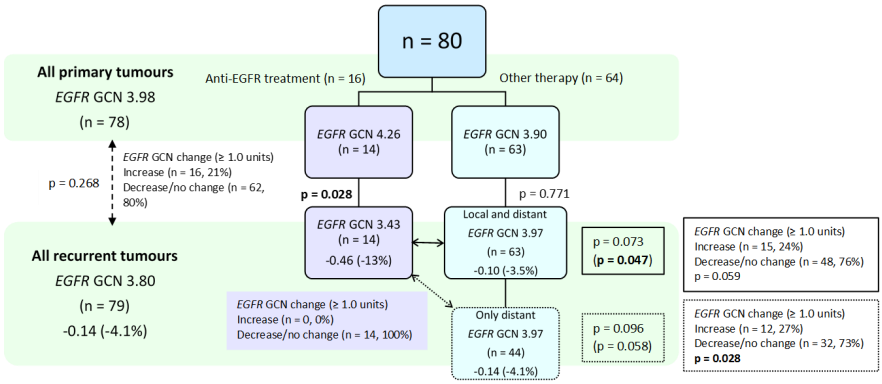

Figure 5. The median *EGFR* gene copy number is presented for each group of tumours. *EGFR* GCN change between primary and recurrent tumours is presented as absolute median values (relative change, %). The number of patients with tumours showing GCN change ≥ 1.0 units is noted on the side. The p values are calculated for comparisons between patients receiving either anti-EGFR treatment or other forms of therapy after primary surgery. The comparisons between patients with or without anti-EGFR treatment are presented both for all recurrent tumours and for distant metastases only (all anti-EGFR treated patients had distant recurrencies). Among the anti-EGFR treated patients, one patient was excluded because the sample taken before anti-EGFR treatment was obtained from the metastatic site, and one patient was excluded because the primary tumour was *KRAS* mutated.

Among the whole study population, the *EGFR* GCN did not change during disease progression when analysed as a continuous variable (p = 0.268). However, there was a significant decrease in *EGFR* GCN between the primary and recurrent tumours among the anti-EGFR-treated patients (p = 0.028) but not among patients without anti-EGFR therapy (p = 0.771). Also the relative GCN decreased significantly among the anti-EGFR-treated patients in comparison to the other group (p = 0.047). When *EGFR* GCN change of ≥ 1.0 was used as a cut-off value, GCN tended to decrease or stay stable among the anti-EGFR-treated patients in comparison to patients not treated with anti-EGFR therapy (p = 0.059), and particularly so when analysing only patients with distant metastases (p = 0.028). None of the anti-EGFR-treated patients experienced GCN increase ≥ 1.0 units between the primary and recurrent tumours. In contrast, *EGFR* GCN values among patients not treated with anti-EGFR therapy after primary surgery increased in 15/63 (24%) patients among all recurrences and in 12/44 (27%) patients with distant metastases (p = 0.028) (Figure 5).

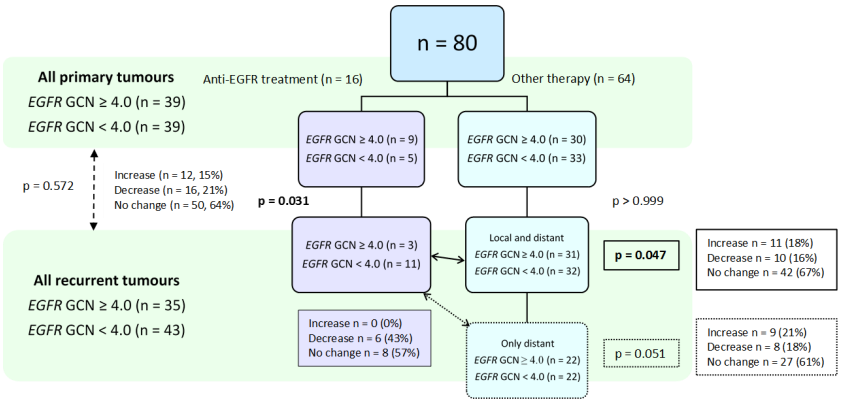

Figure 6. *EGFR* gene copy number change with regard to the cut-off value 4.0 in primary and recurrent tumours. Among the anti-EGFR treated patients, one patient was excluded because the sample taken before anti-EGFR treatment was obtained from the metastatic site, and one patient was excluded because the primary tumour was *KRAS* mutated.

When *EGFR* GCN change was analysed as a categorical variable using GCN 4.0 as a cut-off value, *EGFR* GCN did not change during disease progression among the whole study population (p = 0.572) (Figure 6). With regard to the cut-off value 4.0, discordant EGFR GCN was detected in 36% (28/78) of the primary – metastasis tumour pairs among the whole study population. Among the primary tumours, the *EGFR* GCN did not differ between patients treated later with anti-EGFR therapy (n = 14) and patients receiving other forms of therapy (n = 63, p = 0.588). Similarly, the *EGFR* GCN of the recurrent tumours did not differ between patients having received anti-EGFR therapy (n = 14) and patients not treated with anti-EGFR therapy (n = 64, p = 0.123).

However, among the anti-EGFR-treated patients, 43% (6/14) of the tumour pairs were discordant, and the recurrent tumours had more often GCN < 4.0 than the primary tumours (p = 0.031). Among the patients not treated with anti-EGFR therapy, the number of discordant tumour pairs (33%, 21/63) was not significant (p > 0.999). None of the anti-EGFR-treated patients experienced GCN increase during anti-EGFR therapy, while the GCN values among patients not treated with anti-EGFR therapy were either stable or even increased (33%, 21/63; p = 0.047). (Figure 6).

5.3.2 EGFR *gene copy number of the primary tumours in relation to selected clinicopathological variables*

The relationship between *EGFR* GCN and *KRAS* status, tumour stage and lymph node status of the primary tumours is presented in more detail in the respective publication (IV). In brief, *EGFR* GCN was observed to be higher in *KRAS* wt tumours (p = 0.019), patients with stage III disease (p = 0.024) and patients with N1–2 lymph node status (p = 0.018) in comparison to *KRAS* mutated

tumours, stage I–II disease and N0 lymph node status, respectively. Similar associations were observed between *EGFR* GCN ≥ 4.0 and *KRAS* wt tumours (p = 0.021), stage III disease (p = 0.037) and N1–2 lymph node status (p = 0.039). No significant associations were seen between *EGFR* GCN and patient sex, patient age at diagnosis, location of primary tumour (colon *vs* rectum or left-sided *vs* right-sided), depth of tumour invasion (pT), histological differentiation grade or location of recurrent tumour (local *vs* distant).

6 DISCUSSION

Gastrointestinal adenocarcinomas have been extensively studied with various molecular characterisation methods (TCGA 2012; TCGA 2014; Wang *et al.* 2014; Cristescu *et al.* 2015; Secrier *et al.* 2016; TCGA 2017) with the aim of identifying distinct molecular subtypes that could be utilised in cancer diagnostics and treatment of patients. (Liu *et al.* 2018). The result from all of these analyses is a multitude of information about gene expression, mutations, chromosomal alterations and other features that needs to be converted into relevant knowledge of practical use.

In this thesis, the main focus has been on biomarkers associated with signalling or regulatory processes known to be altered in cancer cells. EGFR is already utilised in the form of anti-EGFR antibodies in the treatment of metastatic colorectal cancer but it does not (yet) play any role in the treatment of oesophagogastric adenocarcinomas. In the future, the usage of new cancer therapies will likely require more sophisticated tumour characterisation than the detection of single biomarkers. This demands methods that are suitable for routine clinical use: easy to perform, inexpensive and straightforward to interpret. Furthermore, the utilisation of traditional therapies such as RT might also profit from molecular markers that could aid in selecting patients likely to benefit from the treatments.

6.1 EGFR and HER2 in oesophagogastric cancer (I)

The prevalence of *EGFR* amplification in gastric cancer has previously been reported to be 2.3–4.9% among all histological subtypes (Kim *et al.* 2008; Kandel *et al.* 2014; Nagatsuma *et al.* 2015), whereas the prevalence of *HER2* amplification has been reported to vary from 7 to 17% (Takehana *et al.* 2002; Tanner *et al.* 2005). In study I, the working hypothesis was that *EGFR* amplification might be detected more commonly among the intestinal-type tumours than what has been reported for gastric cancer in general. The rationale behind this originated in earlier reports according to which RTK gene amplifications are prevalent particularly in the CIN subtype of gastric adenocarcinomas, the majority of which (80%) have intestinal-type histology (TCGA 2014). This observation also implies that there might exist a subgroup of tumours that could be targeted by therapeutic agents inhibiting EGFR signalling parallel to the usage of trastuzumab in HER2 overexpressing tumours (Bang *et al.* 2010).

In study I, it was shown that in intestinal-type adenocarcinomas the prevalence of *EGFR* amplification (14%) was comparable with that of *HER2* (13%) and, indeed, not as uncommon as previously reported for gastric cancer in general. Particularly, *EGFR* amplification was most prevalent in tumours of distal oesophagus and GOJ. *EGFR* and *HER2* co-amplification was detected in 3.6% of intestinal-type adenocarcinomas, which is somewhat higher than what has been reported for all histological subtypes (< 0.5%) (Kandel *et al.* 2014; Nagatsuma *et al.* 2015)

No survival benefit has so far been demonstrated in clinical trials for patients treated with anti-EGFR antibodies in comparison to patients receiving standard chemotherapy. A phase III clinical trial has recently been conducted that examined the effect of an anti-EGFR monoclonal antibody (nimotuzumab) in patients with EGFR overexpressing tumours. The study was completed in February 2018 but the results have not yet been published. However, the overexpression of EGFR was only defined by IHC (2+ or 3+) without considering the GCN. (ClinicalTrials.gov NCT01813253).

Overexpression of EGFR has been reported in 24–27 % of all gastric adenocarcinomas (Kim *et al.* 2008; Nagatsuma *et al.* 2015) and specifically in 31% (Kim *et al.* 2008) of intestinal-type tumours. Consistent with these findings, high EGFR IHC staining intensity was detected in 33% of the intestinal-type adenocarcinomas in study I. Notably, only 31/72 (43%) of these contained *EGFR* amplification. The relatively low prevalence of gene amplification among tumours with protein overexpression implies that determining the EGFR status solely based on protein expression may be an inadequate method for selecting patients for anti-EGFR therapy. Similar to the definition of HER2 overexpression (Bang *et al.* 2010), it seems reasonable that the definition of EGFR overexpression might include both the overexpression of EGFR protein and *EGFR* gene amplification. Moreover, it has been observed *in vitro* that patient derived gastric cancer xenografts containing *EGFR* amplification respond better to anti-EGFR therapy than tumours without the gene amplification (Zhang *et al.* 2013).

The infrequency of co-amplification of *EGFR* and *HER2* implicates the presence of two distinct subgroups of patients with either *EGFR* or *HER2* amplification. Similarly, in large-scale sequencing studies *EGFR* amplification has proven to be mutually exclusive with HER2 activation and with activating mutations in *KRAS* or *BRAF* (Sanchez-Vega *et al.* 2018). These findings suggest that the potential candidates for clinical anti-EGFR antibody trials are specifically those patients not eligible for anti-HER2 treatment. The few patients with tumours containing receptor co-amplification might benefit from some kind of treatment targeting both *EGFR* and *HER2* signalling.

EGFR amplification was also found to be associated with decreased TTR and CSS. A similar association with survival has also been observed by others (Kim *et al.* 2008; Kandel *et al.* 2014). Studies examining *HER2* amplification as a negative prognostic factor in gastric cancer have not yielded unequivocal results (Tanner *et al.* 2005; Kandel *et al.* 2014), and in study I the presence of *HER2* amplification was not found to be associated with the survival of patients. Regardless of whether or not *EGFR* amplification proves to have a prognostic role, the main potential advantage to be gained from GCN analysis will most likely be related to the selection of cancer treatment. However, the association with decreased survival could indicate a subgroup of patients in need of a targeted therapy.

Potential weaknesses of the study regarding the survival analyses include the heterogeneity in the treatment regimens and the inclusion of patients with all stages of disease. The primary aim of the study, however, was to examine the prevalence of *EGFR* and *HER2* amplification in intestinal-type oesophagogastric adenocarcinomas and only secondarily to examine their prognostic role. In order

to avoid at least some treatment-related bias, patients treated with trastuzumab were excluded from the TTR and CSS analyses and patients with stage IV disease were excluded from the TTR analysis. With regard to the primary aim, a limitation of the study was the usage of tumour biopsy material instead of surgical specimens for GCN analyses in 22 patients. This could result in misclassifying a tumour, due to tumour heterogeneity, as containing or, more likely, not containing a gene amplification.

6.2 Molecular subtypes of oesophagogastric cancer (II)

In study II, the aim was to apply the findings from the molecular characterisation of stomach adenocarcinomas (TCGA 2014) and to examine the applicability of the proposed categorisation algorithm to clinical diagnostics. A similar approach has been undertaken by several other research groups (Kim *et al.* 2016; Park *et al.* 2016; Setia *et al.* 2016; Ahn *et al.* 2017; Díaz del Arco *et al.* 2018; Huang *et al.* 2019), each of which have utilised some variation of the original theme.

In contrast with the aforementioned publications, in study II the oesophagogastric adenocarcinomas were first divided into two groups based on the Laurén classification and then examined with other methods. Intestinal-type adenocarcinomas have observed to present more diverse molecular profiles than diffuse-type tumours, which predominantly fall under the category of genomically stable tumours (TCGA 2014) or tumours with MSS/EMT features (Cristescu *et al.* 2015). RTK copy number alterations are mainly present in the intestinal-type tumours and thus the histological subtype could also be a relevant factor to take into account when investigating new RTK-targeting therapies for gastric cancer (TCGA 2014). Therefore, the intestinal-type adenocarcinomas were the main focus of our analyses in study II. A small subset of diffuse-type tumours was included to serve as a reference group but not used in statistical analyses to the same extent as the intestinal-type tumours. Therefore, in study II the percentages for different markers for diffuse-type tumours should be considered as approximate.

A summary of the results from previous studies examining the prevalence of the molecular subtypes and related molecular markers among gastric adenocarcinomas is presented in Table 12. The principles for the final division of tumours into each molecular subtype vary in each study. Inevitably, a few tumours in each study could have been sorted into more than one category. Therefore, the classification was usually performed sequentially and one marker was given more significance over another. The percentages for each marker have been derived from the information given in each study or in its supplementary material.

In study II, aberrant E-cadherin expression could be detected in only three intestinal-type tumours, whereas almost all of the tumours with aberrant E-cadherin expression were already categorised as diffuse-type according to the Laurén classification. The proportion of intestinal-type tumours with aberrant E-cadherin is comparable to the frequency of E-cadherin mutations (4.1%) detected in the TCGA study (TCGA 2014). These findings imply that the Laurén classification could be used as an

approximate marker for tumours characterised by EMT and thus loss of E-cadherin expression. The three intestinal-type tumours with aberrant E-cadherin showed either EBV positivity, MSI or TP53 aberration and thus could be classified according to these characteristics. Notably, EBV positivity and MSI were found to be mutually exclusive, which is consistent with other studies (TCGA 2014; Kim *et al.* 2016).

None of the EBV negative, MSS and TP53 wt intestinal-type tumours showed aberrant E-cadherin expression, and inversely, none of the diffuse-type tumours was found to be EBV positive or MSI. Interestingly, diffuse-type tumours are practically always considered poorly differentiated, whereas poor histological differentiation among the intestinal-type tumours was found to be associated with EBV positivity.

These observations imply that the Laurén classification followed by EBER *in situ* hybridisation and MSI IHC could be enough for general tumour characterisation without the need to perform E-cadherin, or even TP53 staining. Detecting TP53 aberration by IHC can be more equivocal than the detection of EBV or MSI-H due to variable staining intensity in TP53 wt tumours. In fact, similarly to the approach employed in study II, a recent study has combined the TCGA and Laurén classification systems, and first separated the EBV positive and MSI-H tumours followed by the division of the remaining tumours into either intestinal- and diffuse-type based on histology (Huang *et al.* 2019). However, some additional biomarker might be needed to detect or characterise those intestinal-type tumours that show neither EBV positivity, MSI nor definite TP53 or E-cadherin aberration.

In contrast with study II, a small proportion of diffuse-type tumours has been reported to be either EBV positive or MSI (TCGA 2014; Cristescu *et al.* 2015; Kim *et al.* 2016; Ahn *et al.* 2017). Somewhat exceptionally, Cristescu *et al.* (2015) reported MSI in 17% of the diffuse-type tumours. The proportion of diffuse-type tumours with TP53 aberration in study II was quite similar to some other reports (TCGA 2014; Cristescu *et al.* 2015) but notably different from the 54% observed by Kim *et al.* (2016). In addition to methodological differences, some discrepancy in the proportions of different markers may result from molecular variation between tumours derived from ethnically diverse patients.

The criteria for differentiating between oesophageal, gastro-oesophageal and proximally located gastric adenocarcinomas have been a subject of debate. Recent molecular analyses have shown, however, that defining the precise anatomical localisation of the tumours could be regarded as less important than their biological and molecular properties as the oesophageal and gastro-oesophageal adenocarcinomas show strong genetic similarities to CIN gastric adenocarcinomas (TCGA 2017). Thus, in study II, tumours of distal oesophagus, GOJ and cardia were also analysed as a single group of proximally located tumours.

Table 12. The distribution of gastric adenocarcinomas examined with the different molecular markers in study II and in other similar studies.

	Study II[a]		TCGA 2014[b]		Cristescu *et al.* 2015[c]		Kim *et al.* 2016[d]		Park *et al.* 2016[e]	Ahn *et al.* 2017[f]		Díaz del Arco *et al.* 2018[g]
Number of patients (%)	244		295		300		438		993	349		206
Intestinal	190 (78)		196 (66)		150 (50)		98 (22)		518 (52)	199 (57)		111 (54)
Diffuse	54 (22)		69 (23)		142 (47)		130 (30)		475 (48)	147 (42)		71 (34)
Mixed	-		19 (6.4)		8 (2.7)		17 (3.9)		-	3 (0.9)		24 (12)
	Int	Diff	Int	Diff	Int	Diff	Int	Diff		Int	Diff	
EBV pos	17		15			13	3	5		19	7	
	(9.1)	0 (0)	(7.7)	5 (7.2)	4 (3.0)	(9.8)	(3.1)	(3.8)	61 (6.1)	(9.5)	(4.8)	ND
EBV neg	169	52	181		131	119	95	125		180	140	
	(91)	(100)	(92)	64 (93)	(97)	(90)	(96.9)	(96)	910 (92)	(90)	(95)	
MSI	19		48			24	9	5				
	(10)	0 (0)	(25)	6 (8.7)	39 (26)	(17)	(9.2)	(3.8)	114 (11)	26 (7.4)		60 (29)
MSS	167	52	148		111	118	89	125				
	(90)	(100)	(76)	63 (91)	(74)	(83)	(91)	(96)	876 (88)	323 (93)		145 (70)
E-cadherin	3	25	8			8						
aberr	(1.6)	(51)	(4.1)	23 (33)		(3.6)		ND	ND	56 (16)		13 (6.3)
E-cadherin	180	24	188			215						
wt	(98)	(49)	(96)	46 (67)		(96)				293 (84)		193 (94)
TP53	103	10	104			37	66	70				
aberr	(55)	(19)	(53)	19 (28)	47 (31)	(26)	(67)	(54)	622 (63)	221 (63)		35 (17)
TP53 wt	83	42	88		103	105	32	60				
	(45)	(81)	(45)	48 (70)	(69)	(74)	(33)	(46)	371 (37)	128 (37)		171 (83)
EGFR	27		9			9						
amplif	(15)	-	(4.6)	3 (4.3)		(3.5)	ND	ND	49 (4.9)	ND		ND
HER2	24		32			17	18	10				
amplif	(13)	-	(16)	3 (4.3)		(6.7)	(7.3)	(4.1)	49 (4.9)	8 (2.3)		ND

[a]183 intestinal-type tumours and 49 diffuse-type tumours could be analysed with all of the molecular markers. Mixed-type tumours were not included. *EGFR* and *HER2* amplifications were detected among 183 intestinal-type tumours analysed with EGFR and HER2 IHC.

[b]Histological subtype could not been determined for 11 tumours. Among the mixed-type TCGA tumours, *EGFR* amplification was also detected in five tumours and *HER2* amplification in three tumours.

[c]For E-cadherin, the number of mutations is reported for all histological types; data available for 223 tumours. Gene amplification data available for 254 tumours; intestinal- and diffuse-type tumours are combined.

[d]Laurén classification was determined for 245 tumours. EGFR was analysed with IHC, HER2 with IHC and SISH.

[e]Intestinal- and mixed-type tumours were analysed together. EBV information was available for 971 and MMR information for 990 tumours. *EGFR* and *HER2* were analysed with SISH.

[f]The frequencies are presented for all histological subtypes except for EBV, the presence of which is shown separately for intestinal- and diffuse-type tumours. HER2 was analysed with IHC.

[g]One tumour with isolated loss of MSH6 expression was scored inconclusive. Twelve tumours with MSI were classified based on either aberrant E-cadherin or TP53 expression.

aberr, aberration; amplif, amplification; diff, diffuse-type; EBV, Epstein-Barr virus; IHC, immunohistochemistry; int, intestinal-type; MMR, mismatch repair; MSI, microsatellite-stable; MSI, microsatellite instability; ND, not determined; SISH, silver *in situ* hybridisation; wt, wild-type.

Indeed, *EGFR* amplifications were observed to be most common in the tumours of distal oesophagus and GOJ/cardia, which are also the main locations for the CIN subgroup (TCGA 2014, 2017, 2018). In accordance with the features of the CIN subgroup (TCGA 2017), the co-localisation of aberrant TP53 expression together with *EGFR* or *HER2* amplification was also noticed to concentrate in the proximally located intestinal-type tumours. The association between *HER2* amplification and intestinal-type histology and the gastro-oesophageal location of tumours has also been observed by others (Tanner *et al.* 2005; Gravalos *et al.* 2008). *EGFR* amplification was infrequent in the tumours of gastric corpus, and its prevalence in antral/pyloric tumours was intermediate to that in other locations. MSS/TP53- tumours according to the ACRG classification

also typically contain RTK gene amplifications and are predominantly situated in gastric antrum (Cristescu *et al.* 2015).

Among the intestinal-type tumours, *EGFR* amplifications were most common in tumours with EBV negativity, MSS and TP53 aberration (17% of these contained *EGFR* amplification) and second most common (15%) in EBV negative, MSS and TP53 wt tumours. The proportion of *HER2* amplification was equal in both subtypes (15%). In the TCGA material, *HER2* amplifications were more distinctly concentrated in the EBV negative, MSS and TP53 aberrated tumours (29/85, 34%) than in the EBV negative, MSS and TP53 wt tumours (6/45, 13%). The prevalence of *EGFR* amplification in the TCGA material was somewhat smaller in both of these tumour subtypes (11%; 9/85 and 5/45) than in study II. (Cerami *et al.* 2012; Gao *et al.* 2013; TCGA 2014). These discrepancies might be related to methodological differences between the two studies (detecting mutations *vs* protein expression; genomic copy number analysis *vs* ISH) and tumour heterogeneity (GCN analysis in random samples *vs* selected areas).

Patients with MSI tumours had longer overall survival in comparison to patients with MSS tumours both in the univariate and multivariate analysis in study II, which is consistent with earlier observations (Cristescu *et al.* 2015; Park *et al.* 2016). The study by Huang *et al.* (2019) could show that patients with either EBV positive or MSI-H tumours had a favourable prognosis in comparison to patients with diffuse-type tumours. Especially, patients with EBV positive lymphoepithelioma-like carcinoma were noticed to have the most indolent disease.

A potential source of error with molecular classifications in general is the usage of surrogate markers. For example, IHC stainings for MSI and TP53 were used in study II and in many other studies instead of direct mutational analyses. In particular, some studies have used only one MSI marker (MLH1) (Ahn *et al.* 2017), while others have used all four markers (Kim *et al.* 2016; Park *et al.* 2016; Díaz del Arco *et al.* 2018). Additional limitations of the study include the TMA method itself because it multiplies the potential selection bias already present in the histological sampling of tumours and may lead to erroneous interpretations. In fact, all studies analysing tissue samples taken at one time point and comprising only a small fraction of the tumour are susceptible to bias caused by spatial and temporal intratumoural heterogeneity (Bedard *et al.* 2013; de Bruin *et al.* 2014; Zhang *et al.* 2014). Thus, the usage of only small tissue cores per each tumour for IHC and ISH analyses may result in over- or underestimating protein expression or RNA transcription levels. Especially gene amplifications may be present in a scattered pattern (Yoon *et al.* 2012).

This bias could partly be alleviated by including several tissue cores from each tumour into the TMA or, in general, by profiling multiple samples from a single tumour (Bedard *et al.* 2013). Also for this reason, whole slide sections were used for detecting *EGFR* and *HER2* GCN in studies I – II. In addition, repeated analyses of circulating tumour cells (CTC) or cell-free circulating tumour DNA (ctDNA) might offer a way to detect emerging genomic aberrations when monitoring treatment response or disease recurrence (Bedard *et al.* 2013). With regard to survival analyses, potential weaknesses in study II are similar to those in study I, that is, the heterogeneity in the treatment regimens and the inclusion of patients presenting all stages of disease.

6.3 CIP2A and radiosensitivity (III)

There is limited information about the association between CIP2A expression and clinical response to (chemo)radiotherapy in rectal cancer patients. In previous studies, CIP2A has been demonstrated to promote resistance to irradiation and other DNA-damaging therapies in intestinal progenitor cells (Myant *et al.* 2015). Similarly, elevated CIP2A expression contributes to radioresistance in head and neck squamous cell carcinoma (HNSCC) through increased cell proliferation and resistance to apoptosis (Ventelä *et al.* 2015), while ovarian (Böckelman *et al.* 2011) and breast (Laine *et al.* 2013) tumours negative for CIP2A respond favorably to cancer therapies. It has been postulated that a stem cell transcription factor, Oct4, could act both as a regulator of stem cells and as a driver of CIP2A expression, and both of these functions could contribute to radioresistance. IHC positivity for both Oct4 and CIP2A has also been associated with poor histological differentiation. (Ventelä *et al.* 2015). Moreover, suppression of *CIP2A* transcription by siRNA results in increased radiosensitivity in cervical squamous cell carcinoma and hepatocellular carcinoma cell lines (Huang *et al.* 2012).

In study III, the hypothetical relationship between CIP2A expression level and radiation response was examined in the context of rectal cancer using the previously collected sample material comprised of patients treated with either short- or long-course RT or not treated with RT at all. As the number of patients treated with long-course (C)RT was limited, supportive studies were carried out *in vitro* by exposing colorectal cancer cells treated with *CIP2A* siRNA to different doses of irradiation.

Among patients treated with long-course (C)RT, low-CIP2A-expressing tumours responded better to preoperative treatment than high-CIP2A-expressing tumours. This is in agreement with previous findings regarding HNSCC (Ventelä *et al.* 2015) and might indicate that the more responsive tumours are those with less stem cell-like properties. However, no significant association was observed between CIP2A expression and tumour differentiation grade.

CIP2A expression could be evaluated only in posttreatment tumour samples because an adequate number of representative pretreatment biopsies were not available, which is a source of uncertainty in interpreting the results. Nevertheless, in support of the finding, reduction of *CIP2A* transcription by siRNA was observed to sensitise colorectal cancer cells to irradiation and decrease their survival. Another *in vivo* study has reported that irradiation did not markedly affect CIP2A transcription or expression in mouse testis during the 144-hour observation period (Ventelä *et al.* 2015). This would be in accordance with the hypothesis that initially low CIP2A expression could associate with a more pronounced response to preoperative (C)RT. In order to confirm the possible association between CIP2A expression and (C)RT treatment response, tissue samples from rectal cancer patients obtained both before and after the treatment should be compared to each other. Moreover, it would be interesting to study whether, in addition to the pretreatment CIP2A expression level of the tumour, the magnitude of change in CIP2A expression during (C)RT affected the radiation response.

Even though low CIP2A expression was found to be more common than high CIP2A expression among the most invasive tumours, low CIP2A expression level was still associated with better treatment response after long-course (C)RT. Low CIP2A expression level also associated with higher 36-month DSS rate of the patients in categorical analysis. The multivariate analysis also suggested that low CIP2A expression level could be an independent prognostic factor for increased DSS.

A prognostic role of CIP2A in various cancers is supported by several previous studies, which have found an association between low CIP2A expression and increased survival (Khanna & Pimanda 2016). Whether or not CIP2A has a role as a prognostic biomarker in colorectal cancer is somewhat uncertain. In one study, CIP2A expression level was not associated with five-year DSS of patients with either colon or rectal cancer. (Böckelman *et al.* 2012). In contrast, high *CIP2A* mRNA levels (Wiegering *et al.* 2013) or CIP2A overexpression (Chen *et al.* 2015) have been associated with reduced overall survival (OS) of colorectal cancer patients. The study by Chen *et al.* (2015) included patients with *KRAS* wt tumours after surgical treatment of liver metastases.

In addition to the inclusion of only posttreatment samples, weaknesses of study III include the relatively small number of patients treated with long-course (C)RT, the lack of clear-cut survival differences between patients with either high or low CIP2A expression levels and the relatively weak overall CIP2A IHC staining intensity, which may make it difficult to consistently differentiate between weakly, moderately and strongly stained areas both within the same tumour and between different tumours. It is also possible that the RT has some unspecific effects on CIP2A expression levels unrelated to CIP2A function or on tumour tissue in general, which could affect the CIP2A staining intensity and thus bias the interpretation of the IHC stainings.

6.4 Primary and recurrent colorectal tumours (IV)

Anti-EGFR antibodies are recommended for the treatment of metastatic colorectal cancer in patients with *RAS* wt tumours (Atreya *et al.* 2015; Sorich *et al.* 2015). As an additional predictive factor, *EGFR* GCN has also been demonstrated to have an impact on the anti-EGFR treatment response (Moroni *et al.* 2005; Sartore-Bianchi *et al.* 2007). Specifically, *EGFR* GCN $\geq$ 4.0 in primary colorectal adenocarcinomas has been associated with a favourable anti-EGFR treatment response in patients with *RAS* wt tumours (Ålgars *et al.* 2011; Ålgars *et al.* 2014; Ålgars *et al.* 2017). In contrast to gastric cancer, elevated *EGFR* GCN in colorectal cancer is observed to be related to Chr7 polysomy, while true gene amplifications are rare (Ålgars *et al.* 2011).

However, little is known about the potential effects of anti-EGFR therapy on *EGFR* GCN in recurrent disease. Some studies have compared EGFR expression levels in primary and corresponding metastatic colorectal tumours (Loupakis *et al.* 2009), but comparative studies detecting *EGFR* GCN both in primary colorectal tumours and their metastases are scarce, and even fewer have made comparisons among patients treated with anti-EGFR therapy. In these studies,

EGFR GCN has been reported to be discordant in 5 – 13% of patients. However, these analyses have been performed with FISH and have not taken into account the *KRAS* status of the tumours. (Cappuzzo et al. 2008; Park et al. 2011). In the study by Molinari *et al.* (2009), the *EGFR* FISH pattern remained stable between the primary and metastatic tumour during the anti-EGFR treatment of the *KRAS* wt patients (Molinari *et al.* 2009).

In study IV, *EGFR* GCN was observed to decrease between the primary and recurrent tumours among the anti-EGFR treated patients but not among patients receiving other treatment regimens after primary surgery. None of the patients whose recurrent tumour showed *EGFR* GCN increase ≥ 1.0 were treated with anti-EGFR antibodies before obtaining the sample from the recurrent tumour. Similarly, the shift from primary tumours with *EGFR* GCN ≥ 4.0 to recurrent tumours with GCN < 4.0 occurred more often among the anti-EGFR-treated patients. *EGFR* GCN increase between the primary and metastatic tumour was only observed in patients not treated with anti-EGFR antibodies also in the study by Molinari *et al.* (2009).

The association between anti-EGFR treatment and GCN decrease became more evident when analysing only patients with distant metastases. This observation may be related to the phenomenon that cancer cells are known to accumulate molecular changes in order to acquire metastatic capability, whereas local recurrences tend to remain genetically similar to the primary tumour (Hanahan & Weinberg 2011; Vakiani *et al.* 2017). In contrast, some evidence shows that primary sporadic colorectal tumours might have more similar mutational profiles to their liver metastases than primary tumours with CIMP or Lynch syndrome –associated features (Hühns *et al.* 2018). This implies that discrepancies between the primary and metastatic tumour might result rather from the genetic properties of the primary tumour than from the metastatic process itself.

The tendency to *EGFR* GCN decrease during anti-EGFR therapy is interesting given the predictive value of *EGFR* GCN ≥ 4.0 in the primary tumour with regard to anti-EGFR antibodies (Ålgars *et al.* 2011; Ålgars *et al.* 2014; Ålgars *et al.* 2017). The specific mechanism for this predictive association is unknown, whereas the constantly active RAS–RAF–MAPK signalling pathway in *RAS* mutated tumours is known to be responsible for the resistance to anti-EGFR therapies (Ciardiello et al. 2008). In study IV, the positive predictors, *KRAS* wt and *EGFR* GCN ≥ 4.0, tended to occur in the same tumours, which has also been observed by others (Personeni *et al.* 2008; Sanchez-Vega *et al.* 2018). Higher *EGFR* GCN was also more often detected in lymph node positive (stage III) than in lymph-node negative (stage I – II) tumours. Thus, increased *EGFR* GCN might indicate tumours with higher invasive potential, which is in accordance with the known cancer promoting effects of EGFR signalling (Ciardiello & Tortora 2008), and could further underline those patients who especially might benefit from the anti-EGFR treatment.

One explanation for the observed *EGFR* GCN decrease in study IV could be that a selective pressure exerted by the antibody treatment leads to the survival of cancer cells with smaller GCN. In general, this kind of selective pressure has been proposed as one of the mechanisms that leads to the survival of cancer cell subclones with genetic properties protecting them against the antibody and thus results in acquired resistance (Misale *et al.* 2014). Acquired resistance to anti-EGFR

therapies is known to occur in a substantial proportion of patients, and several studies have been conducted in order to unravel the mechanisms contributing to this process (Diaz *et al.* 2012; Misale *et al.* 2012; Misale *et al.* 2014; Bertotti *et al.* 2015; Bronte *et al.* 2015). Amplifications of RTK genes such as *HER2* and *MET*, *KRAS* mutations and amplifications as well as *NRAS* and *BRAF* mutations have been observed to associate with acquired anti-EGFR antibody resistance (Yonesaka *et al.* 2011; Bardelli *et al.* 2013; Pietrantonio *et al.* 2017).

Whether *EGFR* GCN decrease during anti-EGFR therapy has any effect on clinical treatment response could not be properly examined in study IV due to the limited number of patients. It could be hypothesised that the exposure to anti-EGFR antibodies could result in decreased *EGFR* GCN and thereby contribute to acquired treatment resistance. However, potential effects of *EGFR* GCN change on acquired resistance during disease progression have not been reported. The few studies reporting any findings regarding *EGFR* GCN change in relation to anti-EGFR treatment response have had too small a study population to yield clinically useful information (Molinari *et al.* 2009).

Potential weaknesses of study IV include the relatively small number of primary – metastatic tumour pairs and especially the limited number of patients treated with anti-EGFR antibodies between sampling the primary and metastatic tumours. In addition, the heterogeneity in the locations of the metastatic tumours and in the administered therapies as well as the usage of biopsy material for the *EGFR* GCN analyses may also add some uncertainty in the interpretation of the results.

Cancer genomes are not only complex but also diverse even among tumours arising from the same cell type. This inter-tumour heterogeneity can make it difficult to predict how an individual tumour will progress over time or respond to different therapies. (Eifert *et al.* 2012). Nevertheless, the large-scale characterisation studies, combined with histological data, have been able to elucidate different carcinogenic mechanisms, untangle various signalling pathways underlying the malignant processes, and provide some information about the prognosis and suitable treatments for individual patients (Liu *et al.* 2018).

Most of the data collected and analysed, for example by the TCGA project, is from primary tumours. Subsequent large-scale projects, such as the Cancer Moonshot Initiative and the Human Tumor Atlas Network, aim to characterise not only metastatic tumours but also premalignant neoplasms, and they will also conduct analyses regarding treatment sensitivity and resistance (Ding *et al.* 2018). In order to yield clinically useful applications, forthcoming studies also need to address the difficulties related to interpreting the findings from various genome-wide analyses as well as from an enormous amount of studies examining the significance of single proteins or mutations. It needs to be determined whether any recurring individual alteration is functionally important to the tumour (driver *vs* passenger mutations, for example) and if so, whether the alteration is required only during carcinogenesis or even in the fully developed tumour (Eifert *et al.* 2012).

To address this problem with data overload, several different approaches have been developed that utilise different computational and screening methods as well as techniques based on genome-wide comparisons. However, more advanced methods will be required to characterise individual tumours in order to acknowledge the complex interactions between different molecules as well as the influence on carcinogenesis exerted by the tumour microenvironment or gut microbes. (Eifert *et al.* 2012). It has been suggested that transcriptome-based, rather than mutation-based, analyses might provide more functionally relevant information by allowing for acknowledging also the effects of various signals from the tumour surroundings (Bijlsma *et al.* 2017). Furthermore, an important source of information is the medical records, the integration of which with the biological data derived from tumour samples is a task suitable for organisations such as the different biobanks. Regardless, the unique properties of cancer cells will still continue to challenge the attempts to eradication, and it may be that in the future the management of at least some cancer types will concentrate on tumour containment rather than total annihilation.

7 CONCLUSIONS

Based on the studies included in this thesis, the following conclusions can be made:

1. *EGFR* amplifications in oesophagogastric cancers are concentrated in the intestinal-type tumours in which they are not uncommon. Amplified *EGFR* also associates with decreased survival of these patients. Including *EGFR GCN* analysis in prospective trials warrants further consideration as it could be used to identify patients with adverse prognosis and to improve the specificity of patient selection when investigating anti-EGFR therapies in the treatment of intestinal-type oesophagogastric adenocarcinomas.

2. Oesophagogastric adenocarcinomas can be classified into biologically and clinically relevant subgroups by straightforward methods based on the Laurén classification together with immunohistochemistry and *in situ* hybridisation. In future clinical trials, the application of new classification algorithms combining both histological and molecular information will be necessary in order to improve the clinical benefit obtained from new targeted therapies.

3. Low CIP2A protein expression level in post-treatment tumours is associated with a favourable response to long-course (C)RT in rectal cancer patients. In support of the finding, suppression of *CIP2A* expression by siRNA increases the radiosensitivity of colorectal cancer cells *in vitro*. Low CIP2A expression level might also prognosticate increased survival of patients after long-course (C)RT.

4. *EGFR* GCN tends to decrease between the primary and recurrent tumours among those colorectal cancer patients who have been treated with anti-EGFR antibodies after primary surgery. In contrast, among patients not exposed to anti-EGFR treatment, *EGFR* GCN of the recurrent tumour tends to stay stable or even increase in comparison to the *EGFR* GCN of the primary tumour. High *EGFR* GCN is associated with *KRAS* wt status and lymph node positivity (stage III) in primary colorectal tumours.

The biomarkers examined in this thesis have established functions as key participants in diverse intracellular signalling and regulatory pathways involved in the pathogenesis of malignant tumours, and some of them are used as predictive biomarkers in their special fields of application. As it becomes increasingly evident that even a particular type of cancer located in a specific organ and with defined histological features may behave and respond to cancer treatments in divergent ways in different patients, the molecular characterisation of tumours becomes ever more important. The challenge is and will be to distinguish the functionally meaningful information among all the data acquired from both the small-scale studies examining single biomarkers and the large-scale studies analysing hundreds or thousands of tumour samples and to convert it into clinically relevant knowledge.

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