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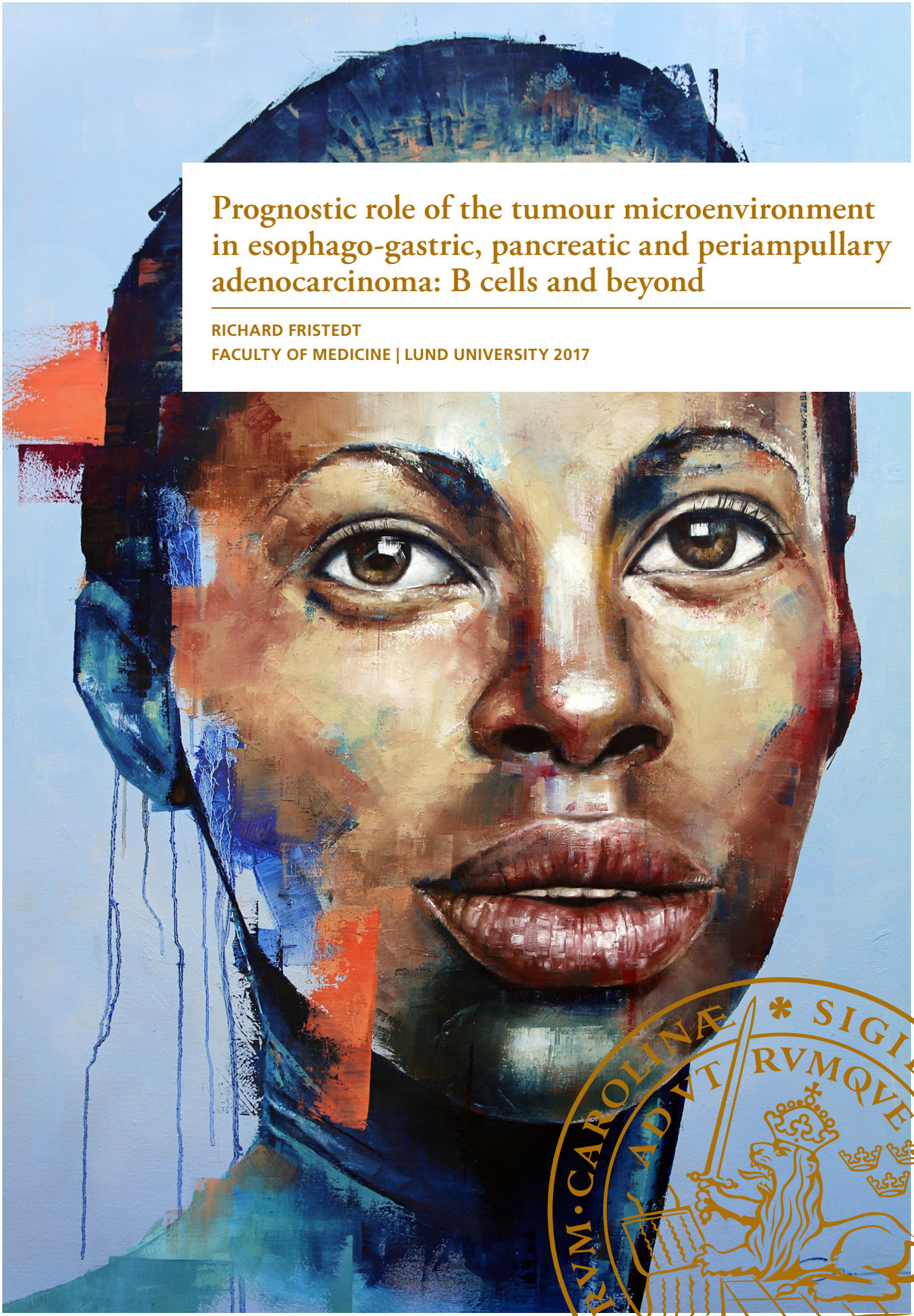

Prognostic role of the tumour microenvironment in esophago-gastric, pancreatic and periampullary adenocarcinoma: B cells and beyond

RICHARD FRISTEDT
FACULTY OF MEDICINE | LUND UNIVERSITY 2017

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DOCTORAL DISSERTATION

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Title and subtitle: Prognostic role of the tumour microenvironment in esophago-gastric, pancreatic and periampullary adenocarcinoma: B cells and beyond

Abstract: Cancer development depends on cells aquiring a skillset of limitless growth and finally invasive properties to allow for metastasis, as summarised by the six hallmarks of cancer. Recently four more hallmarks have been added: genomic instability, deregulating cellular metabolism, tumour-promoting inflammation and avoiding immune destruction. This thesis focuses on the latter two: immune system and cancer cell interactions. Oesophageal and gastric cancer present late and only 25% and 30%, respectively, will be subjected to curative treatment. Periampullary cancer includes: pancreatic cancer (PDA), biliary, ampullary and duodenal cancer. Periampullary cancer is often dichotomised into intestinal (I)-type and pancreatobiliary (PB)-type. Overall survival for PDA is 5%, whilst I-type periampullary cancer has a better prognosis.

The aim of this thesis was to analyse the expression of the polymeric immunoglobulin receptor (pIgR), and the abundance of B cells and plasma cells in oesophago-gastric, pancreatic and periampullary adenocarcinoma, and to explore their potential prognostic and predictive value.

To this end, two patient cohorts were used The first cohort encompassed 174 patients with surgically resected oesophago-gastric adenocarcinoma, who had not received neoadjuvant treatment. The second cohort encompassed 175 patients treated with pancreatoduodenectomy for pancreatic and periampullary adenocarcinoma. Tissue microarrays were constructed from primary tumours and selected metastases and immunohistochemistry (IHC) was applied with validated antibodies against pIgR, $CD20^+$ B cells, $CD138^+$ and $IGKC^+$ plasma cells. Light microscopy and/or digital image analysis was used for detection. In addition, *PIGR* gene expression was assessed by quantitative polymerase chain reaction.

High expression of pIgR, a transporter protein that transcytoses antibodies across epithelial cells to mucosal surfaces and has been associated with improved survival in many cancer types, was associated with a prolonged time to recurrence (TTR) and overall survival (OS) in oesophageal and gastric junction adenocarcinoma. Furthermore, reduced expression of pIgR was associated with decreased OS in I-type periampullary adenocarcinoma.

In oesophageal and gastric adenocarcinoma, high density of $IGKC^+$ plasma cells was associated with a prolonged TTR and OS. No association was seen for $IGKC^+$ plasma cells in I-type or PB-type periampullary adenocarcinoma but a, high density of $CD20^+$ B cells was associated with both a prolonged TTR and OS. In addition, there was a significant treatment interaction in relation to OS between high density of $CD20^+$ B cells and adjuvant chemotherapy in patients with PB-type periampullary adenocarcinoma.

In conclusion, high pIgR expression is associated with improved outcomes in oesophageal, GE junction and I-type periampullary adenocarcinoma. $IGKC^+$ plasma cells are associated with a prolonged survival in esophageal and gastric adenocarcinoma. $CD20^+$ B cells are associated with a prolonged survival in I-type and PB-type periampullary adenocarcinoma, and, possibly, with an improved response to adjuvant chemotherapy in PB-type tumours. The underlying mechanistic principles remain to be deciphered.

Key words: PDA, periampullary, oesophago-gastric, adenocarcinoma, pIgR, B cells, plasma cells, prognosis, IHC

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Science bestowed immense new powers on man, and, at the same time, created conditions which were largely beyond his comprehension. *(Sir Winston Churchill)*

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Original papers

The basis for this thesis is the following papers, referred to by the Roman numerals throughout the text:

I. Fristedt R, Gaber A, Hedner C, Nodin B, Uhlén M, Eberhard J, Jirström K. Expression and prognostic significance of the polymeric immunogobulin receptor in esophageal and gastric adenocarcinoma. *Journal of Translational Medicine* **12**:83 (2014)

II. Fristedt R, Elebro J, Gaber A, Heby M, Yudina Y, Nodin B, Uhlén M, Eberhard J, Jirström K. Reduced expression of the polymeric immunoglobulin receptor in pancreatic and periampullary adenocarcinoma signifies tumour progression and poor prognosis. *PLOS ONE* **9**:e112728 (2014)

III. Fristedt R, Borg D, Hedner C, Berntsson J, Nodin B, Eberhard J, Micke P, Jirström K. Prognostic impact of tumour-associated B cells and plasma cells in oesophageal and gastric adenocarcinoma. *Journal of Gastrointestinal Oncology* **7**: 848-859 (2016)

IV. Fristedt R, Lundgren S, Elebro J, Karnevi E, Nodin B, Warfvinge C F, Micke P, Eberhard J, Tingstedt B, Jirström K. The prognostic role of B cells and plasma cells in pancreatic and periampullary adenocarcinoma differs by adjuvant chemotherapy and morphological type. (Submitted)

Related papers not included in the thesis:

- Elebro J, Heby M, Gaber A, Nodin B, Jonsson L, Fristedt R, Uhlén M, Jirström K, Eberhard J. Prognostic and treatment predictive significance of SATB1 and SATB2 expression in pancreatic and periampullary adenocarcinoma. *Journal of Translational Medicine* **12**:289 (2014)

- Borg D, Hedner C, Gaber A, Nodin B, Fristedt R, Jirström K, Eberhard J, Johnsson A. Expression of IFITM1 as a prognostic biomarker in resected gastric and esophageal adenocarcinoma. *Biomarker Research* **4**:10 (2016)

Abbreviations

5-FU	5-fluorouracil
ADCC	Antibody dependent cell-mediated cytotoxicity
AF	Activator protein
APC	Antigen presenting cells
BE	Barrett's oesophagus
BLIMP1	B lymphocyte induced maturation protein 1
B_{reg}	B regulatory cells
CA19.9	Carbohydrate antigen 19.9
CD	Cluster of differentiation
CDKN2A	Cyclin dependent kinase inhibitor 2A
CDR	Complementarity determining region
CRT	Classification and regression tree
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen 4
DBA	Diaminobenzidene
DC	Dendritic cells
DNA	Deoxyribonucleic acid
EBRT	External beam radiotherapy
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
ER	Estrogen receptor
ESPAC	European study group for pancreatic cancer

Fab	Fragments of antigen binding
FFPE	Formalin-fixed paraffin embedded
FOLFIRINOX	Folinic acid, 5-FU, irinotecan, and oxaliplatin
FOLFOX	Folinic acid, 5-FU and oxaliplatin
GEJ	Gastroesophageal junction
GEMCAP	Gemcitabine and oral 5-FU prodrug capecitabine
GERD	Gastro oesophageal reflux disease
GIST	Gastrointestinal stromal tumour
HDGC	Hereditary diffuse gastric cancer
HER2	Human epidermal growth factor receptor 2
HLA	Human leucocyte antigen
HNPCC	Hereditary nonpolyposis colorectal carcinoma
HP	Helicobacter Pylori
HPA	Human protein atlas
I-type	Intestinal type
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGKC	Immunoglobulin kappa C
IHC	Immunohistochemistry
IM	Intestinal metaplasia
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
J-chain	Joining-chain
JAK	Janus kinase
KRAS	Kirsten rat sarcoma viral oncogene homolog
LMP	Low molecular mass protein
MAGIC	Medical research council adjuvant gastric infusional chemotherapy

MDSC	Myeloid derived suppressor cells
MHC	Major histocompatibility complex
MSI	Microsatellite instability
NET	Neuroendocrine tumour
NK cells	Natural killer cells
OS	Overall survival
PAMP	Pathogen-associated molecular patterns
PB-type	Pancreatobiliary type
PD-1	Programmed death 1
PDA	Pancreatic ductal adenocarcinoma
PDL-1	Programmed death ligand 1
pIgR	Polymeric immunoglobulin receptor
PrEST	Protein epitope signature tag
PRSS1	Protease serine 1
qPCR	quantitative polymerase chain reaction
RCC	Regionala Cancercentrum i samverkan
RFS	Recurrence free survival
SCC	Squamous cell carcinoma
SPINK1	Serine peptidase inhibitor Kazal type 1
STAT	Signal transducer and activator of transcription
SWOG	Southwest oncology group
TAM	Tumour associated macrophages
TGF-β	Transforming growth factor beta
Th cells	T helper cells
TI	Thymus-independent
TIL	Tumour infiltrating lymphocytes
TMA	Tissue microarray
TME	Tumour microenvironment
TNF-α	Tumour necrosis factor alpha

TNM	Tumour Node Metastasis
ToGA	Trastuzumab for gastric cancer
TRAIL	TNF related apoptosis inducing ligand
T_{reg}	T regulatory cells
TTR	Time to recurrence
URF	Upstream regulatory factor
VEGF	Vascular endothelial growth factor
VEGFR2	VEGF receptor 2

Introduction

Cancer incidence has been predicted to increase by 20% over the next decade accounting for a total of 1.9 million annual cases in America (1), largely due to an ageing population and other demographic changes and over the past two decades cancer treatment costs have doubled (2).

Around 95% of all human cancers are linked to somatic mutations, leaving 5% of hereditary cancer (3). Epidemiological studies have found the aetiology of cancer to be linked to smoking (30%), diet (35%), infections (18%), obesity (17%), pollution and radiation (7%) (4). Lung cancer is the most common type of cancer, both with regards to incidence and cancer-related death, followed by (incidence) breast, colorectal, prostate, gastric and hepatic cancer, these six cancers accounting for 55% of the global incidence burden (5).

Cancer development is in essence cells acquiring a skillset that will enable them to grow without limitation and then finally spread via lymph or blood vessels to distant organs, i.e. metastasise. These skills are sometimes called the "six hallmarks of cancer" and the concept has, since its introduction in 2000 by Hanahan et al., been widely used (6). The original six features of the concept (self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis) were further supplemented in 2011 by four emerging hallmarks – genomic instability, deregulating cellular metabolism, tumour-promoting inflammation and avoiding immune destruction (7). This thesis focuses on the latter two.

The immune system

Normal immune system

The immune system provides the defence for human homeostasis by identifying and eliminating foreign elements such as viruses, bacteria and mutated cells, and is divided into the innate and adaptive immune responses. The innate immune cells consist of neutrophils, natural killer (NK) cells, basophils, mast cells, eosinophils, monocytes, macrophages and dendritic cells (DC), which provide a non-specific and broad protection. The use of pattern recognition receptors, e.g. toll-like receptors that recognise pathogen-associated molecular patterns (PAMPs), is one way by which innate immune cells detect and eliminate pathogens. Other elements of innate immunity are epithelial cell barriers, complement, chemical barriers and cytokines (8).

The adaptive immune response, on the other hand, sometimes further subclassified into cell-mediated and humoral, recognizes the foreign elements with a high specificity and needs to be activated by means of antigen processing and subsequent antigen presentation on MCH molecules, in order to recognise and eliminate foreign antigens. Antigen presentation is best achieved by expert antigen presenting DCs but macrophages and B cells also fulfil this task (8).

Under acute inflammatory conditions, antigen presenting cells (APC) process and present foreign antigens on MHCII molecules to $CD4^+$ helper T (Th) cells and on MHCI molecules to $CD8^+$ T cells. The activation of $CD8^+$ T cells is co-stimulated by $CD4^+$ Th cytokines and eventually leads to cytotoxic activity of $CD8^+$ T cells. B cells also become activated and undergo differentiation to become antibody-producing plasma cells with specific antibodies that opsonise pathogens for phagocytosis via Fc-binding receptors or neutralise them via exclusion (9). Antibodies also activate complement-mediated elimination of pathogens. When this has been achieved and homeostasis is restored, the inflammatory immune response dissolves (8).

If, however, the immune system cannot resolve the infection or eliminate the mutated cells, a chronic inflammation often with both pro- and anti-inflammatory properties takes over. This situation is optimal for the process of carcinogenesis (10).

The inflammatory tumour microenvironment

The theory of immunosurveillance, i.e. that the immune system is providing the host with a defence against neoplastic transformation, has been refined and supplemented by Schreiber and colleagues who introduced the concept of immunoediting (11). It states that the immune pressure of an immunocompetent host will sculpt the emerging tumour cells, thus selecting the clones with reduced immunogenicity, either through poor recognisability or by having acquired immunosuppressive functions (11). Immunoediting is, furthermore, divided into three stages: elimination, equilibrium and escape.

The interferon (IFN)-γ signalling pathway has been proposed as being key to the elimination stage of immunoediting (12). The cytokine IFN-γ is secreted by T, NK and NKT cells (13), and the pathway involves binding of IFN-γ to the IFN-γ receptor, which is present in almost all human cells. The activated receptor is then coupled with Janus kinase 1 (JAK1), JAK2 and the transcription factor signal transducer and activator or transcription (STAT) 1 (14), leading to the up-regulation of antigen expression on the tumour cell surface by means of MHCI. IFN-γ is however also essential for the presentation of antigens on MHCII molecules (12).

Immune evasion (intrinsic)

Tumour cells have been shown to impede their detection by means of diminishing cell surface expression of MHC/HLA1 molecules (15). Proteins involved in neo-antigen processing and presentation on HLA1 molecules e.g. transporter associated with antigen processing 1 (TAP1), low molecular mass protein 2 (LMP2), LMP7 and tapasin have also been shown to be down-regulated with the result that $CD8^+$ T cells are unable to recognise the tumour cells (15, 16). The down-regulation of the IFN-γ receptor by tumour cells, important for induction of neo-antigen presenting proteins, is yet another example of this (12). Furthermore, overexpression of the serine-protease inhibitor PI9 blocks granzyme-B-perforin pathway and decoy receptors for TNF (tumour necrosis factor) related apoptosis inducing ligand (TRAIL), both make the tumour cells resistant to $CD8^+$ T cell mediated killing (17, 18).

Immune subversion (extrinsic)

Tumour cells actively subvert and suppress the immune system e.g. by indoleamine 2,3-dioxygenase (IDO) which is constitutively expressed in many

human cancers. Tumour cells can reduce the T cell stimulant tryptophan and generate kynurenines that are toxic to $CD8^+$ T cells (19). In addition, IDO blocks $CD8^+$ T cell proliferation and can induce apoptosis in $CD4^+$ T cells (20). Another death-receptor pathway, CD95-CD95L, also known as FAS-FAS ligand, can also be expressed by tumour cells and induce apoptosis in $CD8^+$ T cells (21).

Overall the polarisation of the tumour microenvironment (TME) into a chronic inflammatory or Th_2 state is achieved by the production and secretion of cytokines e.g. IL-4, IL-6, IL-10 and TGF-β (22). The immune cell profile of the Th_2 skewed TME is composed of tumour associated macrophages (TAM), myeloid derived suppressor cells (MDSC), B regulatory cells (B_{reg}) and T regulatory cells (T_{reg}), immature DCs, favouring inactivation and depression of immune effector cells and thereby promoting tumour development and progression (10).

The Th_2 TME can be created by tumours secreting thymic stromal lymphopoietin prompting DC to produce OX40 ligand, which in turn drives $CD4^+$ T cells to diversify into Th_2 cells (23). Other Th_2 polarising tumour-derived factors are granulocyte-macrophage colony stimulating factor (GM-CSF) and CSF-1. TNF-α largely seen as Th_1 cytokine, can also be secreted by Th_2 cells which also produce IL-4 and IL-13 (24) and together with GM-CSF and CSF-1 polarise macrophages into a TAM state. M1 macrophages and TAM produce inducible nitric oxide synthase (iNOS) but the latter also secrete arginase, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF), in addition to the immunosuppressive cytokines IL-10 and TGF-β (25, 26), thereby blocking CTL mediated tumour elimination and promoting tumour growth (27, 28). IL-13 can also be produced by NKT cells, making MDSC to also produce TGF-β that upregulates T_{reg} (29). Furthermore, TAMs promote epithelial mesenchymal transition (EMT) (30). The improved understanding of TAM signalling pathways have opened up for novel therapeutic approaches to circumvent the immunosuppression and they involve CSF-1 receptor inhibitors and inactivating TAM monoclonal antibody (mAb) (30, 31).

Immune checkpoint inhibitors

Over the past several years there have been great advances in cancer immunotherapy, aiming at lifting the suppressive effect of immune checkpoint inhibitors, and thereby reinstating the tumouricidal capacity of $CD8^+$ T cells (32). The cytotoxic T lymphocyte antigen 4 (CTLA-4) is present on T cells and bind to B7, the same ligand as for the co-stimulatory CD28. By competitively binding to B7, CTLA inhibits the proliferation of activated T cells and with it $CD8^+$ T cell cytotoxicity (33). Two humanised mAb of immunoglobulin (Ig) G2 type are available, ipilimumab and tremelimumab, which prevents CTLA-4 from binding

B7 (34). Similarily, the programmed death 1 (PD1), transiently expressed on activated B cells, T cells, and myeloid cells and its ligand PD1-ligand (PD1-L) constitutively expressed on e.g. APC or tumour cells, is another inhibitory ligand:receptor pair (35). Pembrolizumab and nivolumab are humanised IgG4 monoclonal antibodies that bind PD1 and atezolizumab, pidilizumab and durvalumab bind to PD1-L (36), thereby preventing their co-inhibition of $CD8^+$ T cells with a subsequent increase in CTL antitumour response (9).

Investigated biomarkers

Polymeric immunoglobulin receptor

The polymeric immunoglobulin receptor (pIgR) is part of the immunoglobulin superfamily and is a transmembrane protein found on mucosal surfaces of the gastrointestinal tract and exocrine glands (37). It binds to dimeric IgA at the joining (J)-chain, which is a small polypeptide segment, and to a lesser extent to the J-chain of pentameric IgM, on the basolateral aspects of epithelial cells. It is then transcytosed through the cell onto the apical part of the epithelium, where its extracellular part is cleaved off to form the secretory component (SC) (38). The SC can remain bound to or detach from dimeric IgA, the former state protecting IgA from proteolytic degradation (39). IgA then binds to infectious agents such as bacteria and viruses to opsonise or complex-bind them and thereby preventing them from passing through the epithelial lining. Figure 1 illustrates pIgR Ig transport.

pIgR expression is regulated by upstream regulatory factor (USF) 1 and 2 and activator protein (AP) 2 (40) and dysregulation of USF and AP2 have been shown to lead to downregulation of pIgR expression in NSCLC (41). Bacterial and viral infections can upregulate pIgR expression via proinflammatory cytokines such as IL-1, TNF and IFNγ that induce interferon regulatory factor (IRF) 1 and IL-4 that induces STAT-6 (42). There is also evidence of post-transcriptional regulatory mechanisms (38).

Prior to this thesis work, the expression and prognostic significance of pIgR has been examined in several types of cancer. High pIgR expression has been linked to a favourable prognosis in epithelial ovarian cancer (43) and bladder cancer (44). Similarily, low pIgR expression has been linked to poor prognosis in colorectal cancer (45) and with progression from colon adenoma to carcinoma (46). Moreover, loss of pIgR expression has been shown to correlate with tumour progression in non-small cell lung cancer (NSCLC) (41), and pIgR negativity was found to be significantly associated with lymph node metastasis in oesophageal and gastro-oesophageal junction (GEJ) adenocarcinomas (47). One study, however, on hepatitis B derived hepatocellular carcinoma, demonstrated a link

between high pIgR expression and greater metastatic potential and poor prognosis (48).

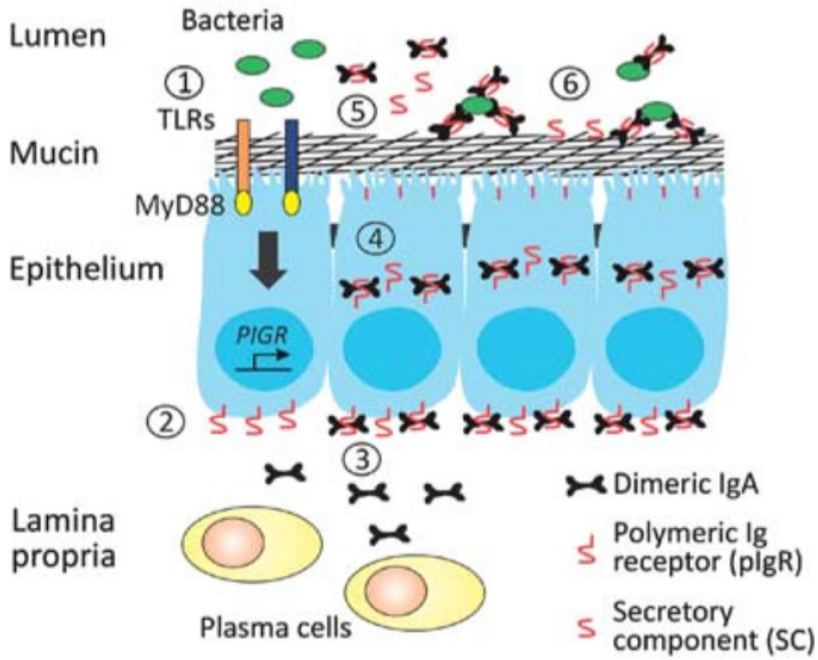

Figure 1. Regulation of production of secretory IgA (SIgA) and free secretory component (SC) in mucosal epithelia. The single-layered epithelium is covered with a thick mucus layer that physically excludes members of the resident microbiota. (1) Stimulation of epithelial Toll-like receptors (TLRs) with microbial-associated molecular patterns activates MyD88-dependent signaling pathways that trigger upregulation of *PIGR* gene transcription. Activation of TLRs may also stimulate polymeric immunoglobulin receptor (pIgR) transcytosis. (2) Newly synthesized pIgR molecules are sorted to the basolateral surface of epithelial cells. (3) Dimeric IgA secreted by lamina propria plasma cells binds to pIgR on the epithelial membrane and stimulates transcytosis. (4) IgA-bound and unoccupied pIgR are transcytosed through epithelial cells. (5) Proteolytic cleavage of pIgR at the apical surface releases SIgA and free SC. (6) Binding of SIgA and SC to luminal bacteria promotes association with the mucin layer and biofilm formation, and prevents direct access of bacteria to the epithelial surface. Reprinted by permission from Macmillan Publishers Ltd: Mucosal Immunology (49) copyright (2011).

B cells

B cells, the name deriving from the Bursa of Fabricius, a gland which is essential for B cell maturation and development in birds (50), are an essential part of the immune system, principally by producing antibodies when activated. In humans, B cells mature in the bone marrow and they then circulate out into lymph nodes and Peyer's patches where they await activation. Apart from producing immunoglobulins when activated, B cells can also act as APC (8). $CD20^{+}$

expression is generally used to define B cells, but the function of $CD20^+$ has not been characterised. As a part of the adaptive immune response, B cells present antigens on the MHC class II molecules to $CD4^+$ Th cells. The $CD4^+$ Th cells then begin to express CD40L on their cell surface, which ligates to the constitutively expressed co-stimulatory CD40 receptor on the B cell surface. The ensuing IL-4 production from the $CD4^+$ Th cell and intrinsic signalling of the B cell makes it active, i.e. initiates proliferation, Ig class switch and antibody production (8), leading to the transition from B cell to plasma cell (or memory B cell). This process usually takes place in the lymph nodes or Peyer's patches of the intestine.

B cells can however produce antibodies in a thymus-independent (TI) manner. The response is faster but the antibodies produced have less affinity for these TI antigens (9). The TI-1 antigens, such as lipopolysaccharide and bacterial DNA, cause proliferation and differentiation of B cells, known as polyclonal activation. The structure of bacterial capsular polysaccharides is highly repetitive and these TI-2 antigens can only activate mature B cells (specifically B1 or marginal zone B cells) as immature B cells become inactive when they encounter repetitive epitopes. TI-2 antigens activate mature B cells by simultaneous crosslinking of B cell receptors (9). The principal antibody isoform for TI antigens is IgM, which provides a swift and non-specific response to many types of bacteria. The thymus-dependent B cell response takes more time to activate and involves affinity maturation and antibody class-switching into IgG (9).

There are various reports on the prognostic value of tumour-infiltrating B cells in solid cancers. In breast, colorectal, cervical cancer and NSCLC, tumour infiltrating B cells have been associated with an improved prognosis (51-54), but an association with poor prognosis has also been reported (55). Recently, a new subset of B cells called regulatory B cells (B_{reg}) has been identified in animal models (56), and tumour-infiltrating B_{reg} have also been associated with unfavourable tumour characteristics in different human cancers (57-64). The exact human B_{reg} surface antigens have yet to be identified, but this B cell subset seems to act via induction of IL-10, which induces the transformation of resting T cells to T_{regs} ($CD4^+CD25^+FoxP3^+$), which are known to weaken the innate immune response, e.g. NK cells. In another report, a B_{reg} subset ($CD19^+CD24^{hi}CD38^{hi}$) inhibited $CD4^+$ T cell IFN-γ and TNF-α in vitro, in an IL-10, CD80 and CD86 dependent manner, the latter two being co-inhibitory ligands for the immune check-point inhibitor CTLA-4 (65). Other proposed mechanisms for B_{regs} to attenuate the anti-tumour immune are by activating STAT-3 (pro-angiogenic transcription factor) (66) and IL-35 secretion, which has been shown to inhibit Th1 and Th17 in addition to promote T_{regs} (67).

Plasma cells

B cells mainly undergo Ig class-switch recombination and somatic hypermutation of the variable regions of Igs in germinal centres, which are highly specialised regions of lymph nodes (68). Plasma cells are believed to derive from the precursor plasmablasts, which in turn derive from the B cells, and plasmablasts and plasma cells indeed express similar markers e.g. IRF4, BLIMP1 and XCB1. However, there is still uncertainty as to the exact sequence of plasma cell maturation (68).

CD138$^+$

CD138 (syndecan-1) is used as a plasma cell marker, but it can also be expressed in stromal fibroblasts and tumour cells (69). Syndecan-1 is part of the transmembrane heparan sulfate proteoglycan (HSPG) family, the members of which act in cell to cell-matrix adhesion as well as being co-receptors for e.g. basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) when binding to their tyrosine kinase receptors. HSPGs have hence been implicated in the mediation of the effect of these mitogenic and angiogenic growth factors (70). It is the posttranslationally added polysaccharide heparin sulfate that is mainly responsible for this co-receptor activity (70).

There are some data on the relationship between CD138$^+$ plasma cell infiltration in solid tumours and prognosis. In NSCLC and colorectal cancer tumour infiltrating CD138$^+$ plasma cells were associated with an improved prognosis (52, 71), whereas associations with poor prognosis were reported in epithelial ovarian cancer (EOC) (69, 72, 73) and breast cancer (74). In gastric cancer, only the stromal expression (no specified cells) has previously been reported (75).

Immunoglobulin kappa C$^+$

Immunoglobulins come in five classes, defined by their constant Fc region. Igs are composed of two heavy chains and two light chains joined together by disulphide bridges, and the light chains come in either of two forms: κ and λ with a ratio of 2:1 in humans (9). The underlying reason for using immunoglobulin kappa C (*IGKC*), or indeed IGKC at the protein level, as a plasma cell marker is that it is expressed in abundance on plasma cells, whilst there is no co-localisation with CD20$^+$ expression, thereby distinguishing plasma cells from B cells (76). Plasma cells are surface Ig-negative but have a strong cytoplasmic staining of IGKC, whereas B cells are surface IGKC positive and have a weak cytoplasmic staining

of IGKC. In addition, IGKC is neither expressed by tumour cells nor by epithelial cells (76). The 1/3 of plasma cells that express IgλC will not be detected using IGKC.

$IGKC^+$ plasma cells has been linked to favourable prognosis in several types of cancer; colorectal, NSCLC, and breast cancer, and has also been shown to predict response to chemotherapy in the latter (52, 54, 71, 76).

Upper gastrointestinal tract cancer

Oesophageal cancer

Oesophageal cancer normally presents at an advanced stage, with dysphagia and weight loss. The late onset of symptoms and the early spread to regional lymph nodes explain the poor survival rate of 15-25% (77, 78). Squamous cell carcinoma (SCC) incidence has remained constant worldwide, but the sharp increase in adenocarcinoma in Europe and North America (79), where it now surpasses squamous carcinoma incidence, is responsible for oesophageal cancer now being the 8th most common cancer worldwide (5, 77, 80, 81) and the 6th most common cancer related death (5).

In Barrett's oesophagus (BE), the squamous epithelium of the oesophago-gastric junction is replaced with an intestinal columnar epithelium, but the metaplastic cells are different from those found in the cardia of the stomach, and it is hence not just an upward migration (82). This is referred to as intestinal metaplasia and can occur with or without dysplasia. The risk of malignant conversion (dysplasia turning into invasive adenocarcinoma) is approximately 0.3% per year (83). Gastro-oesophageal reflux disease (GERD), associated with smoking, alcohol and overweight, is the main cause of intestinal metaplasia. Helicobacter pylori (HP) infection has been reported to incur less risk of developing oesophageal adenocarcinoma, potentially by causing atrophic gastritis with loss of parietal cells leading to less GERD and BE (84, 85). Smoking is also a risk factor in oesophageal cancer (86), as is alcohol, but mainly for SCC development (87). The progression to SCC has been proposed to follow this sequence: basal cell hyperplasia, dysplasia and cancer, whereas adenocarcinoma follows the metaplasia, low-grade dysplasia, high-grade dysplasia and cancer sequence (88, 89). The different T stages of oesophageal cancer is shown in Figure 2, and important genetic alterations in the development of oesophageal adenocarcinoma have been characterised and are shown in Table 1.

Table 1. Important genetic alterations for the development of oesophageal adenocarcinoma (90).

Gene	Function	Protein
CDH1	Cell adhesion	E-cadherin
CDKN2A	Stabilise p53, cell cycle control	p16, p14
GPX3	Inactivate hydrogen peroxide	Glutathione peroxidase 3
CTNNB1	Regulate cell growth and adhesion	β-cathenin
NOX5	Superoxide generation	NADPH oxidase 5
TP53	Apoptosis induction, DNA repair	p53

For all tumours of the oesophagus, apart from Siewert type II, an oesophagectomy, transthoracic or transhiatal, usually with a laparoscopic gastric tube reconstruction and a feeding jejunostomy for alimentation purposes, is required. The transthoracic approach is the most common (91). Siewert type II tumours, even though treated as a part of the oesophagus, will be dealt with by an extended gastrectomy, reconstructed with Roux-en-Y.

Median survival times, with surgery alone, range between 11-19 months (92, 93) with the transthoracic approach. The CROSS study (94) randomised 366 patients to either pre-operative chemoradiotherapy (carboplatin + paclitaxel and 41.4 Gy) followed by surgery within 4-6 weeks or surgery alone. The results showed a median overall survival of 49.5 months in the chemoradiotherapy group compared to 24.0 months in the surgery alone group.

Non-surgical curatively intended chemoradiotherapy can also be a first-hand option but mainly for squamous cell carcinomas in the upper oesophagus (95).

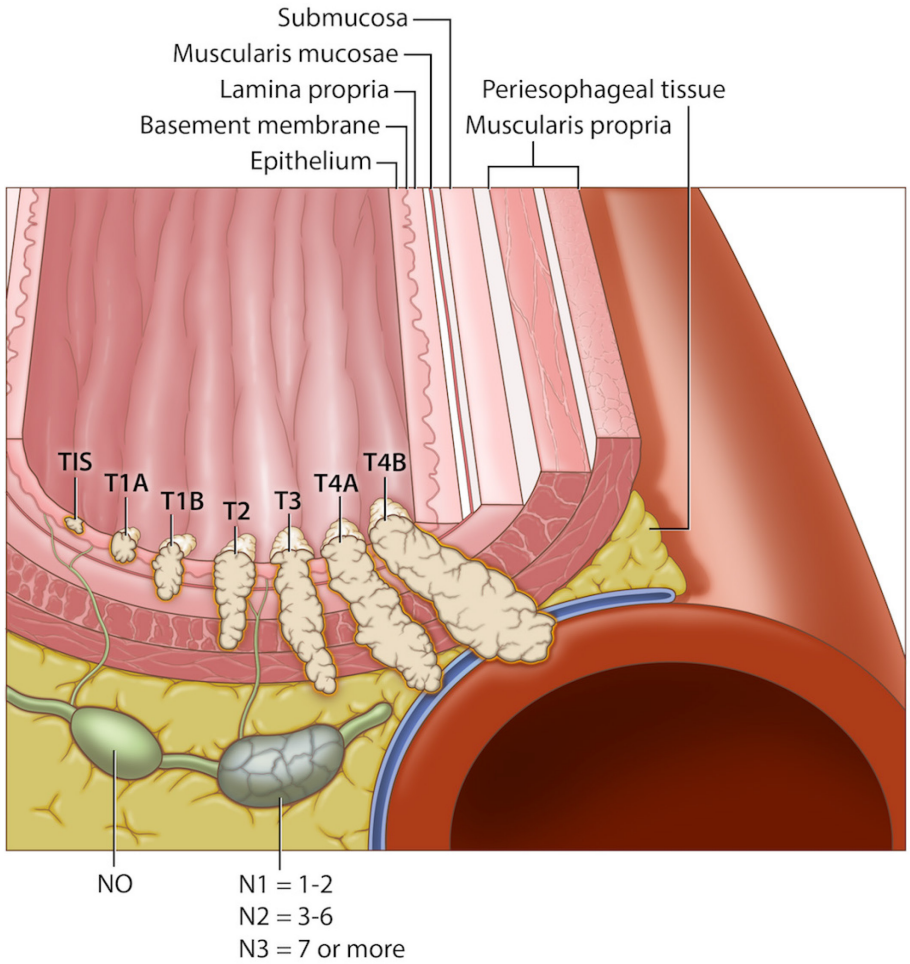

Figure 2. Anatomy of the oesophageal wall and T and N stages of oesophageal cancer. Reprinted with due permission of Visual Art: © 2017 The University of Texas MD Anderson Cancer Center.

Gastroesophageal junction adenocarcinoma

The gastroesophageal junction is sometimes used to characterise the region with the largest increase in adenocarcinomas of oesophago-gastric origin. As shown in Figure 3, it spans from 5cm proximally to 5cm distally of the anatomical cardia and is subdivided into three parts according to the German surgeon Siewert (96). Over the last decades there has been a 2.5 time increase in the incidence of GE junction adenocarcinoma (86).

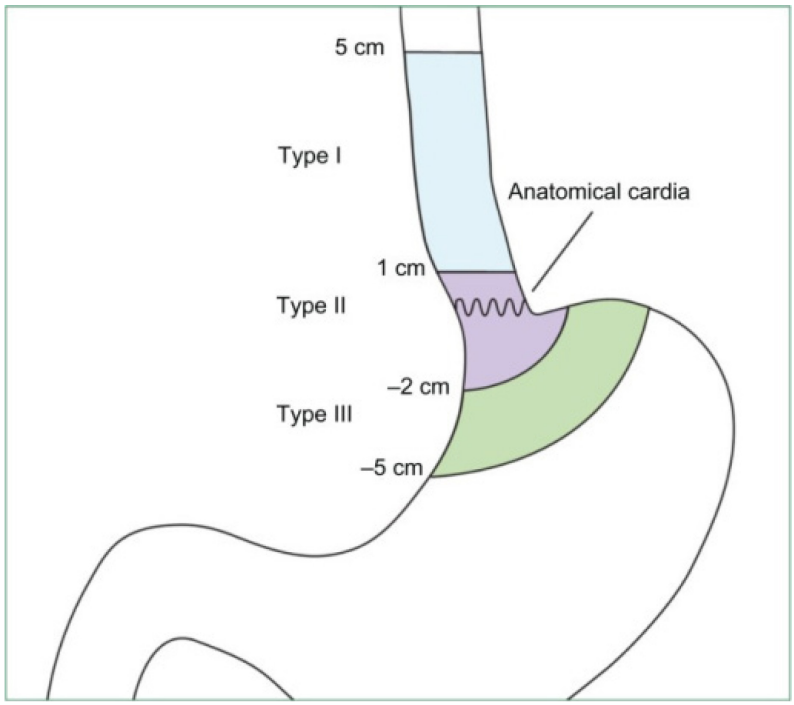

Figure 3. Gastro-oesophageal junction division according to Siewert. Reprinted by permission from PubMed.

Gastric cancer

The incidence of gastric adenocarcinoma is declining, but there are still 950000 new cases yearly worldwide, making it the 5th most common cancer and the 3rd most common cancer related death (5). The steep decline seen in the Westernised world can largely be explained by the diminished prevalence of HP infection, which was previously responsible for approximately 50% of all gastric cancer cases (97). The exposure to other risk factors, such as smoking, salted and smoked foods has also diminished (98).

The vast majority of gastric cancers (95%) are adenocarcinomas (99), the rest being lymphomas, neuroendocrine tumours (NET) and sarcomas including gastrointestinal stromal tumours (GIST). The Lauren classification introduced in 1965 is still in use and it is based on the growth pattern of the malignant cells, distinguishing an intestinal type, with cohesive cells that form gland-like structures, and a diffuse type, with non-cohesive cells infiltrating the stroma as single cells, sometimes shaped like a signet ring, or as small groups of cells (100). There is also a category with a mix of intestinal and diffuse growth. It is the less

deadly intestinal type (101), associated with HP infection, that has shown a marked decrease in incidence over the past decades (99). The suggested order of progression to cancer in the intestinal type is superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and cancer (102). For the diffuse growth pattern no suggested progression model has been proposed as of yet.

A familial clustering of gastric cancer is seen in approximately 10% of cases. Hereditary diffuse gastric cancer (HDGC) and other known gene mutations e.g. familial adenomatous polyposis (FAP), Peutz-Jeghers and hereditary nonpolyposis colorectal carcinoma (HNPCC), are responsible for 1-3% of gastric cancer cases. HDGC, an autosomal dominant disorder that in 30% of the cases is associated with a germline mutation of the tumour suppressor epithelial calcium-dependent adhesion (E-cadherin) (103). E-cadherin is responsible for adhesion junctions between epithelial cells, crucial for epithelial function (90).

Symptoms of gastric cancer are usually subtle such as abdominal discomfort, iron deficiency anaemia, weight loss, nausea and early satiety. It can however present as with acute symptoms of an upper gastrointestinal bleed including hematemesis and black stools (104).

For gastric cancer, the surgical options are partial gastrectomy (Billroth II), complete gastrectomy and, if the tumour is located in the cardia region, the total gastrectomy has to be extended to include part of the esophagus. For intestinal type tumours, 20mm distance to the resection margin is needed (105), while 50mm is practiced for the diffuse type (106). Reconstruction is made by Roux-en-Y loop (104).

Systemic therapies

The prolonged survival seen with chemotherapy treatment in non-resectable or metastatic gastric cancer prompted the Medical research council adjuvant gastric infusional chemotherapy (MAGIC) trial. It compared surgery alone with surgery + perioperative (3 cycles pre and 3 cycles post operatively) chemotherapy (5-FU + cisplatin + epirubicin) in gastric, GEJ and oesophageal adenocarcinoma (lower third) (107). The median overall survival in the surgery alone group was 23% and 36% in the surgery + chemotherapy group, which led to a rapid change in the standard of care.

Targeted therapies

Trastuzumab

The human epidermal growth factor receptor 2 (HER2), also known as c-erbB-2, is a transmembrane tyrosine kinase receptor involved in regulating cell signalling related to proliferation, survival and differentiation (108). HER2 gene amplification is found in approximately 25-30% of breast cancers (109) and in 9.5-18% of gastric cancers (non GEJ) (110). Trastuzumab (Herceptin) is a humanised monoclonal antibody that both blocks HER2 signalling and induces antibody dependent cell-mediated cytotoxicity (ADCC). It has been shown to improve outcomes in breast cancer and is now part of the standard of care (109, 111). The trastuzumab for gastric cancer (ToGA) trial also showed an improvement in the median overall survival when comparing trastuzumab + chemotherapy (13.8 months) with chemotherapy alone (11.1 months).

Ramucirumab

The VEGF receptor 2 (VEGFR2) and its ligand VEGF are involved in angiogenesis, which is important for cancer growth and metastasis. Ramucirumab is a recombinant human monoclonal antibody that binds to the extracellular part of VEGFR2 thereby impeding signal transduction (112). The RAINBOW trial (113) that enrolled patient having received first line chemotherapy, showed a survival benefit for ramucirumab + paclitaxcel as compared to paclitaxcel alone (9.6 months versus 7.4 months) and is now at the doorstep of becoming part of second line standard therapy for advanced gastric cancer.

Periampullary adenocarcinoma

Pancreatobiliary and intestinal type tumours

Periampullary adenocarcinoma mainly encompasses four different histopathological types of adenocarcinomas: pancreatic ductal, cholangio (biliary), ampullary (Vater) and duodenal, which will be described below. There are other, rare, types of tumours originating from the pancreas: acinar cell carcinoma, cystadenocarcinomas and neuroendocrine tumours, which will not be covered. It has been shown that the histopathological type, rather than anatomical centre, is key to determining prognosis and, hence, the division into pancreatobiliary (PB-type) or intestinal type (I-type) is now widely used (114-117). Figure 4A. illustrates PB-type and I-type tumour dichotomisation and B. the four main types of periampullary tumours.

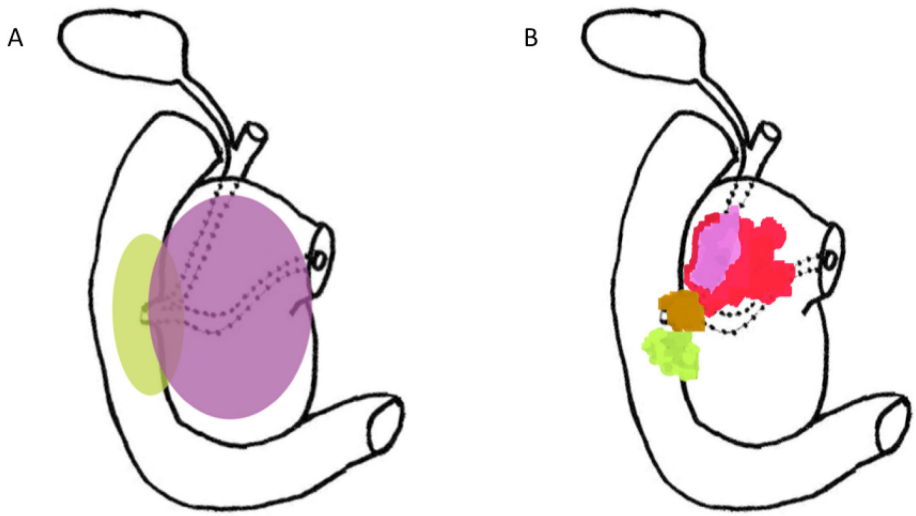

Figure 4. A. Pancreatobiliary type tumours in purple and intestinal type tumours in green. B. Duodenal (green), ampullary (brown), biliary (purple) and PD adenocarcinoma (red). Reprinted with courtesy of Dr. Jacob Elebro.

Assessment of operability

For a periampullary tumour to be readily resectable there must not be any contact between the tumour and the caeliac axis, superior mesenteric artery or the common hepatic artery. Furthermore, no contact must exist with the superior mesenteric vein or the portal vein or ≤180° contact without vein contour irregularity (118). Patients with evidence of distant metastasis upon diagnosis are not eligible for surgery. Figure 5 illustrates the normal anatomy of the pancreas and duodenum.

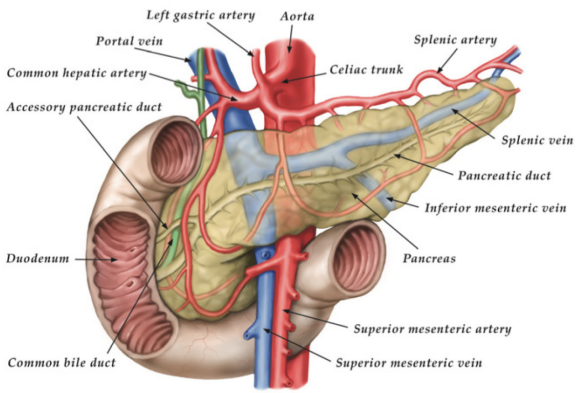

Figure 5. The anatomy of the pancreas and duodenum including major vessels. Reprinted with courtesy of Dr. Daniel Ansari (119), illustration by Anders Flood, copyright.

Pancreatoduodenectomy (Whipple)

Since its introduction by Kausch in 1912 (120) and further popularisation by Allen Oldfather Whipple in 1935 (121), pancreatoduodenectomy for periampullary adenocarcinoma has developed extensively. Initially a two stage procedure, weeks between the primary decompression and the resection of the tumour, with high per and post operative mortality to the modern era with a one stage operation and low post operative mortality (122, 123). Figure 6 illustrates the reconstruction after a pancreatoduodenectomy. It is still, however, associated with significant morbidity, with complications occurring in 50% of patients, however only 15-20% have grade 3a or above according to Clavien-Dindo (122). The slight alteration of the standard Whipple procedure, with the preservation of the pylorus, is faster and has the same oncological results and the same rate of serious complications. However, an increased occurrence of delayed gastric emptying is seen. (124).

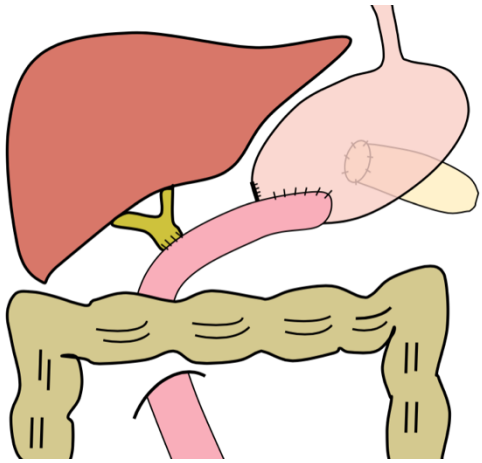

Figure 6. Reconstruction after pancreatoduodenectomy. Reprinted with courtesy of Dr. Caroline Williamsson.

Pancreatic ductal adenocarcinoma

Arising from ductal cells of the exocrine pancreas approximately 90% of PDA cases are sporadic and the incidence in Sweden for 2015 was approximately 12-13 per 100000 (122). The known risk factors for developing PDA are smoking, chronic pancreatitis and obesity (125-127). Diabetes mellitus type II with an onset after 50 years of age might be a clinical manifestation of PDA (128). There are some precursor lesions that are known to develop into PDA: intraductal papillary mucinous neoplasm (IPMN), pancreatic intraepithelial neoplasia (PanIN) and mucinous cystic neoplasm (MCN). It is generally believed that the sequence of genetic alteration begins with KRAS, then CDKN2A, followed by TP53 and SMAD4 (129).

Hereditary PDA is responsible for about 10% of cases and some genetic alterations have been characterised and are displayed in Table 2. Malaise, cachexia, midepigastric pain and jaundice are common initial symptoms of pancreatic cancer, the latter normally only when tumours are located in the head of the pancreas (75% of all PDA) (131).

Table 2. Hereditary genetic mutations with a high risk of developing PDA (130).

Syndrome or condition	Gene	Function/product
Familial adenomatous polyposis	*APC*	Tumour suppressor
Familial atypical multiple mole melanoma	*CDKN2A*	Tumour suppressor
Hereditary breast ovary cancer	*BRCA1 & BRCA2*	DNA repair
Hereditary nonpolyposis colon cancer	*MLH1 & MSH2*	DNA repair
Hereditary pancreatitis	*PRSS1*	Trypsinogen
	SPINK1	Trypsin inhibitor
Peutz-Jeghers syndrome	*STK11*	Tumour suppressor

The carbohydrate antigen 19.9 (CA19.9) has been used in the patient work up and has a sensitivity and specificity of approximately 80% (132). False positives exist when using CA19.9 in cholestasis and a lack of the sialylated Lewis A blood group antigen (not expressed in 5-10% of Caucasians) will lead to false negative results (133). Apart from helping the diagnosis, CA19.9 is used to monitor disease progression.

PDA prognosis is dismal, with 5 year OS for all cases combined still at 5% (134, 135). For metastatic disease, OS is about 2% with a median survival of 7-8 months (with chemotherapy), and for regionally/locally advanced disease OS is about 10%, with a median survival of approximately 14 months (136). The outcome of the ESPAC-4 trial, for localised resectable disease, where gemcitabine monotherapy, the current standard adjuvant chemotherapy treatment, was compared to gemcitabine and capecitabine (oral 5-FU prodrug) in combination yielded an estimated OS of 16.3% for gemcitabine and an estimated OS of 28.8% for GEMCAP (137). Of note, the median survival for patients with R0 resection post operatively receiving GEMCAP was 39.5 months. There are interesting neo-adjuvant trials for borderline-resectable tumours on the way (ESPAC-5F and NEPAFOX) and for resectable tumours (ESPAC-6), that will advance our knowledge further.

Cholangiocarcinoma

Cholangiocarcinomas can be intra- or extrahepatic, the latter divided into perihilar (Klatskin) and distal bile duct tumours. Cholangiocarcinomas are rare, with an incidence of 16.3 per 1000000 per annum (extrahepatic, non-gallbladder) in the Nordic countries (138), and known risk factors are a history of sclerosing cholangitis, ulcerative colitis, choledochal cysts and infection with Chlonorchis siensis (139).

Surgical resection is by means of pancreatoduodenectomy (rarely with a local resection of the distal extrapancreatic bile duct and lymph nodes). Adjuvant treatment with external beam radiotherapy (EBRT) has been shown to yield a 24 month OS of 65% in the SWOG S0809 trial (GEMCAP followed by ERBT and concurrent capecitabine in 54 cases of extrahepatic cholangiocarcinomas) (140). A preplanned subgroup analysis in the ESPAC-3 (141) trial (428 periampullary adenocarcinomas including 96 cases of extrahepatic cholangiocarcinomas) found median survival to be 18 months for adjuvant 5-FU/leukovorin, 20 months for gemcitabine and 27 months for observation alone for the extrahepatic cholangiocarcinomas.

Ampullary adenocarcinoma

Tumours of the ampulla, including the papilla of Vater that bulges into the second part of the duodenum, are very rare. The high frequency of resectability reported (85%) (142) can at least in part be explained by the early onset of jaundice, and hence early diagnosis, in this subset of periampullary adenocarcinoma. The histopathological subdivision is into PB-type or I-type tumours.

The ESPAC3 (141) trial's non-PDA arm was not conclusive regarding the adjuvant treatment choice but gemcitabine overall is a good option, with the possibility of using 5-FU particularly in I-type ampullary adenocarcinoma.

Duodenal adenocarcinoma

The incidence of cancer in the small bowel is 6.2 per 1000000 (138) in northern Europe, and the majority (50-75%) are found to be duodenal. Recent data from Regionala Cancercentrum i samverkan (RCC) show that in 2015 there were 99 cases in total in Sweden, yielding an incidence of approximately 1 per 100000 (122) for duodenal adenocarcinoma. Approximately 75% of duodenal tumours are found in the second part (duodenum descendens) (143) in close proximity of the papilla Vater (and ampulla).

Not much is known about the best adjuvant treatment regimen, if any, for duodenal tumours. The ongoing ESPAC-4 trial's non-PDA arm will hopefully bring some answers. Currently, extrapolation of data from colorectal cancer is used by some centres, favouring adjuvant treatment with fluoropyrimidine ± oxaliplatin (144).

Early detection

Overall survival is 5% for PDA, 10% for oesophageal cancer and 25% for gastric cancer (104, 124). While early detection is needed in all these cancers, this section is focused on PDA and oesophageal adenocarcinoma, since their outcomes are particularly poor.

It has been estimated that the development of PDA takes approximately 22 years; 19 years from the time of the first genetic event to invasion and another 3 years to metastasis and death (145), with similar findings in models for oesophageal cancer (89). Symptoms of disease, typically dysphagia in oesophageal cancer and non-colicky jaundice in PDA, come late and a large proportion of patients present with non-resectable disease, 75% of oesophageal cancers present with stage IV (99) and 68% of PDA in stage III-IV (122). Hence, there is a window of opportunity and an obvious need, in both upper gastrointestinal and periampullary cancer, for early detection, in order to radically change the long-term survival for these patients.

A summary of Japanese case reports estimates the OS for very early PDA, T<10mm, to be 57%, which is an impressive figure for this aggressive type of cancer (146). These cases were however found by coincidence. As there are no ways of screening the entire population, the current focus is instead set on individuals with a high risk for familial or hereditary PDA (147). For other high risk groups for the development of sporadic PDA, there is no consensus on how surveillance should be performed on individuals with e.g. chronic pancreatitis and new onset diabetes type II (148). Interestingly, the paraneoplastic form of DM type II, present in around 50% of PDA (149), has been reported to be caused by paracrine signalling of PDA secreted adrenomullin, leading to β cell dysfunction with impaired insulin secretion (150). This may be explored as a candidate future biomarker.

The biomarker CA19.9 has been extensively investigated and is used to follow disease progression, but with regards to early detection, i.e. screening of non-symptomatic individuals, the results have been disappointing. KRAS mutations are early and common events in PDA but are also found in many benign conditions, e.g. chronic pancreatitis which incurs an increased risk of PDA, and are therefore not suited for early detection (151). Interesting data on the development of PDA, challenging the notion of slow disease progression with genetic alterations occurring in an independently, step-by-step fashion, was recently published (152).

A fast progression, or even simultaneous, of catastrophic genetic events quickly leads to invasion and metastasis, which also changes the conditions and requirements for early detection and screening programs.

Epigenetic modifications achieve heritable changes in gene expression without changing DNA sequence (153). Epigenetic regulators influence DNA methylation, histone modification, chromatin remodelling and non-coding ribonucleic acid, and mutations in these regulators are commonly seen in PDA and leads aberrant gene and protein expression (153). The aberrantly expressed protein profile of tumours can be analysed with quantitative proteomics and show promising results for new biomarkers in early detection (154).

There are currently no biomarkers available to follow disease progression or, indeed, for the early detection of oesophageal cancer (155). In an attempt to monitor progression from non-dysplastic to dysplastic BE, IHC determination of p53 has been suggested to be included in the biopsy examination (156). Epigenetic biomarkers have been explored such as hypermethylation of the gene for p16 (157).

As PDA and oesophageal cancer are rare diseases, it is a challenge to perform efficient surveillance. Indeed, even for colorectal cancer, which is more common, there is not yet a consensus on surveillance programs. In addition to being rare cancers, particularly high specificity is needed to ensure that false-positives are kept to a minimum to prevent patients undergoing unnecessary major surgery. In familial types of PDA, surveillance is recommended (147).

Methods for biomarker discovery

Tissue microarray

Tissue microarray (TMA) is a high throughput technique, introduced in 1998, whereby tissue cores from many donor blocks of formalin fixed paraffin embedded (FFPE) tumour tissue are punched out and inserted into a common receiver paraffin block (158). The cores inserted are typically 0.6-2mm and receiver blocks are sliced in 4 μm layer and mounted on slides that are then subjected to IHC and examined. TMA construction is illustrated in Figure 7.

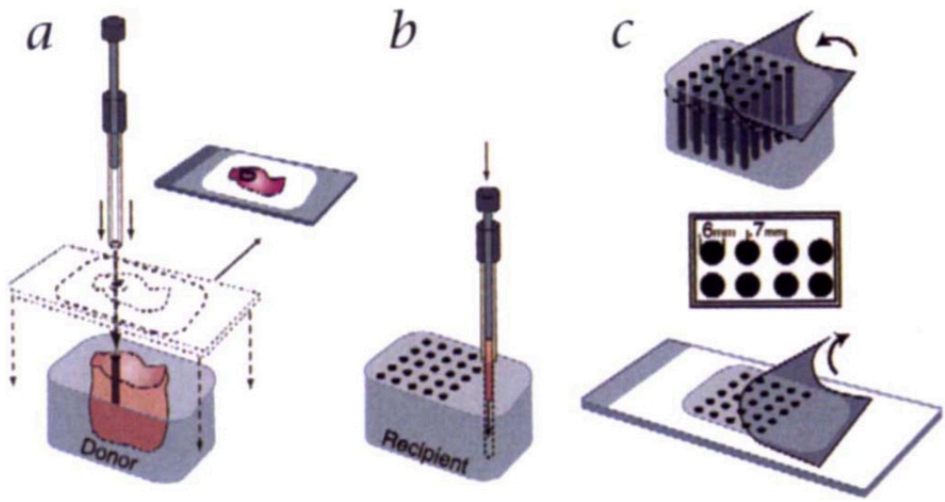

Figure 7. TMA construction. Tumours from multiple donor block are inserted into one recipient block. The block is then cut in 4 micrometer thin sheets that are then subjected to IHC and mounted on a slide. Reprinted by permission from Macmillan Publishers Ltd: Nature Medicine (158) copyright (1998).

The small width of the tissue cylinders means that a very small quantity of the donor tumour block is consumed, which is beneficial since standard full-face

tissue blocks are limited to approximately 300 sections before the tumour block is spent (159). In addition, the antibody consumption is much lower as compared to analysis of full-face tissue sections (159). The issue of tumour heterogeneity might be a concern with the TMA technique (158, 160). Hence, the use of more than one tumour core may circumvent this problem, although this does not fully deal with the issue of tumour heterogeneity. However, the problem is present in full-tissue sections as well and is important to take into account whenever using IHC.

Immunohistochemistry

In the early 1940s, professor Albert H. Coons introduced IHC, that is the use of colour tagged antibodies for the detection of antigens in tissues, and his technique used immunofluorescence (fluorescein detected by ultraviolet light) (161). A further important development was the introduction of enzymes as marked antibodies, by Nakane in the 1960s, moving the detection into a normal light microscope, thereby making it widely available outside of research institutions (162).

The golden standard for subtype categorisation of different tumours are gene expression assays (163). They are, however, currently not readily available in an economical and fast format (164). IHC, which is an omnipresent and well-established technique, is therefore used for diagnosis and subclassification of cancer as well as for quantification of the expression of different prognostic and predictive markers e.g. HER2, Ki67, ER in breast cancer (165). They are in that sense used as surrogate markers for the identification of different cancer subtypes (166).

The sequence for the slide preparation is generally the following: antigen retrieval, non-specific site block, endogenous peroxidase block, primary antibody incubation, detection systems, revealing, counterstaining, slide mounting, interpretation and quantification (167). As is evident from the number of steps involved, there are a lot of potential issues to be aware of, the most important downfalls being the selection of the antibody panel and the interpretation of the reaction (168). Antibodies can be either polyclonal i.e. multiple antibodies binding different epitopes of the same antigen, or monoclonal, i.e. a single antibody binding only one epitope. All antibodies need to be validated regarding their specificity and sensitivity, so as to ensure accurate results (167), and external and internal controls should be used. It is also advised that a specialised technician fulfil this work to optimise results (169).

The most widely used fixation agent is formalin, but alcohol and acetone have also been used to stop autolysis (167). Formalin binds to proteins, crosslinking them with methylene bridges and thereby stabilizing the tissue. It can however also cause conformational changes to epitopes (165). The final part of tissue preparation is dehydration followed by embedding in paraffin.

When performing IHC, the first part of the process is antigen retrieval from FFPE tissue, whereby the methylene bridges are broken down and the epitopes of the antigens are made available again. This is achieved in a multistep process of heating and cooling (165). The antibody system can then be applied, either in the form of a labeled primary antibody, or with the use of an additional secondary enzyme antibody for detection when a chromogen, e.g. diaminobenzidene (DBA), is added (165).

Quantitative polymerase chain reaction

Real time, or quantitative, polymerase chain reaction (qPCR) is the further development of reverse transcriptase PCR, combining gene amplification and detection into one step (170). qPCR is used to assess the quantity of a DNA segment, especially in gene expression analysis, by initially breaking up the DNA chain into a single strand by heating and cooling. A DNA primer for the gene segment is introduced and a heat stable polymerase performs the amplification that is then detected by a fluorescent stain after each cycle (170). qPCR was used to assess *PIGR* gene expression in Paper II.

Digital image analysis

Digital image analysis (DIA) of IHC reactions has been around since the 1980s (171) and when used by many observers, it has been shown to be superior to manual scoring techniques (172, 173). The main drivers for DIA development have been reducing intra-observer variability, increasing analysis speed and improving the quantitative accuracy, i.e. moving away from manual quantitative and semi-quantitative scoring (174).

The sequence of IHC slide reading by DIA can be summarised in: scanning and transferred into digital form, defining of the region of interest in the case of full-face tissue sections and fitting the reading mesh in the case of TMA, and finally the application of the relevant algorithm, potentially with minor alterations depending on the marker being investigated.

There are, however, several caveats when using DIA. The staining colours in IHC were not developed for DIA, which is more sensitive to staining quality, as most computers cannot compensate for variations in staining intensity as human brains do naturally. In addition to problems with folds and sections of unequal thickness, DIA struggles with indistinct cell borders when counting cells (174). Therefore, there has been a push toward changing the colours to provide a better contrast for DIA (175). The algorithm used is man made and it usually has to be altered slightly to make it fit the stained slide and the area of interest has to be defined. This individual fitting, in addition to the man made algorithm, has been shown to present a significant both inter-observer and, interestingly, intra-observer variation with repeated selection of the regions to be assessed (176). DIA was applied to assess B cell and plasma cell density in the pancreatic and periampullary adenocarcinoma cohort used in Paper IV.

The Human Protein Atlas

The Human Protein Atlas (HPA) project was initiated in Sweden in 2003 and is the continuation, at the protein level, of the work of the Human Genome project. The current 16th version encompasses 25000 antibodies targeting 17000 genes, representing approximately 86% of all human protein-coding genes (177). Its main objectives are to provide validated antibodies for the human proteome as a whole and to explore the distribution and relative abundance of the proteome in normal and malignant human tissues and in various types of cell lines (178).

The HPA as such exists in the form of validated antibodies towards a fragment of each protein, called a protein epitope signature tag (PrEST). The PrEST's coding region on the gene is selected based on low homology to other proteins, i.e. low risk of antibody cross-reactivity, and ruling out transmembrane regions since these are less immunogenic (179). The gene segment chosen is amplified by rT-PCR, and then transferred via an expression vector into *Escherichia coli* that produce the small protein fragments in the form of PrESTs (recombinant protein expression). Polyclonal antibodies are subsequently derived by immunizing New Zealand rabbits with the PrEST protein fragments (179).

The derived polyclonal antibodies are affinity purified by using the PrESTs as antigens and then validated by means of protein array assay and Western blot (180). Cellular protein localization in normal and tumorous tissues is assessed by IHC (179). All information including antibody specificity/sensitivity, PrEST gene sequence, and high definition photos of all of the tumour tissues is freely available to the public online (177).

The HPA can be utilised as a tool for biomarker discovery (181). Using this approach, pIgR emerged as a differentially expressed protein in gastric cancer with the selection criteria “negative expression in >40% of tumours and strong expression in >30 % of tumours”, in addition to having a validated antibody (Figure 8).

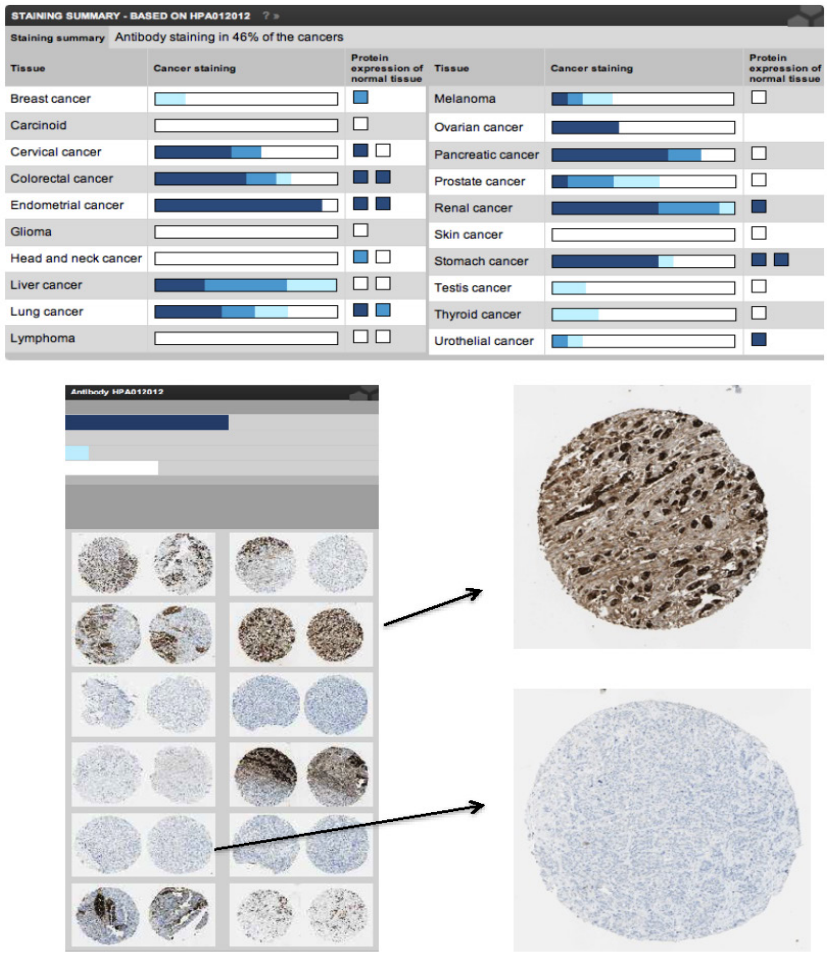

Figure 8. Using the following selection critera for gastric cancer in HPA: negative expression in > 40% of tumours, strong expression in > 30%, in addition to having a validated antibody, pIgR emerged as a potential biomarker. 12 cases of paired primary tumours with varying staining intensity, and enlarged examples of strong and negative pIgR expression. Dark blue: strong stain, medium blue: medium stain, light blue: weak stain and white: negative (177).

The present investigation

Aims

The aims in paper I and II were to determine the expression and prognostic relevance of pIgR in primary tumours and paired lymph node metastases in the oesophago-gastric adenocarcinoma and pancreatic and periampullary adenocarcinoma cohorts. In paper III and IV the aims were to explore the presence and prognostic significance of $CD20^+$ B cells and $IGKC^+$ or $CD138^+$ plasma cells in primary tumours in the same cohorts.

Cohorts

Paper I & III

The cohort used for paper I and III is a retrospective consecutive series of patients with chemo-/radiotherapy-naive oesophageal and gastric adenocarcinoma subjected to surgical resection at Skåne University Hospital between the January 1 2006 and December 31 2010. All cases were histopathologically re-evaluated and 129 cases were excluded from the original cohort of 303 cases, which yielded a cohort of 175 cases. A flowchart is illustrated in Figure 9. Subsequently, one further case was found to have received neoadjuvant chemotherapy and was therefore excluded. Primary tumours, lymph node metastases and benign appearing tumour adjacent tissue were selected for TMA construction. Tumours were re-examined and classified according to TNM7 and medical charts were used to obtain clinical data, time of recurrence and cause of death. Data on survival were gathered from the Swedish National Civil Register.

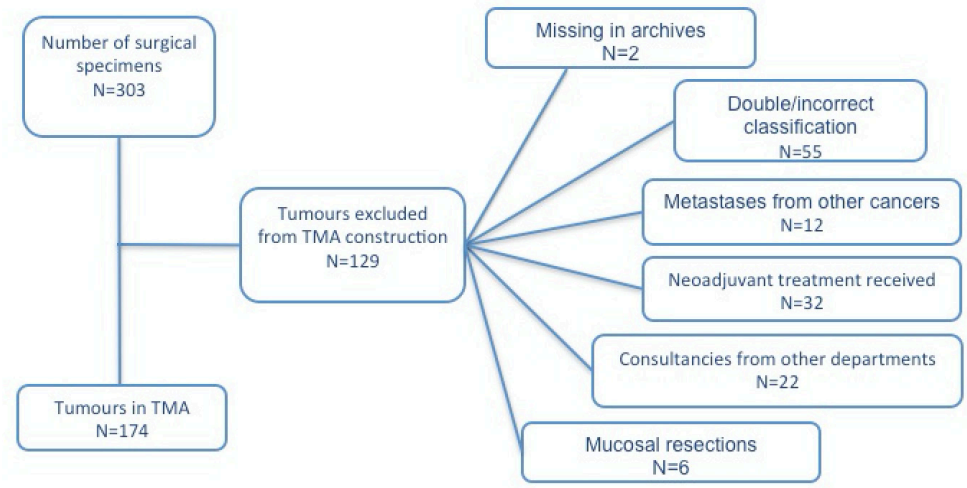

Figure 9. Flow chart of inclusion/exclusion of the oesophago-gastric cohort, with courtesy of Dr. Charlotta Hedner.

Paper II & IV

Paper II and IV are based on a retrospective, consecutive cohort of 175 patients with periampullary adenocarcinoma treated with pancreatoduodenectomy at Skåne University Hospital between January 1 2001 and December 31 2011. All cases were histopathologically re-evaluated and 13 cases were excluded (4 non-invasive and 9 non-pancreatoduodenectomy cases) from the original cohort of 188 cases, which yielded a final cohort of 175 cases. Primary tumours, lymph node metastases and benign appearing tumour adjacent tissue were selected for TMA construction. All the tumours were re-examined and classified according to TNM7, in addition to being grouped into either I-type or PB-type. Medical charts were used to obtain clinical data, time of recurrence and cause of death. Data on survival were gathered from the Swedish National Civil Register.

Results

Paper I

All samples of squamous epithelium (n=51) were negative for pIgR expression. Intestinal metaplasia (IM, n=57) had a significantly higher expression of pIgR than all other tissues. In normal gastric mucosa (n=114) pIgR was expressed in both glandular cells and columnar epithelium in various fractions but with an overall weaker intensity than IM. pIgR could be evaluated in 173/175 of the primary tumours and in 75/81 of the lymph node metastases. A total of 47/173 of the primary tumours and 32/75 of the lymph node metastases were negative for pIgR expression and there was no heterogeneity between sampled duplicate tissue cores in negative or strongly positive cases. There was no significant difference between the pIgR expression in primary tumours and lymph node metastases, however a trend was seen (p=0.058) towards a lower expression in lymph node metastases. Finally, there was no difference in pIgR expression between primary tumours and lymph node metastases with or without associated IM.

For the entire cohort there was a significant association between reduced pIgR expression and a more advanced T-stage (p=0.002) as well as involved resection margins (p=0.034). In oesophageal adenocarcinoma there was a significant association between reduced pIgR expression and T-stage (p=0.006) and for gastric adenocarcinoma with a more advanced N-stage (p=0.043).

For the entire cohort, Kaplan-Meier analysis showed that in primary tumours with R0 and M0 resection there was a significant association between high pIgR expression and an improved RFS (p=0.002). In cases with R0 resection, high pIgR expression was associated with an improved OS (p=0.030) and RFS (p=0.015). The association with RFS (R0/M0) was confirmed in univariable Cox regression analysis (HR 0.37, 95% CI 0.19-0.72), and remained significant in the multivariable model (HR 0.32, 95% CI 0.15-0.69). The association with OS (R0) was confirmed in in univariable analysis (HR 0.58, 95% CI 0.36-0.96), and remained significant in multivariable analysis (HR 0.60, 95% CI 0.36-0.99).

Kaplan-Meier analysis for subgroups revealed a significant association between high pIgR expression and OS in cases with R0 resection for oesophageal adenocarcinoma (p=0.003). For GE junction and gastric adenocarcinoma there was no significant association (p=0.175 and p=0.953 respectively). With regards to RFS and high pIgR expression the association was significant for oesophageal (p=0.002) and GE junction adenocarcinoma (p=0.003) but not for gastric adenocarcinoma (p=0.772).

Paper II

pIgR expression could be evaluated in all 50 samples of non-malignant tissue, in 172/175 of primary tumours and in 96/105 of sampled lymph node metastases. A total number of 25 (14.5%) primary tumours and 20 (20.8%) metastases were completely negative for pIgR expression and pIgR was expressed in various fractions and intensities in the other cases. PIGR mRNA was extracted from FFPE tissue in two cases and the results showed a good correlation between gene and protein expression levels including the downregulation of PIGR/pIgR from primary tumour to lymph node metastasis in one case. pIgR expression was significantly higher in benign pancreatic tissue compared to primary tumours and lymph node metastases both for the entire cohort and in I-type and PB-type tumours. pIgR expression was significantly lower in lymph node metastases compared to primary tumours in the entire cohort and in PB-type tumours but not in I-type tumours.

pIgR expression was significantly associated with tumour origin ($p=0.033$), with the highest expression in tumours of duodenal origin and the lowest expression in PDA. There were significant associations between pIgR expression and perineural invasion ($p=0.027$), tumour differentiation ($p<0.001$) and lymphatic ($p=0.016$), vascular ($p=0.033$) and peripancreatic fat growth ($p=0.039$).

Kaplan-Meier analysis revealed a significantly reduced OS for patients with tumours displaying low pIgR expression (logrank $p<0.001$) in the entire cohort and in I-type tumours (logrank $p=0.003$), but not in PB-type tumours. Similar trends were seen for RFS. Cox regression analysis in the entire cohort confirmed the association with OS in univariable analysis (HR 2.99, 95% CI 1.71-5.25), which also remained significant in multivariable analysis (HR 1.98, 95% CI 1.10-3.57). In I-type tumours, the association of low pIgR expression with OS were significant in univariable (HR 3.90, 95% CI 1.49-10.21) and multivariable analysis (HR 3.76, 95% CI 1.27-11.11). For PB-type tumours low pIgR expression was not significantly associated with decreased OS for the dichotomised variable but there was a borderline significant result when pIgR expression was used as a continuous variable (univariable HR 0.76, 95% CI 0.57-1.01, multivariable HR 0.75, 95% CI 0.55-1.03). The continuous variable was also significantly associated with decreased HR for OS in the entire cohort and I-type tumours. There was no significant interaction between pIgR expression and adjuvant chemotherapy in relation to OS or RFS.

Paper III

$CD20^+$ expression could be evaluated in 170/174 of the cases and $CD138^+$ in 172/174 of the cases, while $IGKC^+$ could be assessed in 173/174 of all cases. In oesophageal adenocarcinoma $CD20^+/IGKC^+$ cells were intercorrelated with a coefficient of 0.431 and $CD138^+/ IGKC^+$ cells with a coefficient of 0.459. For gastric adenocarcinoma the intercorrelation of $CD20^+/IGKC^+$ was 0.431 and for $CD138^+/ IGKC^+$ the coefficient was 0.425.

In patients with oesophageal adenocarcinoma, Kaplan-Meier analysis revealed a significantly prolonged TTR and OS in cases with a high $IGKC^+$ expression (p=0.003 for both). In patients with M0/R0 disease, the association between high IGKC+ expression and a prolonged TTR remained significant in both univariable and multivariable Cox regression analysis (HR 0.20, 95% CI 0.06-0.65 and HR 0.15, 95% CI 0.03-0.71), which was also true for OS (HR 0.21, 95% CI 0.08-0.60 and HR 0.10, 95% CI 0.02-0.57).

There was a significantly prolonged OS for patients with gastric adenocarcinomas displaying high $CD138^+$ expression (p=0.002), and a non-significant trend towards an improved OS for patients with tumours displaying high $IGKC^+$ expression (p=0.083). The association between high $CD138^+$ expression and a prolonged OS was confirmed in Univariable Cox regression analysis, but did not remain significant in multivariable analysis. On the other hand, a high $IGKC^+$ expression was significantly associated with a prolonged TTR in multivariable analysis (HR 0.45, 95% CI 0.21-0.98) and with a prolonged OS in both univariable and multivariable analysis (HR 0.55, 95% CI 0.31-0.99 and HR 0.46, 95% CI 0.26-0.87).

$CD20^+$ B cell TLS were present in 6.3% (11) of cases, and there was a non-significant trend towards an improved survival in the entire cohort, but not in strata according to tumour location.

Paper IV

The density of $CD20^+$ and $CD138^+$ cells could be evaluated in 171/173 cases and in 168/173 cases for $IGKC^+$ cells. The intercorrelation between $CD20^+/IGKC^+$ was 0.481, 0.239 for $CD20^+/CD138^+$ and 0.134 for $IGKC^+/CD138^+$. In I-type adenocarcinoma there was a significant association between high CD20+ density and less advanced T-stage (p=0.002), low-grade tumours (p=0.045), free resection margins (p=0.008), absence of perineural growth (p=0.006), vascular invasion (p=0.016) and growth in peripancreatic fat (p=0.001). In PB-type adenocarcinoma a high $IGKC^+$ density was found to be significantly associated with low grade tumours (p=0.007) and absence of vascular invasion (p=0.023), whilst high

density of $CD20^+$ was only significantly associated with adjuvant chemotherapy ($p=0.032$).

Kaplan-Meier analysis revealed significant associations between high density of $CD20^+$ lymphocytes and a significantly prolonged TTR and OS in I-type tumours ($p<0.001$ and $p=0.001$, respectively) but not in PB-type tumours. These associations were confirmed in univariable analysis and remained significant in multivariable analysis for both TTR (HR 0.25, 95% CI 0.12-0.54 and HR 0.31, 95% CI 0.12-0.79, respectively) and OS (HR 0.29, 95% CI 0.14-0.62, and HR 0.32, 95% CI 0.12-0.86, respectively). High $CD138^+$ density was significantly associated with both a prolonged TTR and OS in univariable Cox regression analysis (HR 0.45, 95% CI 0.21-0.97 and HR 0.43, 95% CI 0.21-0.90) but not in multivariable analysis.

In PB-type tumours, high $CD20^+$ expression was not prognostic in univariable analysis, but was significantly associated with a prolonged OS in multivariable analysis (HR 0.38, 95% CI 0.23-0.66). There was a significant association between $CD138^+$ expression and a prolonged OS in univariable Cox regression analysis (HR 0.63, 95% CI 0.40-0.97) that remained significant in the multivariable model (HR 0.51, 95% CI 0.32-0.82).

There was a significant interaction between adjuvant chemotherapy and $CD20^+$ B cells in relation to OS and a borderline significant interaction in relation to TTR in patients with PB-type tumours ($p_{interaction}=0.027$ and $p_{interaction}= 0.053$, respectively). There was no significant interaction between $CD20^+$ B cells and adjuvant chemotherapy in I-type tumours and no significant interaction was observed between $CD138^+$ or $IGKC^+$ cells and adjuvant chemotherapy regardless of morphological subtype.

$CD20^+$ B cell TLS were present in 13.9% (15) of PB-type and 7.9% (5) I-type tumours and were not associated with survival.

Discussion

Polymeric immunoglobulin receptor

The results from Papers I and II show that high pIgR expression is associated with an improved OS in patients with oesophageal and GE junction adenocarcinoma

and that low pIgR expression is associated with a decreased OS in patients with I-type periampullary adenocarcinoma. Our findings are in line with previous studies on colorectal, bladder, epithelial ovarian, oesophageal and gastric cancer as well as NSCLC (41, 43-45, 47).

However, conflicting data have been reported. In hepatocellular carcinoma, high pIgR expression was found to be associated with early recurrence (182). In addition, pIgR was found to induce EMT both *in vitro* and *in vivo* via TGF-β and Smad signalling suggesting a role for pIgR as a mediator of inflammation-induced EMT (182). In PDA, results from both cell lines and a TMA of human PDA demonstrated that high pIgR expression is predominantly seen when large amounts of stellate cells are present, and is then inversely correlated with E-cadherin expression (183), potentially further implicating pIgR with EMT. Interestingly, in a more recent study by Kocher and colleagues (184), there was no pIgR expression in normal pancreatic tissue and the expression did not influence survival outcomes. pIgR expression is indeed negative in acinar cells which compose majority of normal pancreatic tissue. Normal pancreatic tissue stained for pIgR was examined for Paper II and stains are also available from the HPA, and both sources show that the ductal cells, representing approximately 5% (177) of normal pancreatic tissue, express pIgR. If indeed the ductal cells were negative for pIgR expression in the study by Kocher and colleagues, an investigation into the validity of the antibody used might be of value.

pIgR expression can be upregulated via proinflammatory cytokines such as IL-1, IL-4, TNF-α and IFN-γ in the event of bacterial and viral infections (42). In a non-tumorous setting, free SC or bound to polymeric Ig (pIg) can complex bind unwanted elements such as bacteria and viruses. There are also reports of pIgR bound to pIg eliminating lipopolysaccharide intracellularly during transcytosis (185). In patients with Crohn's disease reduced expression of pIgR and other biomarkers have been linked to more severe chronic disease and non-response to immunosuppressive therapies and anti-TNF therapy (186).

In a Th_2 skewed microenvironment the tumour cells potentially interfere with the proinflammatory cytokines to decrease pIgR expression. The findings in paper I and II, and for other types of cancers as well, are opposite from Ai et al. and Kadaba et al. The underlying mechanistic benefit for the tumour cells in decreasing pIgR expression remains to be elucidated.

Plasma cells

$IGKC^+$ plasma cells have been associated with an improved survival in colorectal, NSCLC, ovarian and breast cancer (52, 54, 71, 187, 188), including being predictive of chemotherapy response in the latter (187). The results of paper III

show an improved TTR and OS in cases with high $IGKC^+$ plasma cell infiltration for both oesophageal and gastric adenocarcinoma.

One plausible reason for the improved prognosis seen with high $IGKC^+$ plasma cell tumour infiltration is activation of ADCC, the principal effector cells being NK cells and $CD8^+$ T cells (8). Furthermore, plasma cells can activate the complement system (189) and have also been reported to competitively inhibit MDSC and thereby influencing the TME in a positive (Th_1 skewed) direction (190). However, in paper IV, on pancreatic and periampullary adenocarcinoma, there was no association between $IGKC^+$ plasma cells and prognosis, whilst there was a significant association between $CD138^+$ plasma cells and OS in PB-type adenocarcinoma. The weak correlation between $IGKC^+$ and $CD138^+$ plasma cells, as well as the strong background stain of the $CD138^+$ TMA, makes the association unreliable.

B cells

The results of Paper IV show a strong association between $CD20^+$ B cell expression and an improved TTR and OS in I-type and PB-type adenocarcinoma. B cells have previously been associated with an improved prognosis in a number of cancers (51-54). Previous studies on high $CD20^+$ B cell expression in PDA, have however shown decreased OS (191, 192), and only one study have shown an association between high density $CD20^+$ B cells and improved survival, but it had to be related to the ratio of neutrophils/B cells (193). In Paper III, on oesophageal and gastric adenocarcinoma, $CD20^+$ B cells did not confer an improved prognosis.

A possible explanation for the positive effect on survival might be the antigen-presenting role of B cells, thereby facilitating the activation of the cell-mediated anti-tumour immune response (194), more specifically cytotoxic T cells (195).

The significant interaction seen in PB-type tumours between adjuvant chemotherapy and high $CD20^+$ B cell expression in relation to an improved survival merits further investigation. Previous reports have shown that gemcitabine and 5-FU, independently, induce apoptosis in MDSC but also increase $CD8^+$ T cell cytotoxicity, IFN-γ signalling and NK cell activity (196, 197). Gemcitabine can furthermore decrease T_{reg} density (198). High $CD20^+$ B cell density as a positive predictor of chemotherapy is not a game changer when the OS for the disease is 6% even with gemcitabine, but an increased understanding of tumour cell and immune system interactions will enable us to find new ways of treating this disease.

Correlation between pIgR, B cells and plasma cells

As shown in Table 3 and 4, the correlation between pIgR and B cells and $IGKC^+$ plasma cells was weak in oesophageal adenocarcinoma, but moderate in gastric and I-type periampullary adenocarcinoma. In PB-type periampullary adenocarcinoma pIgR showed a moderate correlation with $IGKC^+$ plasma cells, but a very weak correlation with B cells. The correlations were generally not strong, but overall moderate. Hypothetically, pIgR expression could be upregulated when high numbers of $IGKC^+$ plasma cells are present producing large amounts of antibodies. However, the link between an increased intraductal presence, after pIgR transcytosis, of antibodies and improved survival seen for $IGKC^+$ plasma cells in Paper III and $CD20^+$ B cells in Paper IV is not obvious.

Table 3. Spearman's correlation coefficient for the investigated markers in oesophageal and gastric adenocarcinoma.

	Oesophageal adenocarcinoma			**Gastric adenocarcinoma**		
	pIgR	**CD20**	**IGKC**	**pIgR**	**CD20**	**IGKC**
pIgR						
R		0.111	0.180		0.287**	0.321**
p		0.373	0.141		0.005	0.001
n		67	68		94	96
CD20						
R	0.111		0.431**	0.287**		0.460**
p	0.373		<0.001	0.005		<0.001
n	67		70	94		100
IGKC						
R	0.180	0.431**		0.321**	0.460**	
p	0.141	<0.001		0.001	<0.001	
n	68	70		96	100	

Table 4. Spearman's correlation coefficient for the investigated markers in I-type and PB-type periampullary adenocarcinoma.

	I-type adenocarcinoma			PB-type adenocarcinoma		
	pIgR	**CD20**	**IGKC**	**pIgR**	**CD20**	**IGKC**
pIgR						
R		0.384**	0.333*		0.101	0.308**
p		0.002	0.010		0.299	0.001
n		61	59		108	107
CD20						
R	0.384**		0.507**	0.101		0.437**
p	0.002		<0.001	0.299		<0.001
n	61		59	108		108
IGKC						
R	0.333*	0.507**		0.308**	0.437**	
p	0.010	<0.001		0.001	<0.001	
n	59	59		107	108	

PB-type versus I-type

Currently, the dichotomisation of periampullary adenocarcinomas into PB-type or I-type is commonly used because histopathological origin rather than anatomical centre is more relevant for clinical outcome (114-117). Recently a report critical of this categorisation was published (199), raising issues of reproducibility and better prognosis PB-type ampullary tumours compared with other PB-type tumours. The results for the herein used pancreatic and periampullary cohort is shown Figure 10, where PB-type ampullary tumours had as poor prognosis as pancreatic adenocarcinoma and distal cholangiocarcinoma, PB-type ampullary tumours did however receive chemotherapy less frequently (data not shown). This finding is compatible with the view that tumours of pancreatic origin have a worse prognosis than cases of bile duct or PB-type of ampullary origin. This fact is important to keep in mind but does not necessarily change the usefulness of the dichotomisation into I-type and PB type periampullary adenocarcinomas.

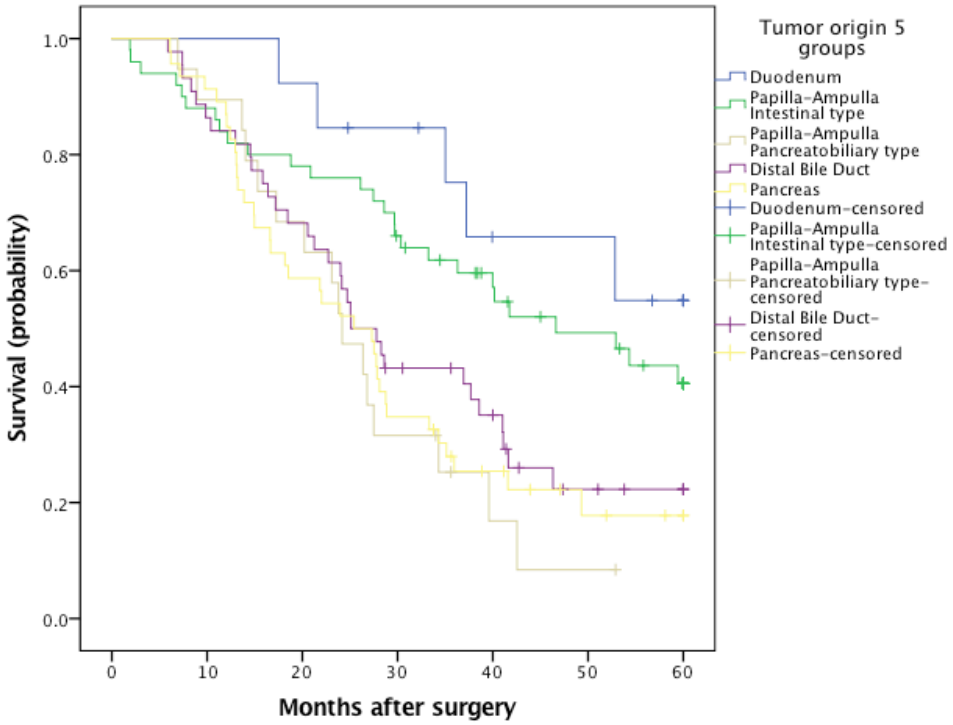

Figure 10. OS in relation to different tumour categories in the cohort of periampullary adenocarcinoma.

Strengths and Limitations

The major strengths of this thesis work are the oesophago-gastric and periampullary adenocarcinoma cohorts used. As two consecutive, retrospective series they were subjected to blind re-evaluation and have been extensively characterised and validated, providing excellent datasets for biomarker discovery.

The four papers of this thesis are of an exploratory nature and when making many statistical tests on a limited amount of data there is a risk of making type I statistical errors, i.e. finding significances by coincidence. One way of trying to correct that is the use of Bonferroni correction method that divides the p-value with the amounts of test performed, thereby increasing the significance level by diminishing the p-value of statistical significance. A similar way to prevent type I errors is to increase the limit of statistical significance from the commonly used

p=0.05 to p=0.001. There is however a balance, setting the significance levels too high might make some associations undetectable with the emergence of type II errors, that is especially important to consider in non-confirmatory research. Throughout this work p=0.05 was used as the significance level.

Classification and regression tree (CRT) analysis (200) was used in paper I, II and IV to decide the prognostic cut-off. Using the CRT analysis to find the mathematically most optimal cut-off value between two groups exposes your data to the risk of overfitting of the model. It is therefore important to ensure that the dichotomised groups are of similar size and the results should be regarded as descriptive and need to be validated in independent patient cohorts using the same cut-off values.

The use of the TMA technique raises the potential issue of representativity, especially of heterogeneously expressed biomarkers. To reduce this risk of sampling bias multiple cores from different donor blocks were used. It should however be pointed out that the use of full-face tissue sections, as being the alternative to TMA, will not fully circumvent the problem of representativity since it also only represents a part of the tumour, however much larger. It also introduces a lot more tissue that needs to be assessed, and stained with costly antibodies. While the use of TMA appears to be problem when analysing large diffuse B-cell lymphoma (160), there have not been many other reports on tissues unsuitable for analysis with the TMA technique.

Conclusions and Future perspectives

High pIgR expression is associated with an improved prognosis in oesophageal, GE junction and I-type periampullary adenocarcinoma.

High density of $IGKC^+$ tumour infiltrating plasma cells is associated with an improved prognosis in oesophageal and gastric adenocarcinoma.

High density of $CD20^+$ tumour infiltrating B cells is associated with an ameliorated prognosis in both I-type and PB-type periampullary adenocarcinoma.

The break through with check-point inhibitors has provided proof of principle that targeted immune therapy works in cancer and has lead to a surge in the search for other treatable immune targets.

The work of this thesis on the antibody transporter pIgR, B cells and plasma cells and their expression and relation to prognosis in oesophageal, gastric, pancreatic and periampullary adenocarcinoma is hopefully a small piece in the intricate puzzle of the interaction between cancer cells and the immune system. However,

the underlying mechanistic principles, and in a next stage targets for new drugs, still have to be deciphered. In that respect, a further characterisation of different B cell subpopulations, in particular B_{reg}, and how these contribute to immune system subversion will be of great value and an interesting avenue for future investigations.

Both cohorts used in this thesis work represent cancers that frequently present at a late tumour stage, often too late for curative intent treatment strategies. Unfortunately there are, as of yet, no biomarkers for the early detection of these cancers, that in addition are not as common as e.g. breast and colorectal cancer, further complicating the establishment of efficient screening programs. Even though there are many hurdles to overcome in finding good screening methods that will allow for early detection of these cancers, it is probably the most important area of research.

Populärvetenskaplig sammanfattning

Cancerutveckling uppkommer när kroppens normala celler börjar dela sig obegränsat, oberoende av omgivande celler och deras signaler. Cancercellerna tillägnar sig sedan olika egenskaper som ökar deras förmåga att växa till och så småningom kunna sprida sig till andra organ. Kroppens eget immunförsvar spelar en fundamental roll i kampen mot elakartade tumörer. En viktig mekanism bakom cancerutveckling är att cancercellerna tillägnar sig egenskaper som gör att immunförsvaret inte längre kan känna igen dem som främmande och eliminera dem. Immunförsvaret ska under normala förhållanden oskadliggöra bakterier, virus och cancer celler för att upprätthålla kroppens balans. I försvaret ingår ett snabbt, brett och ospecifikt skydd genom bl.a. barriärer och immunceller såsom neutrofiler, makrofager och dendritceller. Det finns också ett anpassat eller specifikt försvar som först behöver aktiveras innan det kan utöva sin effekt, det utgörs framförallt av T-celler, B-celler och antikroppar. I den inflammatoriska cancermiljön formar immunförsvaret cancercellerna så att det blir en selektion av cancerkloner som inte känns igen av eller som har lärt sig att hämma immunförsvaret. Denna process liknar till viss del den vid antibiotikaresistens där det också blir en selektion av de bakteriestammar som tål antibiotikan eller har lärt sig att försvara sig mot den.

I denna avhandling har jag studerat hur B-celler och antikroppsproducerande plasmaceller, samt den polymeriska immunglobulinreceptorn (pIgR), påverkar prognosen hos patienter med cancer i matstrupe, magsäck och i den s.k. periampullära regionen, d.v.s. området där bukspottkörtelgång och gallgång mynnar ut i tolvfingertarmen. Av de sistnämnda utgör bukspottkörtelcancer den vanligaste typen, cirka 65%,

Samtliga dessa cancerformer har en hög dödlighet och det finns ett stort behov av nya biomarkörer för att bättre kunna individualisera behandling samt för att finna nya behandlingsstrategier.

pIgR är ett protein som transporterar antikroppar genom ytceller, framförallt i tarmen. Väl ute på den andra sidan binder antikropparna in till bakterier och virus för att förhindra att de infekterar ytcellerna och tar sig in i kroppen. Ett högt pIgR uttryck har i tidigare studier kopplats till förlängd överlevnad i olika cancerformer såsom äggstock och urinblåsa, medan ett lågt pIgR uttryck har kopplats till

försämrad överlevnad i tjocktarms- och icke-småcellig lungcancer. I levercancer har dock ett högt uttryck av pIgR visat sig vara kopplat till försämrad överlevnad.

En del är känt om hur immunförsvaret interagerar med cancerceller och det finns till och med mediciner, antikroppsbehandling riktade mot T-celler, som stärker immunförsvarets förmåga att reagera mot cancercellerna,. Hittills har det dock funnits relativt beskedligt med data om B-celler och plasma celler. B celler bildas i benmärgen och tar sig sedan till lymfkörtlar där de väntar på att bli aktiverade. När detta sker utvecklas de till plasmaceller och börjar producera antikroppar, vilket är deras viktigaste funktion. En hög andel B-celler har visats leda till förlängd överlevnad i bröst-, livmoderhals, tjocktarm samt icke-småcellig lungcancer. Försämrad överlevnad har dock rapporterats i äggstockscancer.

Plasmaceller bildas från B-celler och kan ses som kroppens ”antikropps-fabriker”. Vi har använts oss av två olika markörer för att identifiera plasmaceller. De har något olika profil där CD138, ett protein som är involverat i cellers bindning till varandra, även kan finnas på andra av kroppens celler och tumörceller. CD138-positiva plasmaceller har blivit kopplade till både god och dålig prognos i olika cancerformer. Immunoglobulin kappa C (IGKC), som är den vanligaste av två möjliga lätta kedjor på en antikropp (2 tunga och 2 lätta), har kopplats till förbättrad överlevnad i tjocktarm-, bröst- och icke-småcellig lungcancer.

Den här avhandlingen omfattar fyra stycken delarbeten och undersökningarna har utförts i tumörer från två olika patientgrupper. Den första gruppen omfattar 174 patienter som opererats för cancer i matstrupe eller magsäck vid universitetssjukhuset Lund/Malmö mellan den 1 januari 2006 och den 31 december 2010. Den andra tumörgruppen omfattar 175 patienter som opererats för periampullär vid universitetssjukhuset Lund/Malmö mellan den 1 januari 2001 och den 31 december 2011. För att på ett snabbt och säkert sätt kunna analysera förekomsten av olika biomarkörer skapades vävnadsmatriser, s.k. tissue microarrays, med tumörer från de olika patientgrupperna. Förekomsten av B celler och plasmaceller i tumörernas omgivning samt uttrycket av pIgR i tumörcellerna analyserades med hjälp av immunohistokemi, en teknik där man låter antikroppar binda in till det ämne man vill undersöka. Bindning ger sedan, via ytterligare en antikropp, en färgreaktion som kan bedömas i ljusmikroskop.

I delarbete I undersökte vi hur pIgR uttrycks och hur det är kopplat till prognos vid matstrups- och magsäckscancer. När vi tittade på gruppen som helhet fann vi minskad risk både för återfall samt förlängd överlevnad hos patienter vars tumörer hade ett högt uttryck av pIgR. När vi tittade i undergrupperna matstrupe, magsäck samt övergången mellan dem båda, den övre magmunnen, så fann vi dock ingen skillnad i överlevnad kopplat till uttrycket av pIgR.

I delarbete II undersökte vi hur pIgR uttrycks och hur det är kopplat till prognos vid periampullär cancer. Vi studerade dels hela gruppen och dels två olika undergrupper; tumörer av s.k. pancreatobiliär typ (PB-typ) respektive intestinal typ (I-typ). I hela gruppen samt i intestinal typ fann vi att ett lågt uttryck av pIgR var signifikant kopplat till såväl förkortad tid till återfall som överlevnad. Detta samband sågs inte i tumörer av PB-typ.

I delarbete III undersöktes relationen mellan densiteten av B-celler och plasmaceller och prognos vid matstrups- och magsäckscancer. Vid matstrupscancer fann vi att patienter vars tumörer hade en hög andel plasmaceller som uttryckte IGKC hade en signifikant längre tid till återfall och längre överlevnad. Vi fann inget samband mellan andelen plasmaceller som uttryckte CD138 eller B-celler och tid till återfall eller död.

I delarbete IV undersöktes relationen mellan densiteten av B-celler och plasmaceller och prognos vid periampullär cancer. Analysen gjordes den här gången dator-assisterat med s.k. digital bildanalys. Det fanns en stark korrelation mellan plasma celler av IGKC typ och B-celler i både I-typ och PB-typ av periampullär cancer, alla övriga korrelationer var dock svaga. Det var en signifikant association mellan B-celler och förlängd överlevnad både i PB-typ och I-typ av periampullär cancer. Vidare fann vi att den gynnsamma prognostiska effekten av en hög andel B-celler i tumörer av PB-typ endast gällde patienter som fått cellgiftsbehandling efter operation.

Sammanfattingvis vet vi nu mer om hur pIgR, B-celler och plasmaceller uttrycks och är kopplade till prognos i dessa cancerformer. Fynden måste dock bekräftas i ytterligare studier och de underliggande orsakerna bör undersökas vidare, så att vi förhoppningsvis kan hitta nya behandlingsstrategier för att förbättra överlevnaden för dessa patienter.

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