Pancreatic Cancer

Experimental and Clinical Studies

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Pancreatic Cancer
- Experimental and Clinical Studies

A doctoral thesis at a university in Sweden is produced either as a monograph or as a collection of papers. In the latter case, the introductory part constitutes the formal thesis, which summarizes the accompanying papers.

Front cover: Pancreatic duct from 20th US Ed Gray's Anatomy, Lea & Febiger, New York 1918.

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To Elias and Benjamin - the future is so bright

He who loves practice without theory
is like the sailor who boards ship
without a rudder and compass
and never knows where he may cast

– **Leonardo da Vinci**

Abstract

Pancreatic Cancer – Experimental and Clinical Studies

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Background Pancreatic cancer is one of the most lethal of known cancers and the only treatment with possibility of cure is surgery. The costs associated with treatment of pancreatic cancer are reputably high, both in terms of morbidity and financially. To reinforce decision making there is a need to assess the costs and benefits of current treatment. Furthermore, the incitements to develop therapeutic alternatives and biologically characterize individual tumors are considerable.

Methods Evaluation of effects of proteasome inhibition on intracellular signaling systems using *in vitro* and *in vivo* experiments. Estimation of achieved utilities and direct healthcare costs based on a clinical cohort. Assessment of prognostic significance of structural genomic aberrations using comparative genomic hybridization and single nucleotide polymorphism analysis on resected tumor tissue.

Results Proteasome inhibition activated an antiapoptotic and mitogenic therapy resistance response in several mediators (EGFR, JNK, ERK and PI3K/Akt) and the inhibition of Akt and JNK increased the tumoricidal effect of proteasome inhibitors. The activation was EGFR independent and the increased cell death was not NF-κB mediated.
Patients undergoing resections with curative aim and patients receiving palliative care both experienced decreased health related quality of life in all SF-36 dimensions at diagnosis, without apparent improvement over time. The cost of treatment for patients undergoing surgery was two times the cost for the palliative patients (€50,950 vs. €23,701). Interestingly, already after one year the achieved QALY was twice as large in the resection group (0.48 vs. 0.20) resulting in cost per QALY neutralization between groups.
DNA copy number alterations were seen in 2p11.2, 3q24, 8p11.22, 14q11.2 and 22q11.21. No convincing specific aberrations of prognostic value were found. Short survival was however responsible for 67% of total copy number variation and associated with significantly more amplifications, possibly related to alterations in chromosome 2, 11 and 21.

Conclusions Proteasome inhibition is a promising adjunct in horizontal targeted therapy regimens and the effect may be potentiated by simultaneous inhibition of signaling systems. Costs for pancreatic cancer surgery are comparable to other major healthcare interventions and long term survival in a few is effectively increasing cost-effectiveness on patient group basis. DNA from patients with poor prognosis contains more amplifications and seems to be generally more degenerated possibly indicating a greater genomic instability. The pancreatic cancer mutational profile is displaying vast inter-individual heterogeneity and most mutations are probably passengers.

Keywords: Pancreatic Neoplasms; Proteasome Inhibitors; Apoptosis; Intracellular Signaling Peptides and Proteins; Epidermal Growth Factor Receptor; Pancreaticoduodenectomy; Cost and Cost Analysis; Quality-Adjusted Life Years; DNA Copy Number Variations; Comparative Genomic Hybridization

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List of papers

The thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

Paper	Field	Title	Content	Publication
I	Experimental Therapeutics	Proteasome Inhibition Activates Epidermal Growth Factor Receptor (EGFR) and EGFR- Independent Mitogenic Kinase Signaling Pathways in Pancreatic Cancer Cells	*In vitro* and *in vivo* studies of proteasome inhibitors activating EGFR, ERK, JNK and PI3K/Akt mitogenic pathways	Clin Cancer Res 2008;14(16): 5116-5123 doi: 10.1158/1078-0432.CCR-07-4506
II	Health Economy	Cost-Utility Estimation of Surgical Treatment of Pancreatic Carcinoma Aimed at Cure	Clinical outcome, HRQL and hospital-based direct costs for resections	World J Surg 2011;35(3): 662-670 doi: 10.1007/s00268-010-0883-8
III	Health Economy	Cost-Utility Estimations of Palliative Care in Patients with Pancreatic Adenocarcinoma; a Retrospective Analysis	Survival, HRQL, hospital-based and primary health care costs for all diagnosed	World J Surg Aug;37(8):1883-91 doi: 10.1007/s00268-013-2003-z
IV	Biologic Characterization	Sequence-Alterations in Tumor DNA as Related to Short Postoperative Survival in Patients Resected for Pancreatic Carcinoma Aimed at Cure	Comparative genomic hybridization and single nucleotide polymorphism assessment of tumors in short and long term survivors undergoing resection for cure	Manuscript

Abbreviations

ANOVA	Analysis of variance
ASCO	American society of clinical oncology
BCL-2	B-cell lymphoma 2; apoptosis regulatory protein
BRCA1/2	Breast cancer 1/2, early onset
CA19-9	Carbohydrate antigen 19-9 or Cancer antigen 19-9
CBA	Cost benefit analysis
CDKN2A	Cyclin-dependent kinase inhibitor 2A
cDNA	Complementary deoxyribonucleic acid
CEA	Cost effectiveness analysis
CER	Cost effectiveness ratio
CGH	Comparative genomic hybridization
CI	Confidence interval
CIN	Chromosomal instability
CNA	Copy number alteration
CNV	Copy number variation
CSC	Cancer stem cells
CUA	Cost utility analysis
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ERK	Extracellular signal regulated kinases
FAMMM	Familial atypical multiple mole melanoma syndrome
FoSTES	Fork stalling and template shifting
GWAS	Genome wide array study
HNPCC	Hereditary non-polyposis colorectal cancer
HRQL	Health related quality of life
ICER	Incremental cost effectiveness ratio
IPMN	Intraductal papillary mucinous neoplasia
IκB	Inhibitor of kappa B
IQSP	Integrated quality-survival product
JPS	Japan pancreas society
JNK	c-Jun N-terminal kinase
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LD	Linkage disequilibrium
LOH	Loss of heterozygosity
MAPK	Mitogen-activated protein kinase
MCN	Mucinous cystic neoplasm
MLH1	mutL homolog 1, colon cancer, non-polyposis type 2
MSH2/6	mutS homolog 2/6, colon cancer, non-polyposis type 1
MSLN	Mesothelin
mTOR	Mechanistic target of rapamycin
NAHR	Non-allelic homologous recombination
NF-κB	Nuclear factor of kappa light-chain enhancer of activated B cells

NHEJ	Non-homologous end joining
NICE	National institute for health and clinical excellence
OR	Odds ratio
Panel	US panel on cost-effectiveness in health and medicine
PanIN	Pancreatic intraductal neoplasia
PDAC	Pancreatic ductal adenocarcinoma
PI	Proteasome inhibition
PI3K	Phosphoinositide-3 kinase
PMS2	Postmeioitic segregation increased 2
PROM	Patient reported outcome measure
QALY	Quality adjusted life year
RAF	v-raf-1 murine leukemia viral oncogene homolog 1
RalGDS	Ral guanine nucleotide dissociation stimulator
RR	Relative risk
SE	Standard error of the mean
SF-36	Medical outcome study 36-item short form health survey
SF-6D	Short form 6 dimensions
SMA	Superior mesenteric artery
SMAD4	SMAD family member 4 or Mothers against decapentaplegic homolog 4
SNP	Single nucleotide polymorphism
STK11	Serine/threonine kinase 11
TGF-β	Transforming growth factor β
TP53	Tumor protein p53
TSG	Tumor suppressor gene
UICC	Union for international cancer control
UPD	Uniparental disomy
VEGF	Vascular endothelial growth factor
WTP	Willingness to pay

Table of Contents

Introduction

The History of Pancreatic Surgery

Any journey of studying a phenomenon should be embarked in the light of historical efforts. The pancreas was first described by Herophilos, one of the founders of the school of medicine in Alexandria, in the 4th century BC. The name 'pancreas' is Greek for "all flesh" and is traced to the 2nd century AD and another Greek physician, Ruphos. The first demonstration of the pancreas as an exocrine gland was exercised in 1663 in Leiden by Regnier de Graaf and ten years later the first experimental pancreatectomies in animals was performed in Paris by Johann Brunner. The pancreas was however inaccessible to surgeons due to its anatomical position for another two hundred years until the end of the nineteenth century. At that time the inventions of anesthesia, microscopy, infection control and radiology enabled the first attempts at major surgical interventions.

Soon pancreatic tumors with cholestasis could be palliated by biliodigestive bypasses; in 1886 a cholecystogastric anastomosis was established by Felix Terrier in Paris and one year later Kappeler performed a cholecystojejunostomy on this indication in Switzerland. Cesar Roux described the roux-en-Y reconstruction in 1897 and his mentor Kocher developed a method to mobilize the duodenum and the head of the pancreas to facilitate surgery in this region, published in 1902. Already in 1882 Friedrich Trendelenburg, a surgery professor in Bonn, performed the first distal splenopancreatectomy, the patient died however a few weeks after discharge. It lasted four years until his assistant Witzel published the case.

Alessandro Codivilla in Imola, Italy was foremost a pioneer in orthopedic surgery, interestingly he also performed the first pancreaticoduodenectomy in 1898, alas the patient died on the 24th day. Eleven years later in 1909 Walter Kausch in Berlin-Schöneberg performed the first of a series of pancreaticoduodenectomies, the first patient survived several months but was followed by disappointing results in later patients. Due to these poor results only a few further attempts were done until Allen Oldfather Whipple performed his first pancreaticoduodenectomy at a patient with ampullary neoplasia and cholestasis at the Presbyterian Hospital in New York in 1934. This was followed by two other patients after which he described his method, initially a two-step procedure with cholecystogastrostomy and gastrojejunostomy followed by pancreaticoduodenectomy without pancreaticojejunostomy or gastric resection performed several weeks later[1].

The Pancreas

The pancreas is a large compound gland found in vertebrates containing both exocrine cells (forming acini) and endocrine cells (forming islets of Langerhans). It

forms from the embryonic foregut through a ventral and dorsal endodermal bud subsequently fusing. The duct of the ventral bud forms the main duct (Duct of Wirsung) draining the whole pancreas and the duct of the dorsal bud remains as the accessory duct (Duct of Santorini) in two thirds of the population.

The origin of the exocrine and endocrine cells has been shown to be the same carbonic anhydrase II positive ductal progenitor cells that from late gestation to after birth (and possibly lifelong) can differentiate to both acini and islets[2]. The endocrine islet cells constitutes only about two percent of the cell mass but secrete various hormones; insulin and amylin (β-cells), glucagon (α-cells), somatostatin (δ-cells), pancreatic polypeptide (PP- or γ-cells) and ghrelin (ε-cells). The remainder of the gland is arranged in acini where exocrine cells produce digestive enzymes; proteolytic enzymes cleaving proteins to peptides (trypsin, chymotrypsin, carboxypolypeptidase, elastase and nuclease), amylase for carbohydrate cleavage into di- and tri-saccarides and enzymes for fat digestion (lipase, cholesterol esterase and phospholipase). The proteolytic enzymes are held inactive by the trypsinogen inhibitor until reaching the intestine in order to prevent autodigestion of the pancreas.

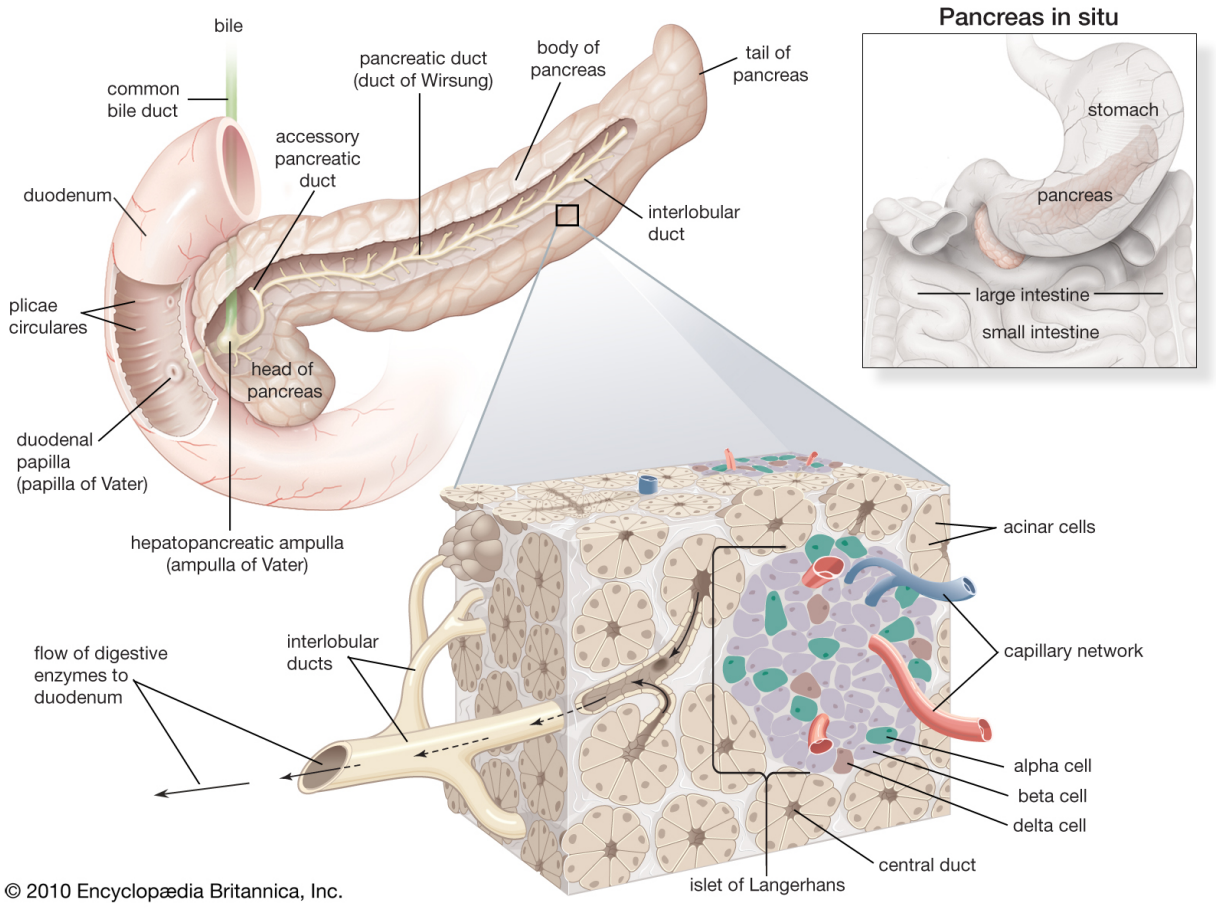


Fig. 1. Pancreas, Art. *Encyclopædia Britannica Online*[3]. By courtesy of Encyclopaedia Britannica, Inc., © 2010; used with permission.

To neutralize acid gastric juice a variable amount of sodium bicarbonate and water is secreted from ductal cells. The regulation of the secretion are from three main stimuli; acetylcholine, cholecystokinin and secretin. The first two stimulate the acinar cells more than the ductal cells yielding large concentrations of enzymes in little fluid; the reverse is true for the latter.

Tumors of the Pancreas

The versatility and activity of the pancreatic cells outlined above may be part of the answer to why pancreatic tumors present in so many forms. Along with the paradigm of the cancer stem cell (CSC) hypothesis the ability of stem cells, progenitor cells and mature cells to alter properties during life most likely results in an ever-increasing heterogeneity in terms of cell properties[4]. A true pancreatic CSC compartment has so far not been found but facultative stem cells, cells with ability to acquire *stemness* through trans- or de-differentiation is possible; one candidate is the centroacinar cells at the junction between acini and the ducts, showing a persistent expression of developmental markers[5,6]. $CD44^{+}CD24^{+}ESA^{+}$cells have been shown to have a 100-fold tumorigenic potential compared to normal tumor cells[7].

The hallmarks of cancer initially described by Hanahan and Weinberg are sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis[8]. The conceptualization is now expanded to include two emerging hallmarks; deregulating cellular energetics and avoiding immune destruction, and two enabling characteristics; genome instability and tumor promoting inflammation[9].

The picture is made even more intricate by the increased understanding of the importance of the stroma; the recruitment of non-epithelial cells to form the tumor microenvironment, clearly playing an important role in the tumorigenesis. Due to the rapid increase of knowledge of developmental and neoplastic cell biology, at present it is required to have a more differentiated view on the classification of tumors than before and guidelines are regularly reviewed. In the formation of a neoplastic lesion there is a continuum of cells with highly individual differentiation where even the lesion itself is heterogeneous containing clonal expansion with disparate genomic mutations and epigenetic alterations[10].

The conceptual framework for determinants of this inter- and intra-individual phenotypic heterogeneity is continuously evolving. The genomic instability and branching evolution is causing genotype diversification, where the interaction of multiple coexisting neutral mutations possibly creates additional phenotype diversification. Exceeding the buffering capacity of the heat shock protein response increases the diversification even more[11]. This genetic heterogeneity is also modulated by an abnormal epigenetic landscape. These factors are causing a deterministic heterogeneity of phenotypes. Apart from this, the stochastic nature of biochemical processes influences, among other things, gene expression patterns

enabling transitions between phenotypic states. Taken together this implies important obstacles in diagnostic accuracy from tissue sampling and causes development of clonal chemotherapy resistance[12].

Classification

Traditionally the term pancreatic cancer is used synonymously with pancreatic ductal adenocarcinoma (PDAC) as it constitutes more than 85 % of pancreatic neoplasms[13]. PDAC develops to about 70 % in the pancreatic head and displays a fulminant clinical course unparalleled by any other solid tumor. In this thesis PDAC will be in focus. The location is however at the crossroad of several epithelial structures; each of them the potential origin of a solid tumor and clinically often indistinguishable. For this reason treatment strategies are affected by the possibility of a less common (and usually less aggressive) tumor.

The main types of periampullar cancer are PDAC, cholangiocarcinoma and duodenal adenocarcinoma. These have been shown to logically intersect in one type based on anatomy, often reported as a separate neoplasm; ampullary adenocarcinoma. In the ampulla (or papilla of Vater) the common bile duct and pancreatic duct epithelium merge with the duodenal mucosa in a transition zone. A thorough assessment of the origin of ampullary tumors was performed by Kimura et al[14]. By histological and immunohistochemical analysis it was concluded that three fourths of the tumors in their material arose from the pancreaticobiliary epithelia (72 %) and the remainder from the duodenal mucosa. These intestinal type tumors show histologic similarities with colorectal cancer with APC mutation and microsatellite instability and have a far better prognosis than the pancreaticobiliary type.

There are rare tumors that do not fit into this classification; these include the undifferentiated adenocarcinoma, sarcomatoid carcinoma, squamous cell carcinoma, colloid carcinoma and medullary carcinoma. Furthermore there are neoplasms displaying a spectrum from pure acinar cell adenocarcinomas transdifferentiating to ductal cell carcinoma most likely involving the centroacinar cells showing many duct cell characteristics[15]. The developmental relation between exocrine pancreas and endocrine pancreas enables tumor formation with various degrees of neuroendocrine cell properties as well as true neuroendocrine tumors along a spectrum from non-functioning to functioning and from poorly differentiated to highly differentiated endocrine tumors, the latter usually with a more indolent clinical course and separate genetic profile[16,17].

Carcinogenesis

At present there are three main PDAC precursor lesions identified. The first and most common, is the pancreatic intraepithelial neoplasia (PanIN) sequence of microscopic lesions usually arising in small branch ducts. PanIN-1, existing in up to 40 % of normal adult pancreata, are papillary or micropapillary, shows minimal

atypia and is subclassified into A or B depending on presence of micropapillary infoldings of the epithelium. PanIN-2 lesions are similar to PanIN-1 but have nuclear abnormalities such as loss of polarity, hyperchromatism and enlarged nuclei. PanIN-3 can display budding off of epithelial cells into the lumen or luminal necrosis, occasionally abnormal mitoses and dystrophic goblet cells. It is seen in only 5 % of pancreata without invasive carcinoma but in 30 to 50 % of those with. This association suggests the higher grades can be associated with pancreatic carcinoma[18].

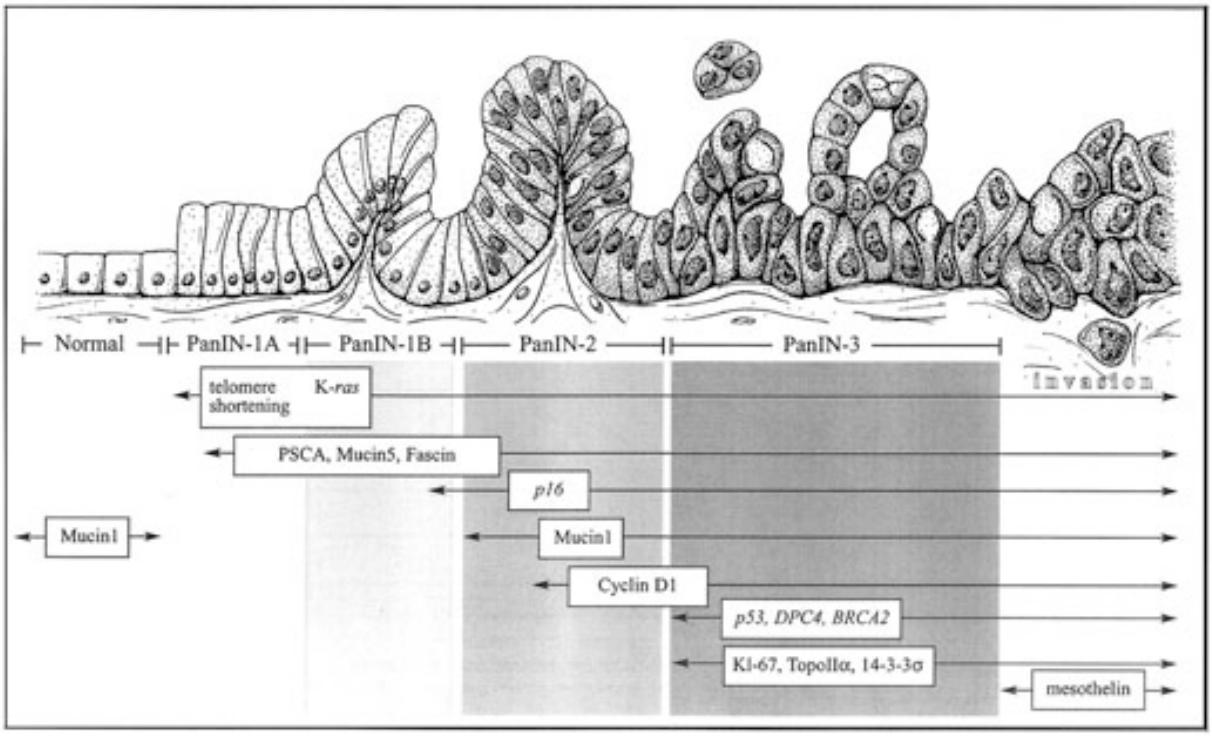


Fig. 2: "PanINgram". Reprinted by permission from Macmillan Publishers Ltd: Modern Pathology, Maitra et al[19]. © 2003.

The other two lesions are often macroscopic and increasingly detected as 'incidentalomas' on computed tomographies performed on other indications. Intraductal papillary mucinous neoplasms (IPMNs) are mucin-producing neoplasms that typically present in the head of the pancreas in communication with the ducts, the common main duct type with greater malignant potential and the less common branch duct type with a more favorable prognosis. It is usually subclassified in an adenoma–borderline–*carcinoma in situ* sequence depending on degree of dysplasia and in a gastric-, intestinal-, pancreaticobiliary- or oncocytic type. Mucinous cystic neoplasms (MCNs) are mucin secreting cystic epithelial neoplasms most often found in the body and tail of pancreas that do not communicate with the duct. They are usually solitary lesions with pseudocapsule to 90 % arising in women. The cysts are lined with columnar epithelia with atypia standing on a characteristic 'ovarian-like stroma'. One third have an invasive component, often focal, demonstrating a significantly worse prognosis[20].

Pathology and Histopathology

PDAC is characterized by early invasion and lymph node metastases[21]. Some reports suggest that as many as 75% of T1 tumors already are metastasized[22]. There is also frequent metastasizing to liver (80 %), peritoneum (60 %), lungs and pleura (50-70 %) and the adrenals (15 %) and sometimes direct overgrowth on stomach, colon or spleen[23]. Cell differentiation can be seen from well to poor and a typical feature is the abundance of desmoplastic stroma, a fibrous reactive tissue putatively produced by pancreatic stellate cells that is mixing with the epithelial cells and extending into surrounding pancreas creating atrophy or ductectasias. The neoplastic cells are usually cylindric with clear cytoplasm, sometimes cubic with reduced cytoplasm and less frequently display goblet cell appearance[13]. Mucin secretion is common. In a proportion of tumors there are a significant amount of endocrine differentiated cells with expression of neuroendocrine markers, the behavior is however dictated by the exocrine component. There is often an unusually aggressive neuronal infiltration even in small tumors indicating that this is an early event in carcinogenesis.

Molecular Biology

During carcinogenesis the vast range of genomic mutations, epigenetic alterations and microenvironmental changes dictate the phenotypic development. Mutations in various genes and regulatory domains cause deregulation of core signaling pathways ultimately affecting most cellular processes. In pancreatic cancer the most commonly mutated genes are KRAS, SMAD4, TP53 and CDKN2A (p16). The mutated oncogene KRAS is upregulating downstream signaling cascades primarily via the Raf/ERK pathway, the RalGDS pathway and the PI3K/Akt pathway, thereby acting on several downstream targets such as the transcription factor NF-κB and mTOR achieving increased proliferation, resistance to apoptosis, angiogenesis and invasion[6]. The PI3K/Akt pathway is of major importance in tumor development and it has been shown that most of the mediators are mutated or amplified in a range of tumors[24]. SMAD4 is a protein binding to phosphorylated R-SMADs after TGF-β receptor tetramerization. This complex is subsequently transferring to the cell nucleus to regulate transcription factors[25]. The tumor suppressor p53 is a crucial component of DNA damage surveillance acting through induction of apoptosis, cell-cycle arrest and repair[26]. Loss of function causes genomic instability. CDKN2A (p16) is also a tumor suppressor that arrests the cell cycle to inhibit cell growth. These are only a few of the myriad alterations reported so far. The pancreatic cancer genome is discussed further on page 29-32.

Epidemiology

Pancreatic cancer is increasingly common, reaching its highest incidence in developed regions of North America, Japan and Europe. It here ranks fourth of cancer death causes and the death rate is close to the incidence. Predicted number

of deaths for 2013 in the EU was 40,069 for men and 40,197 for women, corresponding to an age-standardized death rate of 8 and 5.5 per 100,000 respectively[27]. The reason for differential incidence in sexes is not known. Hormonal factors have not been shown to affect incidence in women[28]. The main non-hereditary risk factors are old age, smoking (OR 3), obesity (OR 1.72) and chronic pancreatitis (OR 26.3)[29,30]. Diabetes with recent onset is probably an early sign of tumor development and the reverse causality is unlikely. Alcohol is arguably not an independent risk factor but conditional on development of chronic pancreatitis. Coffee or tea consumption is not associated with increased risk either.

Current knowledge attributes only 5 % to heredity. The number of affected first-degree relatives is however an important risk factor; two first-degree relatives without known cancer susceptibility gene mutations causes an OR of 4.25[30]. Important cancer susceptibility genes are BRCA1 and BRCA2 in the cancer predisposition syndrome Hereditary Breast and Ovarian Cancer Syndrome, the latter causing a RR of 3.51 for pancreatic cancer through impaired DNA mutation repair. Patients with HNPCC (Hereditary Nonpolyposis Colorectal Cancer) carry mutations in mismatch repair genes MSH2, MSH6, MLH1 and PMS2 causing microsatellite instability, which is resulting in an 8.6 fold increase also for pancreatic cancer compared to the general population. A germline mutation in the CDKN2A (p16) tumor suppressor causes the FAMMM (Familial Atypical Multiple Mole Melanoma) syndrome associated with a 20 % lifetime risk of pancreatic cancer. Individuals with the Peutz-Jeghers syndrome with mutation in the STK11 tumor suppressor gene carry a lifetime risk of 36 %. However, the issue of ascertainment bias has been raised for this group, a common problem when establishing risks in subpopulations[30].

Pancreatic Cancer Staging

Stage	T	N	M	Description
0	Tis	N0	M0	Carcinoma *in situ*, includes PanIN-3
Ia	T1	N0	M0	Limited to pancreas, ≤ 2 cm
Ib	T2	N0	M0	Limited to pancreas, > 2 cm
IIa	T3	N0	M0	Beyond pancreas but no celiac axis or SMA involvement
IIb	T1	N1	M0	Limited to pancreas, ≤ 2 cm, regional lymph node metastasis
	T2	N1	M0	Limited to pancreas, > 2 cm, regional lymph node metastasis
	T3	N1	M0	Beyond pancreas but no celiac axis or SMA involvement, regional lymph node metastasis
III	T4	Any N	M0	Celiac axis or SMA involvement
IV	Any T	Any N	M1	Distant metastasis

From UICC TNM 7th Ed. 2009

Clinicopathological Prognostic Factors

Established clinicopathological factors commonly stated to have relevance for survival are clinical staging according to UICC (Union for International Cancer Control) (Table above) and JPS (Japan Pancreas Society)[31], tumor size[32], node status and node ratio[33,34]. It is interestingly shown that even the number of assessed lymph nodes have prognostic meaning, likely due to being a general quality indicator[35]. The importance of involvement of resection margins are ambiguous with reports of both non-significance[36,37] and significance[38,39]. This unclarity can in part be due to variations in pathological reporting as the introduction of standardized protocols have increased the R1 frequency drastically[40,41]. Obvious signs of advanced disease such as distant metastases and peritoneal engagement carries prognostic value as does extrapancreatic nerve plexus infiltration[31]. The drawback of this information (with the exception of radiological findings) is that it is available only after resection and meticulous pathology and, hence, cannot be utilized in treatment planning at diagnosis.

The only serological marker with some prognostic value that is widely used in clinical practice today is preoperative CA19-9. A finding of >37 U/ml which is a cutoff based on standard deviation in normal population has been shown to be highly prognostic[31]. ASCO (American Society of Clinical Oncology) has stated it has no use in selection of patients accessible to curative surgery but values above 130 in patients with pancreatic head mass without jaundice is highly predictive of systemic spread and should lead to staging laparoscopy[42]. Research to evaluate new molecular markers has so far been disappointing. Winter et al used tissue microarrays from short (<12 months) and long (>30 months) survivors; from 13 putative biomarkers only mesothelin (MSLN) was prognostic in a multivariate analysis adjusting for standard pathological features[43].

Study Background and Theoretical Framework

Pancreatic ductal adenocarcinoma (PDAC) is notoriously biologically aggressive. The overall 5-year survival is as low as 5%[27,44,45] despite considerable development in surgical and oncological treatment over the past decades. Surgery is considered to be the only chance of cure and usually implies a major anatomical reconstruction associated with a non-negligible risk of postoperative morbidity at high expenses. Nevertheless it is only possible to achieve about 20 % 5-year survival in this selected subgroup[38,46-49]. Chemotherapy and radiotherapy, adjuvant or as palliative treatment, have so far proven only marginal effect on survival, adding only one or two months to survival[50-52]. This is a strong incitement for the development of alternative and complementary treatment modalities (paper I). Moreover, to evaluate the burden of this disease on the patient and the healthcare system it is pertinent to perform a cost-utility estimation of palliative care and resections with curative intent (paper II and III). It is also necessary to develop tools to guide the selection of therapy along with the paradigm of personalized medicine (paper IV).

Experimental Therapeutics (Paper I)

In paper I we investigate the mechanisms of action of proteasome inhibition in cell lines *in vitro* and *in vivo.*

Conventional Chemotherapy

The limitations of traditional chemotherapy are evident from a great number of studies, many of which unfortunately underpowered and yielding conflicting results. Gemcitabine has for many years been the mainstay of adjuvant and palliative cytotoxic treatment in PDAC. When administered in an adjuvant setting it has a documented but modest effect on overall survival[50] and the ESPAC-3 trial could not show any difference between treatment with gemcitabine and 5-fluorouracil/folinic acid[53]. It is, however, apparent that single-drug treatment regimens are inadequate to surmount the divergent multitude of pro-survival pathways in the Darwinian selection process of heterogeneous cancer populations. New trials focus on multi-drug treatments; one example is FOLFIRINOX (oxaliplatin, irinotecan, leucovorin and 5-FU) showing a survival advantage vs. gemcitabine in metastatic pancreatic cancer but with increased toxicity[54]; another is the ongoing ESPAC-4 trial evaluating the gemcitabine and capecitabine combination as adjuvant therapy.

Targeted Therapeutics

As our knowledge of cellular molecular biology and cancer aberrations is expanding, opportunities to interfere with the neoplastic cell using biologically

active compounds targeting specific cellular mechanisms are being explored. One such target used in clinical practice is the Epidermal Growth Factor Receptor (EGFR), which is over-expressed in 90% of pancreatic tumors. The antibody cetuximab and the tyrosine kinase inhibitor erlotinib are two of the inhibitors used to suppress EGFR activity, both of which have reached use in the clinic. Erlotinib is approved by the Federal Drug Agency (FDA) and the European Medicines Agency (EMA) for use in combination with gemcitabine for treatment of locally advanced, irresectable or metastatic pancreatic cancer, however with only a marginal improval of overall and progression free survival. This modest response to targeted therapeutics is general, and the initially high expectations have not been met. It is increasingly apparent that the intracellular signaling pathways constitute a network of redundant mediators with complex interactions of forward and backward feedback loops of stimulation and inhibition. This is the foundation of the horizontal signal pathway inhibition strategy striving to counteract the compensatory up-regulation of alternative pathways by simultaneous blocking.

Proteasome Inhibition

One promising target is the 26S proteasome, the most common form of proteasome complex, responsive for degradation of unneeded or damaged intracellular proteins such cyclins, caspases and transcription factors, all of them important in cell homeostasis and frequently dysregulated in neoplasia. The description of this important proteolytic process involving ubiquitinization of proteins destined for degradation in all cells was rewarded the Nobel Prize in 2004. In multiple myeloma proteasome inhibition by bortezomib has been a great success as an effective monotherapy[55] and in solid tumors there is evidence in preclinical models for an additive effect of bortezomib[56], and the second-generation proteasome inhibitor marezomib (NPI-0052)[57], in multi-drug treatments. Disappointingly the results have not translated into significant response in the clinic, sometimes inducing inacceptable toxicity. This unpredictability is perhaps not surprising considering that the action of proteasome is universal and broadly active in all cells. Hence, the antitumoral mechanisms of bortezomib are only slowly being elucidated. One major mode of action seems to be suppression of the transcription factor NF-κB primarily resulting in down-regulation of anti-apoptotic genes[58].

There is a strong rationale for using proteasome inhibitors as chemosensitizers, the concept of combining targeted therapeutics and traditional chemotherapy for an additive effect[59]. Figure 3 illustrates how a stressor, such as chemotherapy, induces a phosphorylation cascade involving the IKK complex and the inhibitor IκB which is neutralized by the proteasome and thereby releasing NF-κB to promote transcription of anti-apoptotic and prosurvival genes. The inhibition of NF-κB by cDNA of super-repressor IκBα in a viral vector potentiated apoptosis by TNFα[60].

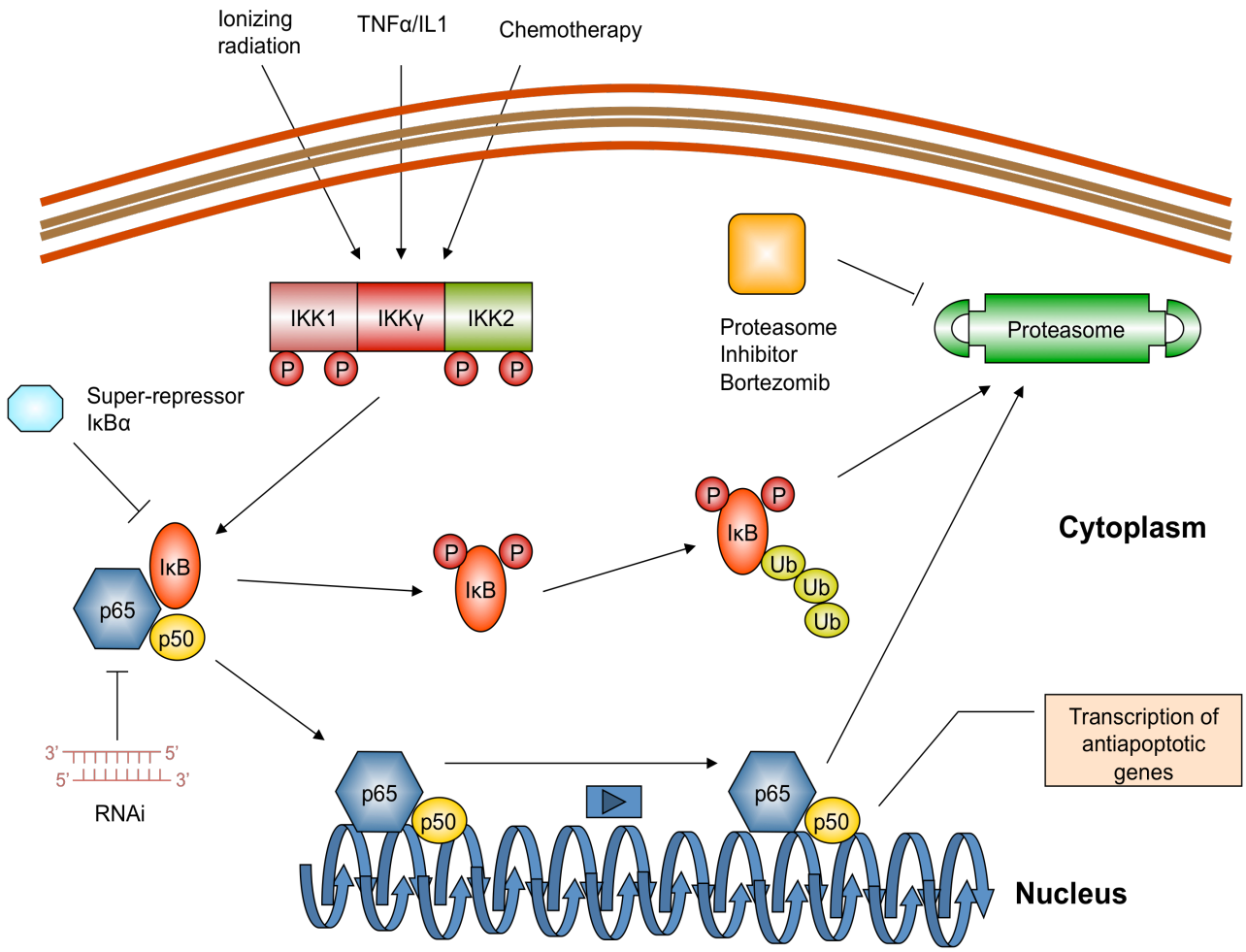


Fig. 3: Proteasome inhibition by bortezomib or marizomib (NPI-0052) induces a transcriptional antiapoptotic response. Own artwork from Cancer Drug Discovery and Development: The Oncogenomics Handbook, Humana Press Inc., Totowa, NJ[61]. With kind permission of Springer Science+Business Media.

This chain of events is also prevented by proteasome inhibition as is supported by findings of induced apoptosis in multiple myeloma cells resistant to dexamethasone[62] and powerful potentiation of irinotecan[56] and gemcitabine[63] respectively in pancreatic cancer xenografts. Results are however conflicting, some have reported activation of constitutive NF-κB but inhibition of induced activation by proteasome inhibition indicating that the relationship is complex[64].

Other observed downstream effects are induction of the caspase-cascade and p53 and a proapoptotic shift involving mitochondrial cytochrome c release and activation of the c-JUN N-terminal kinase (JNK) pathway. Hence, involvement of both the intrinsic BCL-2 mediated pathway and the extrinsic death-receptor mediated apoptotic pathway is apparent (Fig. 4). Apoptosis, programmed cell death, is together with cell division the means by which multicellular organisms maintain cell number homeostasis. Apoptotic dysregulation and immortalization is one of the principle properties of the cancer cell. Furthermore, angiogenesis has been shown

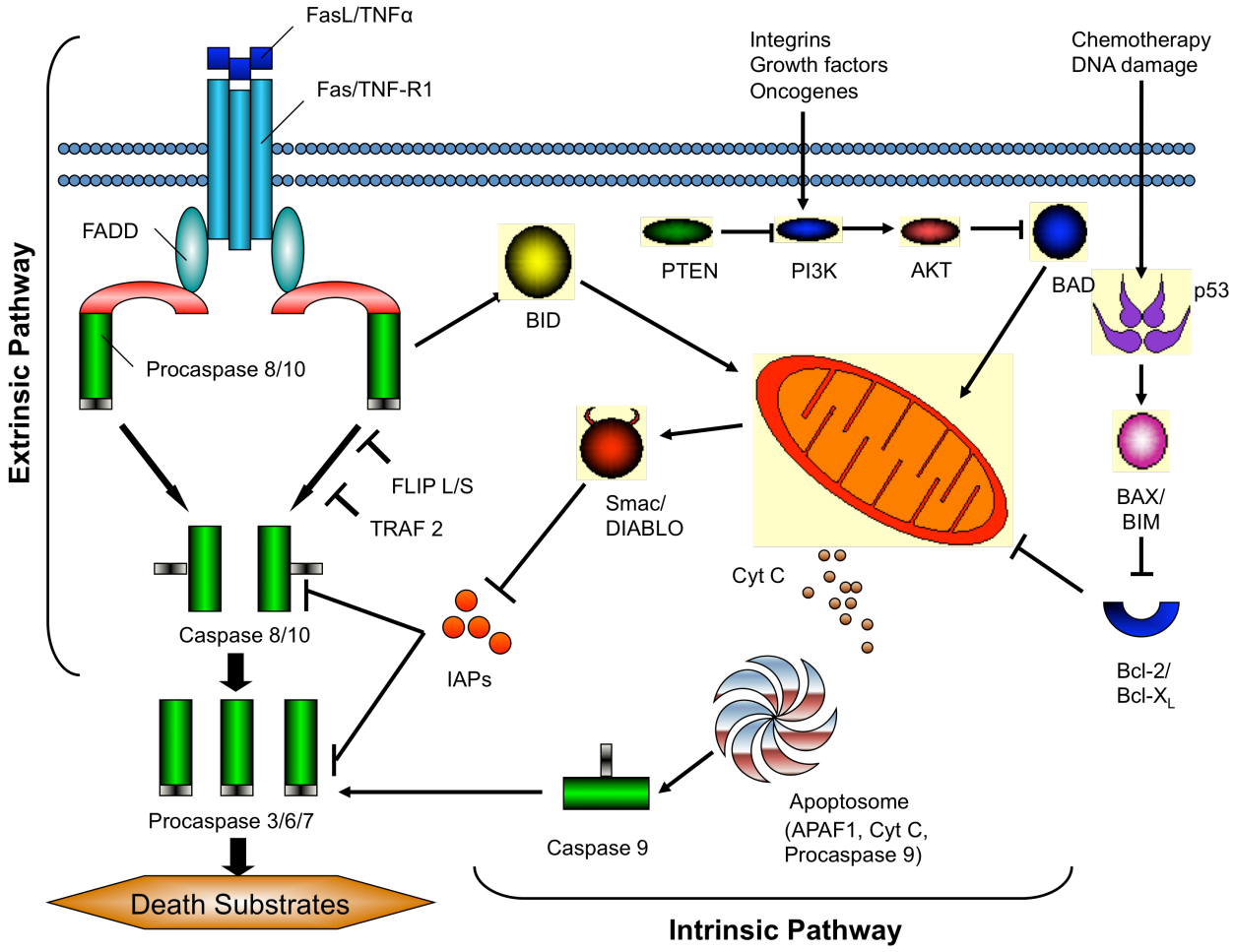


Fig. 4: Key apoptotic pathways. Own artwork from Cancer Drug Discovery and Development: The Oncogenomics Handbook, Humana Press Inc., Totowa, NJ[61]. With kind permission of Springer Science+Business Media.

to be inhibited[65] and virtually every other aspect of cancer dysregulation, i.e. cell cycle control, cell adhesion and migration and DNA damage repair, is affected by proteasome inhibition in pancreatic cancer[66].

This intricacy of the effects led us to investigate the intracellular signaling following proteasome inhibition in pancreatic cancer models, more specifically we hypothesized that proteasome inhibition activates a negative feed-back loop resulting in protection against the apoptotic effects of proteasome inhibition itself. To describe this we assessed four important components of the mitogenic and anti-apoptotic pathways: EGFR, Extracellular regulated kinase (ERK,) and c-Jun N-terminal kinases (JNK), both mitogen activated protein kinases (MAPK) and phosphatidylinositol-3-kinase (PI3K)/Akt. To interfere with cell signaling according to principles of horizontal blockade treatment combinations including EGFR inhibitor erlotinib, vascular endothelial growth factor (VEGF) antibody inhibitor bevacizumab and small molecule selective inhibitors of ERK-kinase (PD98059), JNK (SP600125) and PI3K (LY294002) were used.

Health Economy (Paper II and III)

In paper II and III we report on the direct healthcare costs and achieved utilities as measured by quality adjusted life years (QALYs) in patients treated for pancreatic adenocarcinoma.

In PDAC the dismal prognosis even after resection with curative aim together with a substantial procedure-related morbidity has nurtured a debate of the benefit of pancreatic cancer surgery. Several studies have aimed at evaluating the health related quality of life (HRQL) in this group[67-70] and others have estimated costs for pancreatic cancer treatment[71,72]. To our knowledge no earlier paper is written on costs and utilities combined. Also, in the progressively competitive health care market, where new expensive treatments must fit within a limited budget, it is increasingly important to be able to present good assessments of the cost for achievements, preferably by cost-effectiveness analysis. Therefore, we strive to shed some light on the question to what extent surgery is truly beneficial for the individual.

Health economy is a branch of economics, a social science, with a different conceptual framework where other principles and methodologies apply than in biological medical science. The field is rapidly developing due to the increased need among decision makers to put a value to healthcare interventions to enable prioritization as the available interventions, such as diagnostics and treatments, are increasing in number and cost and the healthcare budget is limited. Several methods have been developed to estimate the costs and effects of interventions; the cost-benefit analysis (CBA) values both costs and effects in monetary terms, the cost-effectiveness analysis (CEA) values effects in natural units (i.e. life years) and the cost utility analysis (CUA) measures outcome in utilities (i.e. a composite metric of life years and health related quality of life, such as a QALY).

A central component in the CEA and CUA is the establishment of a cost-effectiveness ratio (CER), putting a monetary value to the effect, usually QALYs, achieved. Using this information the new intervention can be compared to the standard alternative and an incremental cost-effectiveness ratio (ICER) may be calculated. ICERs are regularly collected in league tables that rank alternative healthcare interventions for use by decision makers for allocation of healthcare resources. Using league tables comparing results from studies in different countries, with different methodology, and from different time points has sometimes been deemed questionable[73]. Nevertheless, it is generally held that in addition to other available information it contributes as a basis in allocation decisions.

Utilities

The QALY is a compound product entity. One of the two factors that build the QALY is time measured in life years. This lifetime is multiplied by a quality factor between 0 that equals death and 1 representing perfect health and is thereby weighted, or

adjusted. The quality factor is called a preference-weighted health state utility because it is valued by HRQL that is adjusted for the preference or desirability of the health state. Consequently, the remaining lifetime QALYs is the sum of the products of each experienced health state multiplied by the time in that state. There are several ways to estimate preference-based health utilities. One is direct inquiry of the study group by using methods such as standard gamble (SG) where participants are asked to choose between a gamble and continuation of the present health state. The gamble is the probability between perfect health and certain death. Through assessing the risk of death acceptable to avoid the evaluated health state a score, or weight, can describe the severity of the health state (Fig 5).

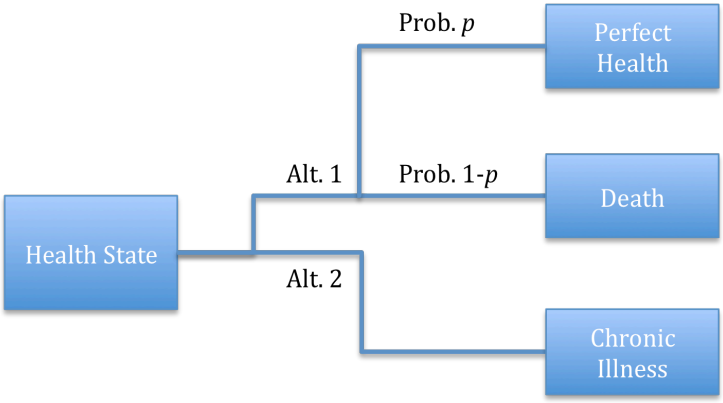


Fig. 5. Standard Gamble

An alternative is the time trade off (TTO) method where individuals are asked how much life in perfect health they are willing to give up to escape the health state in question. Both these are widely used and accepted, the visual analog scale (VAS) is also an option but concerns have been raised about not giving respondents the choice between two alternatives. Direct methods are however often difficult to administer, carries a low response rate and it is sometimes considered unethical to let patients in a difficult situation consider the prospects of death[74]. Therefore, indirect utility assessment methods have been developed in the form of questionnaires with attributes of generic health status. The items are given weight by letting representative samples of a population; healthy, those affected by the health state or professionals, value these different health states in a multi-attribute health status classification system. From this can be derived a utility score. Examples are EQ-5D and HUI.

The main source of HRQL data is however non-preference-based patient reported outcome measures (PROMs). A multitude of questionnaires are designed to cover different aspects of health; there are generic instruments such as SF-36 and EORTC QLQ-C30 assessing global health, disease specific scales such as the lung cancer symptoms scale (LCSS), the gastrointestinal quality of life index (GIQLI) or the pancreatic cancer specific supplementary EORTC module QLQ-PAN26 and finally domain or symptoms scales as the McGill nausea questionnaire (MNQ). The great advantage of generic scales is the theoretical possibility to compare results across diseases and disciplines. Conversely, the main disadvantage is that the items included may not reflect disease specific symptoms. These non-preference-based HRQL instruments ca be used in utility score estimation as in the example of SF-36 data conversion by SF-6D.

Economy

In all economic evaluation the resource spending should be valued by the next best use, the opportunity cost, the cost of the alternative[75]. Common is however to use surrogates as reported estimated market prices. When calculating health care costs it is important to decide upon which perspective is taken; societal, health care provider, payer or individual. The US panel on cost effectiveness in health and medicine (the Panel) and the Swedish Dental and Pharmaceutical Benefits Agency (TLV) recommends a societal perspective if possible[73,76]. However, since 2008 the National institute of health and clinical excellence (NICE) is using the health care perspective, by some considered a logical policy because of difficulties in robust measurements of effects outside healthcare system, additional costs of appraisal and that in most cases no difference would be made to the guidance[77]. It is therefore generally considered that the perspective is depending on the purpose of the evaluation[41]. The perspective taken decides what costs – direct (health care or non-health care) or indirect (dominated by loss of production costs) – are central, i.e. future costs are of considerable importance in some situations but not in others. In short, recommendations from the Panel are that all costs that are small in relation to the cost-effectiveness ratio is to be omitted but large costs are to be subject to a sensitivity analysis to establish their importance[78].

In economics the willingness-to-pay (WTP) is the maximum amount one is prepared to pay to receive a good or to avoid something undesired. Many studies in health economics are referring to an arbitrary WTP threshold, an estimation of what the society is prepared to spend on a QALY. This is usually in the magnitude of $35,000[79] and has been assessed to be originating from a decision by the US government to pass a law of reimbursement for patient with end stage renal disease to have hemodialysis within the Medicare program in 1972[80]. This caused a normative judgment of the societal WTP and was considered being on the limit of cost-effectiveness at that time. The whole concept of putting a monetary value to a human life year or a utility unit is controversial but the need for tools to aid allocation is recognized by most people[81].

QALY Calculations

A central component in health economic evaluation is the sensitivity analysis. The purpose is to reveal how robust assumptions are, in essence how deviation from expected values of input variables are affecting the result. A first order sensitivity analysis is testing uncertainty of external factors and second order sensitivity analysis is variation of assumptions within a model. It can be performed as a univariate (one-way) or multivariate process. Effects on results and conclusions are studied as different possible values are imputed for major assumptions.

Another factor needed to account for is the positive time preference. It is the mechanism by which humans tend to value immediate benefit over future benefit. In line with the opportunity cost rationale it is common to compensate for this in health economical calculations by discounting. Conventional is equal discounting of

both utilities and costs at annual rates of about 5% but a range of rates have been proposed[82]. In the Netherlands utilities are discounted less than costs and there are proponents of no discounting of utilities, as there is an assumption of risk neutrality in the QALY concept.

The criticism towards the use of CUA in resource allocation decisions is mainly based on the ethical problems of using a WTP threshold and the well-known conceptual limitations of QALY. These include strong assumptions that, appropriately or not, simplify reality[83]:

1. Each individual is risk neutral with respect to longevity
2. Utility is additive with time and the positive time preference could be taken into account through discounting
3. Value scores or preferences measured across individuals can be aggregated and used for the group
4. QALYs can be aggregated across individuals

Issues like what method and whose values to be used in forming preference based utility functions remain focus for controversies[84]. Indeed, there are several concerns; the many methods to elicit utility values yield disparate results, patients are not willing to trade lifetime to avoid health states as expected due to adaptation and finally the QALY fails to incorporate two important psychological mechanisms; that humans value depending on reference points and aspirations and that goods tend to have diminishing marginal utility (also when outcomes are uncertain)[85]. Nevertheless, it is important to remember that QALYs provide a convenient yardstick when comparing health interventions in different diseases and can be useful as additional information besides expert opinions in distribution decisions of health benefits. The QALY concept is endorsed by the WHO as the preferred utility unit in CUA[86].

In economic evaluation of healthcare interventions data on costs and consequences are established along trials, through systematic overviews or through modeling. The modeling approach is useful when ethical or logistic reasons make direct comparisons impossible. This is commonly done through Markov decision modeling where probabilities, as in Bayesian statistics, are assigned to health states and cost effectiveness can be established through several assumptions and a stochastic process.

Biological Characterization (Paper IV)

To create further insight into the biology of PDAC we performed a combined CGH-SNP array accounted for in paper IV.

Currently the decision of resectability of pancreatic tumors is based mainly on patient fitness and radiological staging. Preoperative tissue biopsies are not recommended due to uncertainty of representability, fear of tumor seeding and lack of clinically useful information[87]. As survival is limited for most patients also after resections with curative intent the need for better biological characterization is apparent. Along with the paradigm of PDAC as a genetic disease we set out to investigate the relation between long-term survival, expected to represent an inert and truly operable phenotype, and a specific genotype cluster.

The body of information on genetic and epigenetic changes in human cancers is growing rapidly as a consequence of the development of new high throughput platforms and increasing computational power for bioinformatical analysis. This is providing the foundation for linking the genotype to the cancer phenotype through genome annotation (the process of linking biologic information to a sequence) and exploration of the tumor development (carcinogenesis) and progression and metastasis (phylogenetics) of the tumor. As mentioned before the conceptual framework of carcinogenesis is increasingly complex; the cancer phenotype is stipulated to develop through a multistep genomic mutation sequence, in part regulated by epigenetic changes and evidently heterogeneous with subclonal evolution characterized by different mutational profiles and different gene regulatory networks[12,88]. Apart from the epigenetic instability, where changes can cause DNA hypermetylation or histone modification altering the gene expression[89], there is an abundance of genetic changes occurring in carcinogenesis and tumor progression. In this paper we focus on DNA mutations occurring due to the genomic instability of the tumor.

DNA Aberrations

Chromosomal instability (CIN) is a form of genetic instability implying an increased tendency to acquire chromosomal aberrations. This increases the likelihood of copy number alterations (CNA)[90]. Non-disjunction during meiosis is the most common mechanism of rendering the cell aneuploid, one whole extra copy causes trisomy, two tetrasomy and loss of a copy monosomy. There are also structural abnormalities such as rearrangements, deletions, insertions, inversions and amplifications causing segmental aneuploidy. Furthermore, in tumor material it is not uncommon with different cell populations displaying different genomes, mosaicism.

After the completion of the human genome project it has been discovered that there is a normal inter-individual variance of CNA called copy number variation (CNV). These segments ranges from about 1 kb to several Mb constituting about 13% of the

human genome[91]. The CNV is partly inherited through germline mutations, and partly somatic *de novo* mutations. The phenotypic effects are depending on alterations of dosage sensitive genes or regulatory segments and disease can theoretically be caused by interaction between two or more coexisting CNV segments. Evolutionary serial segmental duplications have created low-copy repeats (LCR) constituting up to 5% of the human genome. LCRs over 10 kb and high degree of sequence identity can cause local genomic instability and stimulate CNV formation through nonallelic homologous recombination (NAHR). Two other mechanisms of CNV formation are nonhomologous end joining (NHEJ), responsible for DNA double strand break repair, and fork stalling and template switching (FoSTeS)[91].

Other forms of DNA variation are polymorphic repeats of DNA sequences (microsatellites), single nucleotide insertions and deletions causing frame shift mutations and point mutations causing single nucleotide exchange resulting in single nucleotide polymorphisms (SNP). The latter are DNA sequence variants with a prevalence of >1% in humans[92]. From SNP data it is increasingly evident that human disease is not only inherited, in Mendelian fashion, by gene mutations with near-complete penetrance but also by influence from low penetrant mutations, as illustrated by susceptibility loci in breast cancer (Fig. 6). The concept likely applies also for pancreatic cancer.

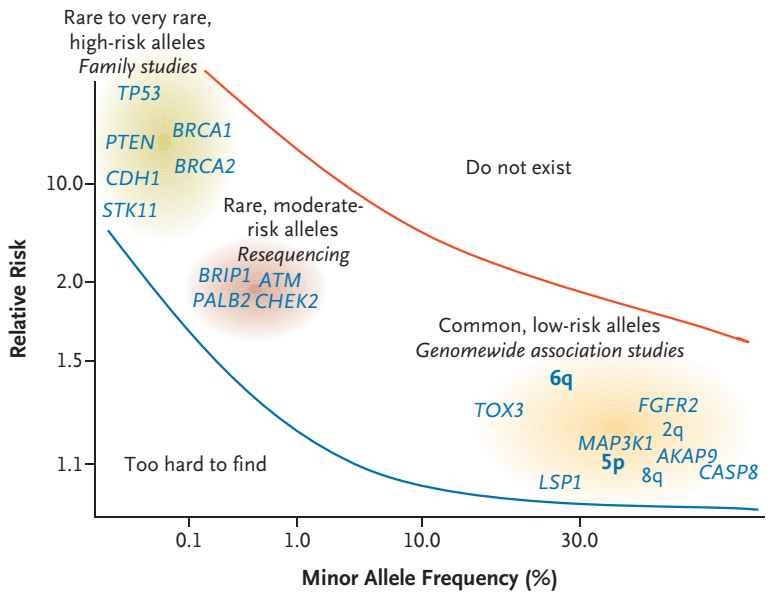


Fig. 6: Susceptibility loci in breast cancer. Reproduced with permission from Foulkes et al[93]. © Massachusetts Medical Society

Genomic Mapping

In the last decade the mapping of known SNPs has seen a great utility in genome wide array studies (GWAS) linking genomic sequence variants to disease. Associating the approximately 10 million known SNPs of a human genome to disease may seem insurmountable. This has however become possible by utilizing knowledge of linkage disequilibrium (LD) implying a non-random association of alleles at two or more loci. This means neighboring SNPs and CNVs are linked in haploblocks showing high degree of conservation between recombination hotspots[94]. Many CNVs are tightly linked to nearby SNPs but as there are others occurring in regions with higher propensity of recombination, hybrid array platforms for simultaneous detection of SNPs and CNVs has been developed. One example is the Agilent SurePrint G3 Human CGH+SNP we have utilized.

One major advantage with the hybrid platforms is the ability to detect not only CNV using comparative genomic hybridization (CGH) but also copy neutral alterations such as uniparental disomy (UPD) causing loss of heterozygosity (LOH) utilizing SNP probes. This is relevant in tumor material considering the established two-hit hypothesis of Knudsen stipulating that tumor suppressor genes (TSGs) are commonly inactivated following sequential mutation of both alleles as they are recessive[95]. An LOH at a tumor suppressor site can be expected to be a susceptibility locus for cancer development. Apart from TSGs, oncogenic mutations arise from gain-of-function mutations activating proto-oncogenes to usually dominant oncogenes. This can occur through various mechanisms such as a point mutation causing a constitutively acting protein product, gene amplification causing overexpression of protein products or translocations changing the regulatory promotor elements leading to increased expression[96].

The Pancreatic Cancer Genome

Pancreatic cancer is characterized by CIN with an unusually high degree of fold-back inversions (ends mapped in reverse direction) and deletions[88]. Breakage-fusion-bridge cycles forming fold-back inversions could indicate dysregulation of the G1-S phase and intact G2-M checkpoint. Also, fusion of chromosome ends is often associated with telomere erosion, a hallmark of malignant cellular replication. One indirect sign of CIN is marked intratumoral mutational heterogeneity. This is particularly evident in PDAC where KRAS activation, loss of TP53 function and defects in the mitotic spindle apparatus likely contribute to this[97].

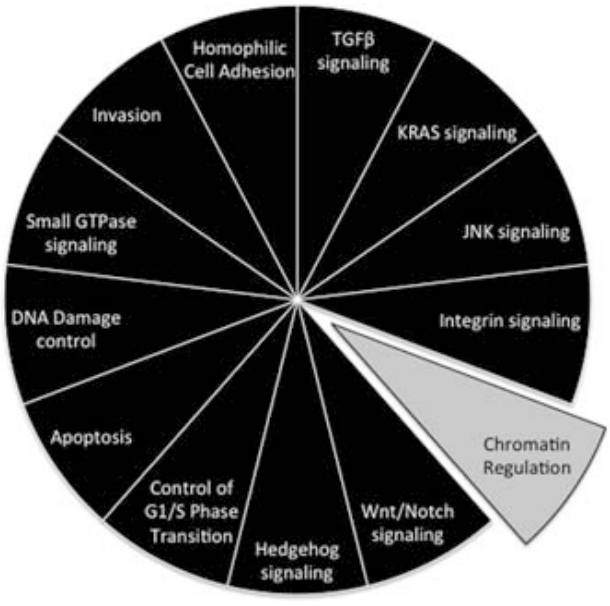


Fig. 7. Core signaling pathways in pancreatic cancer. From Macgregor-Das et al[98]. © 2012 Journal of Surgical Oncology, Wiley Periodicals, Inc. Reproduced with permission of John Wiley & Sons, Inc.

KRAS and TP53 are two of the four known high frequency mutated genes in pancreatic cancer occurring in 95 % and 70 % respectively, the other two are CDKN2A and SMAD4, occurring in 90 % and 55 % respectively[99-103]. These are considered *driver* mutations with own capacity for carcinogenesis[104,105]. Activating point mutation of proto-oncogene KRAS is considered an early event as it is abundant in PanIN1 lesions, inactivation of tumor suppressor gene (TSG) CDKN2A is frequent in PanIN2 and inactivation of TSGs TP53 and SMAD4 in PanIN3. Hence, these are considered later

events in carcinogenesis[98]. These are included in the progression model displayed in figure 2. It is proposed that apart from these aberrational "mountains" there are "hills", genes altered in lower frequency[106]. It has also been put forth that the majority of pancreatic cancers have mutations of genes affecting most of the key signaling pathways and processes[107]. However, the specific components altered in each pathway vary between individual tumors (Fig. 7).

Considering this current knowledge of mutational heterogeneity in PDAC we set out to assess whether distinct aberrational patterns could be linked to survival in patients that had their tumors resected with curative intent. The promise on the other end was increased understanding of the basis for disease, aiding development of tools for early detection and personalized treatment.

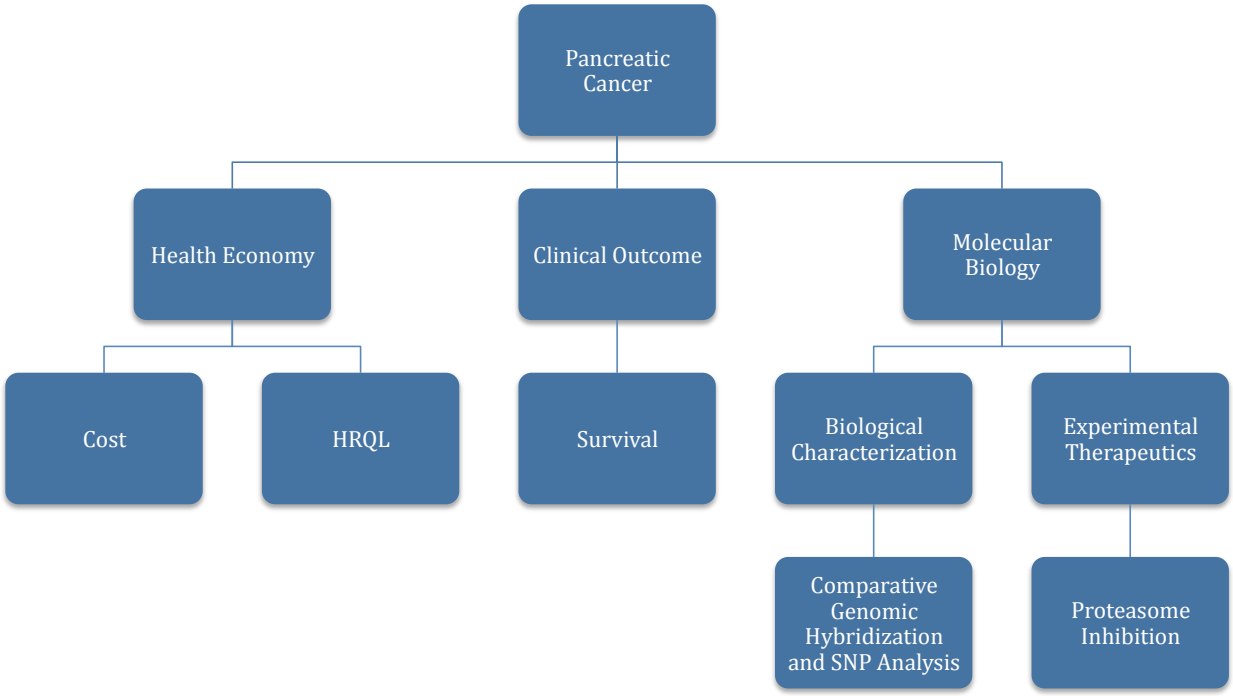
Aims of the Thesis

The relatively high incidence and continuously dismal prognosis of pancreatic cancer poses a significant clinical challenge. We therefore aim to illuminate the burden of the disease on patients and healthcare system under current treatment routines and to strive to enable personalized medicine by biologic characterization and development of targeted therapeutics.

Specifically to:

- Explore the effects of proteasome inhibition on mitogenic signaling pathways in pancreatic cancer cells *in vitro* and *in vivo* (I)
- Estimate the costs and utilities of pancreatic cancer resection and palliation (II and III)
- Put the cost-utility of resective treatment in perspective by comparing with palliative non-curative treatment and other healthcare interventions (II and III)
- Find genomic mutations corresponding to short-term survival in patients that have undergone cancer surgery with curative aim to achieve improved biologic classification (IV)

Structure



Methodological Considerations

Overview

Paper	Study design	Methods	Statistics
I	Experimental	*In vitro* and *in vivo* tumor models ELISA Western Blot Flow cytometry	One-way ANOVA and post hoc Dunnett´s
II and III	Cost-utility estimation	Retrospective medical record review Immunohistochemistry PROMs SF-6D conversion Cost registry mining	Descriptive Non-parametric mean comparison Kaplan-Meier survival QALY calculation
IV	Explorative genetic correlation study	Prospective tumor tissue collection Retrospective medical record review DNA extraction CGH+SNP microarrays	Descriptive Cross tabulation One-way ANOVA Logistic regression Kaplan-Meier survival Cox proportional hazard Clonal fraction ADM-2 aberration analysis Cluster analysis Penetrance analysis Common alteration analysis

Experimental Therapeutics (Paper I)

Cell Culture (I)

Cell cultures and *in vitro* studies are key in exploring tumor biology, enabling investigations that would have been impractical or impossible otherwise. There are usually no major ethical considerations and the experiment time is short providing quick results. However, it is of importance to note that the cell culture as a tumor model have inherent limitations. These include, but are not confined to; non-physiological circumstances such as inadequate electrolyte levels and insufficient oxygen supply, architectural differences with lack of extracellular matrix and diminished cell contacts due to monolayer culture formation leading to impaired intracellular signals and, importantly, only one cell type reducing cell-cell interaction[108]. The cells are monoclonal with a fixed genotype and are further degenerated during maintenance and repeat passages. Rapid growth is promoted through addition of fetal bovine serum with growth factors and together with subclonal selection in culture propagation limiting the alternative of cell

differentiation thus driving cells toward dedifferentiation[109]. Nevertheless it remains an important model for basic science such as experimental therapeutics in oncology.

Cell cultures were obtained from the American Type Culture Collection and cultured according to standard procedures. Panc-1 is an extensively used cell line of pancreatic adenocarcinoma with known mutations in KRAS and TP53. To adjust for cell type specific factors two more cell lines were used; BxPC-3 (wildtype KRAS) and Capan-2 (wildtype TP53). Panc-1 and Capan-2 cells were grown in DMEM and BxPC3 in RPMI - 1640, with 10% fetal bovine serum, 100 µg/mL of streptomycin, and 100 µg/mL of penicillin at 37°C with 5% CO2.

In Vitro Measurement of Apoptosis (I)

Apoptosis was measured through detection of histone-associated DNA-fragments as a late event in apoptotic cells using the Cell Death Detection ELISAPlus kit from Roche Applied Science (Indianapolis, IN). Following the manufacturers protocol the ELISA is rather user-friendly yielding usually consistent and reproducible results. In short the treated cells were centrifuged and supernatant discarded, cells were lysed, centrifuged again and an aliquot of the supernatant exposed to a streptavidin-coated plate, a primary biotin-labeled antihistone antibody and a secondary peroxidase-conjugated anti-DNA antibody. The indirect method with a secondary antibody improves the sensitivity of the test. ABTS (peroxidase substrate) was added after washing with PBS three times and the absorbance was measured at 405 nm. By repeating the experiment several times reproducibility was ensured.

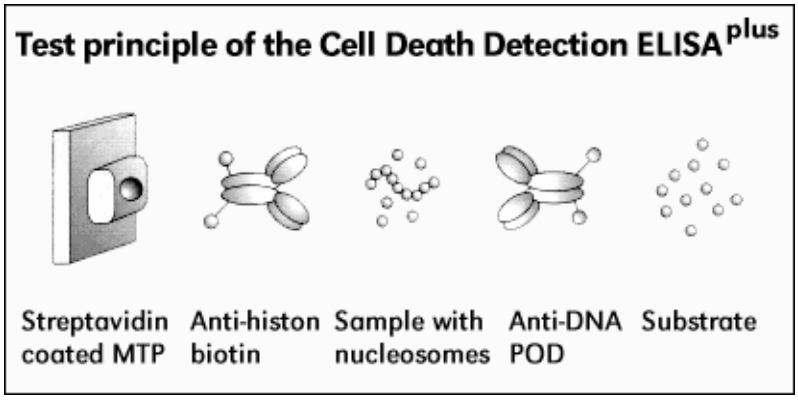


Fig. 8: The mode of action of the Cell death Detection ELISA (Enzyme Linked ImmunoSorbent Assay). Reproduced with permission from Roche Diagnostics.

We also used flow cytometric detection of Annexin-V (BD PharMingen), which is an anticoagulant protein binding to phosphatidylserine (PS) exposed on cellular membrane through phospholipid flipping early in apoptosis, and 7-AAD (BD PharMingen), a fluorescent compound with DNA affinity indicating exposed DNA through membrane rupture in late apoptotic and necrotic cells. The apoptotic fraction was defined as cells with low 7-AAD and high Annexin-V fluorescence. It is central to remember that this depicts only a window of the cell death event, as the

apoptotic bodies resulting from apoptosis will be interpreted as debris once the cell has decompositioned. Therefore what is seen is only the early phase of apoptosis and necrosis. There are numerous examples of pitfalls including cell harvest resulting in exposure of PS or the exposure of PS in healthy cells leading to false positive apoptotic cells and on the other hand there is evidence of programmed cell death without PS exposure yielding false negative cells. It is nevertheless an established and widely used method to compare the apoptotic fractions in different cell groups and gives valuable information if factors of bias are controlled[110].

In short, cells were seeded into six-well plates and allowed to adhere and grow overnight before treatment. After treatment, both floating and adherent cells were collected by trypsinization and washed twice in PBS. Cells (5 x 10^5) were resuspended in 500 µL of Annexin-V binding buffer (BD PharMingen) containing 5 µL of Cy5-Annexin-V. Cells were incubated for 5 min at room temperature in the dark before the addition of 5 µL of 7-AAD and analysis in a BD FACSCalibur flow cytometer (Fig. 9). The sample passes a laser and light is scattered. Assigned channels detect the fluorescence and cells are characterized as Cy5-/Cy3-, Cy5+/Cy3-, or Cy5+/Cy3+ populations representing live, apoptotic or necrotic cells.

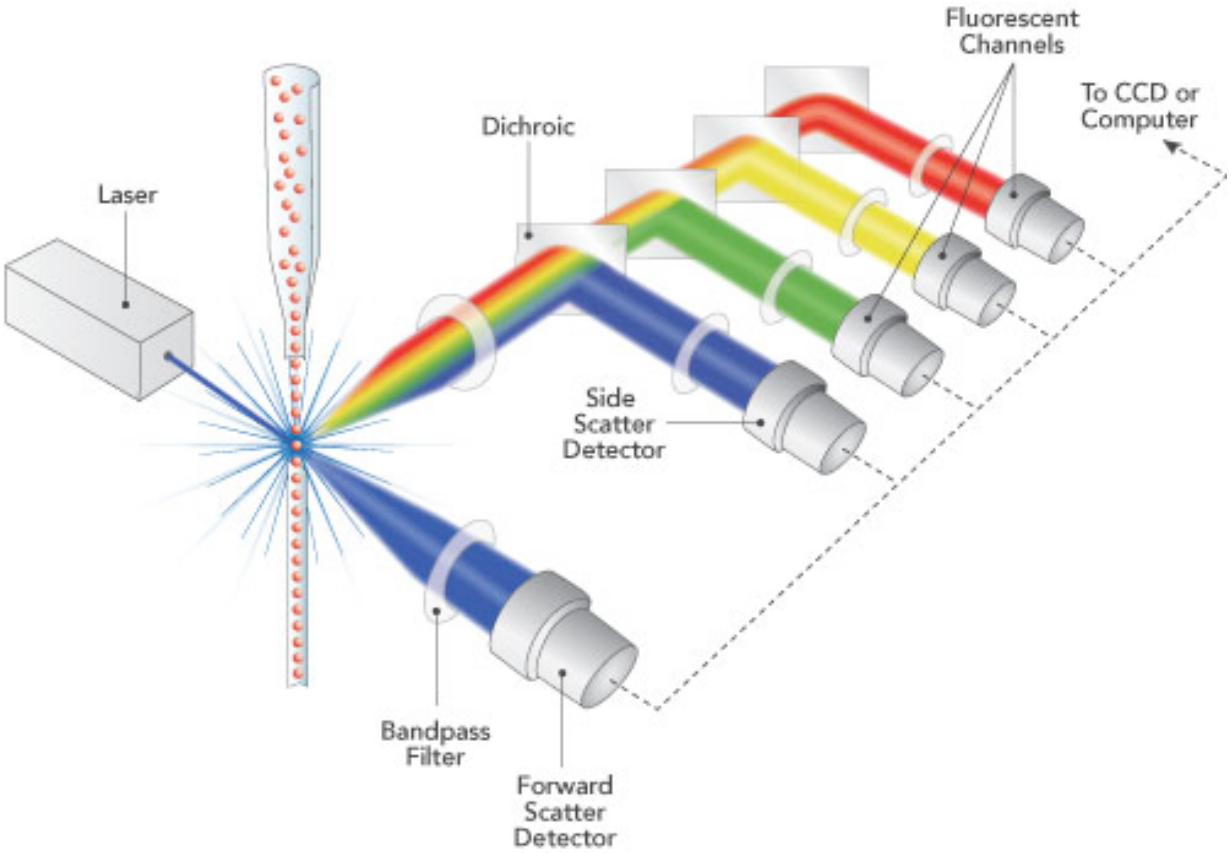


Fig. 9. The mode of action of flow cytometry. Reproduced with permission from Semrock Inc.

Western Blotting (I)

For detection of individual proteins the western blotting technique was used. In comparison with ELISA western blotting carries a higher specificity, thereby requiring less specific antibodies. In short the western blotting involves four main steps; the extraction of the proteins (cell surface receptors or intracellular kinases) through cell lysis, the separation of the proteins by applying an electrophoretic current to a gel where they travel depending on their molecular weight, transfer of proteins to a membrane for specific antibody staining and finally detection of the antibodies by ELISA and chemiluminescence.

Equal amounts of proteins were resolved on denaturing polyacrylamide gels, before being transferred to polyvinylidene difluoride membrane using the Bio-Rad Mini Trans-Blot cell system. Membranes were blocked in 5% nonfat milk dissolved in NaTT buffer [50 mmol/L Tris/HCl (pH 7.4), 150 mmol/L NaCl, 0.02% (v/v) Tween 20] for 2 h. Membranes were incubated with primary antibodies for 16 h at room temperature in NaTT containing 0.5% nonfat milk. Membranes were washed in NaTT before the addition of appropriate horseradish peroxidase – conjugated secondary antibody for 2 h at room temperature in NaTT containing 0.5% nonfat milk. Membranes were again washed using NaTT before visualization using enhanced chemiluminescence (Pierce Biotechnology, Inc.). Figure 10 a-d describes the workflow of western blot.

Antibodies were acquired from Santa Cruz Biotechnology unless otherwise stated and used at the following dilutions: p-EGFR-Y1173 (1:2,000), EGFR (1:1,000; Cell Signaling Technologies), p-ERK (1:5,000), ERK (1:3,300), p-JNK (1:10,000; Biosource), JNK1-FL (1:5,000), p-AKT (1:3,300), glyceraldehyde-3-phosphate dehydrogenase (1:10,000), and rabbit and mouse horseradish peroxidase secondary antibody (1:3,300).

The technique is well established but demanding and the many steps require attention to details. Impurities of sample, inadequate rinsing and blocking of excess protein or overexposure in the chemiluminescence phase can each produce a non-readable result and of course careless culturing of cells or inconsistent exposure to treatments will yield an erroneous result. There is also a possibility of incidental phosphorylation. Therefore at least two runs of each experiment were performed to ensure repeatability.

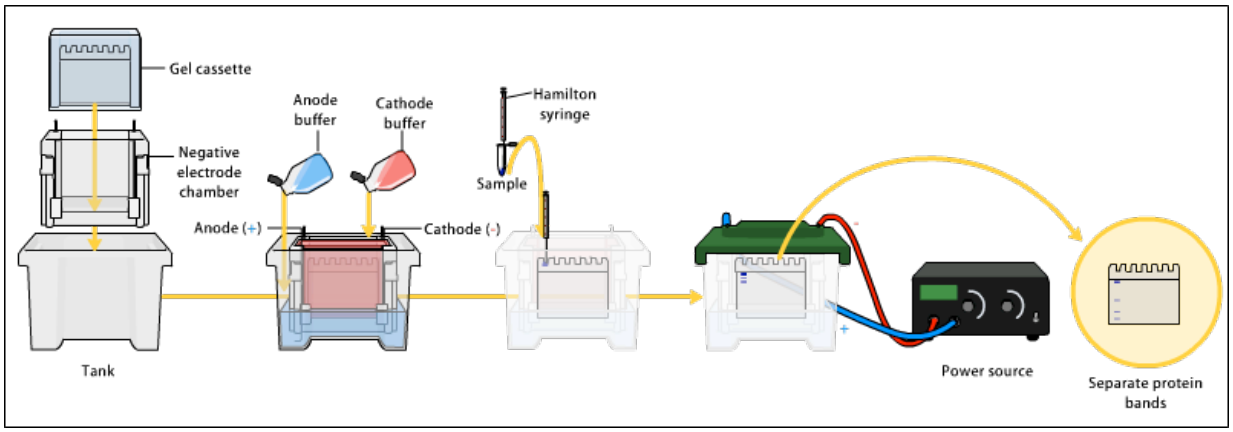


Fig. 10a. SDS polyacrylamide gel electrophoresis in western blot. ©User:Bensaccount. Wikimedia Commons. CC-BY-3.0.

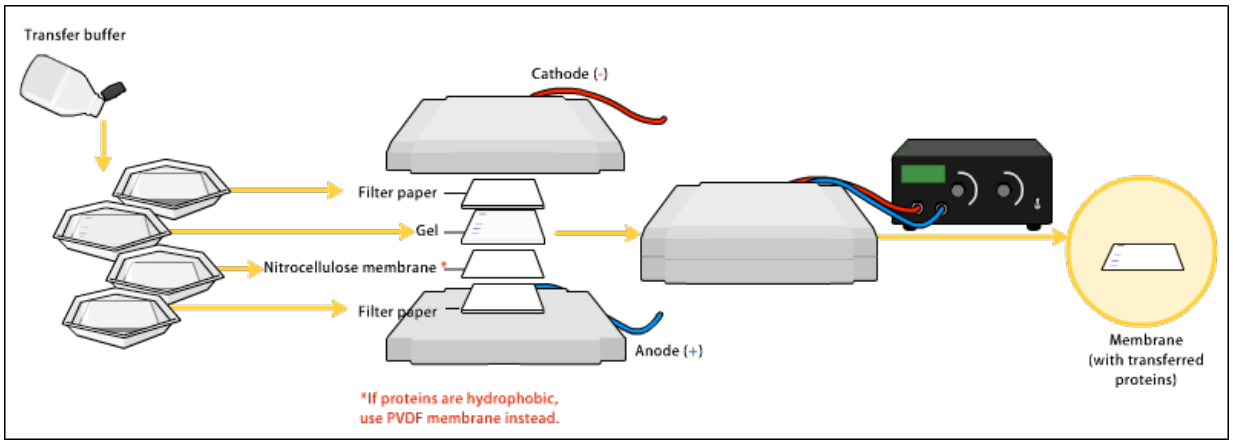


Fig. 10b. Transfer to polyvinylidene difluoride membrane in western blot. ©User:Bensaccount. Wikimedia Commons. CC-BY-3.0.

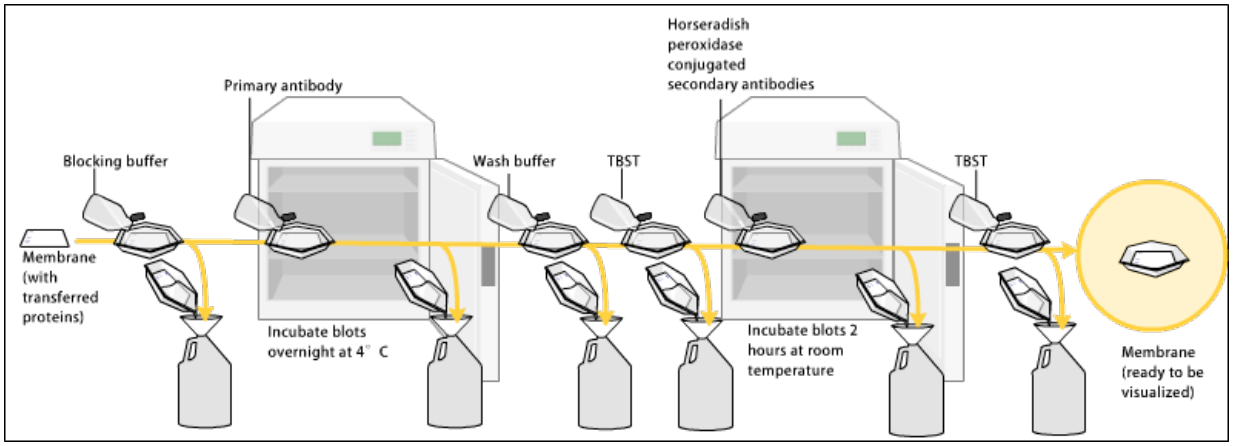


Fig. 10c. Antibody labeling in western blot. ©User:Bensaccount. Wikimedia Commons. CC-BY-3.0.

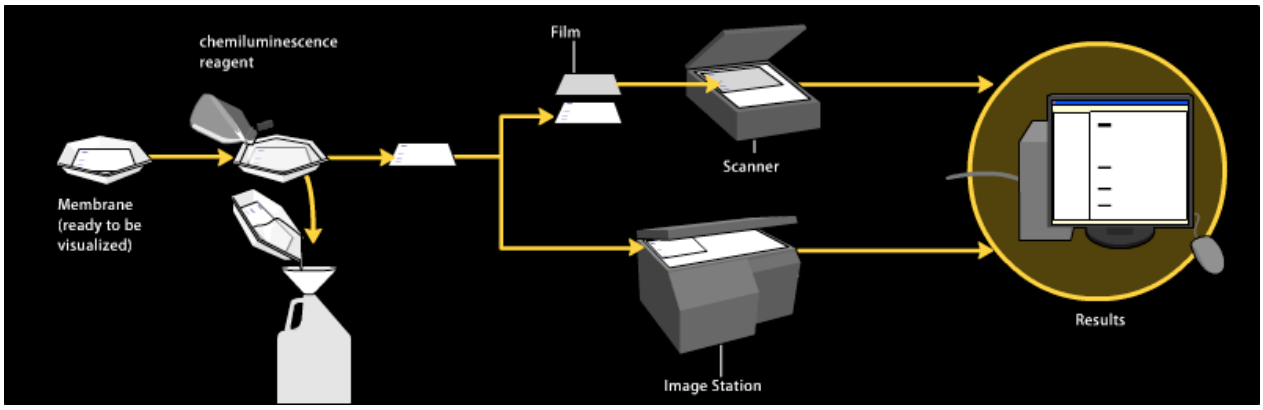


Fig. 10d. Chemiluminescence in western blot. ©User:Bensaccount. Wikimedia Commons. CC-BY-3.0.

In Vivo Evaluation of Tumor Inhibition (I)

An *in vivo* model is usually set up as the next step approaching the clinical situation, commonly in mice. This provides information in a more intricate system with tumor-stroma and tumor-host interactions as well as angiogenic properties since the tumors are dependant on angiogenesis after reaching 1-2 mm in diameter. $5x10^6$ cells were injected in the flank of 6-week-old female athymic immuno-compromised (nu/nu) mice. Hence, it is a xenograft model where the full immunological component of the tumor-host interaction cannot be studied. Female mice were used as they are less aggressive and nu/nu because their T-cell deficiency provides the minimum level of suppression for xenotransplantation. We also explored the possibility to inject cells at the location of the pancreas in order to create an orthotopic xenograft model in line with the rational that the local milieu is important for tumor homeostasis. These models will nevertheless be rough approximations of the tumor biology in humans. One major reason is that we use a monoclonal cell population cultured for many generations in artificial conditions under selection pressure without the heterogeneity normally seen in neoplasias.

The treatment was initiated once the tumors reached a mean diameter of 8 to 10 mm when the viability was certain. Mice were randomized into treatment groups and treated with the various inhibitors either orally (NPI-0052 and erlotinib), via i.p. injection (bortezomib, bevacizumab, cetuximab, PD98059, SP600125, and LY294002), or tail vein injection (NPI-0052 and gemcitabine) twice weekly on days 1 and 4 unless otherwise stated. In earlier experiments the optimal sequence and timing of drug administration had been established. Tumor size was measured by caliper every 4 days and calculated using the formula $TV = 4/3\pi r^3$ (where r is half of the mean tumor diameter, measured in at least two directions). Volumetric measurement is utilized as standard treatment response indicator in tumor *in vivo* models and caliper measurement is an acceptable proxy, particularly if more than one direction is measured[111]. Intraobserver variation is established in one study to be 14%[112]. Lately increased use of microCT and FDG-PET has been motivated by slightly better accuracy. At the time we were interested in the possibility to use

near-infrared fluorescence (NIRF) in apoptosis detection *in vivo*. The experiment was terminated at 20 days and the animals were sacrificed. Reasons to not extend the period were increasing frequency of animal cachexia or tumor ulceration.

Health Economy (Paper II and III)

Patient Material (II and III)

A database was constructed on consecutive patients diagnosed with exocrine pancreatic or ampullary cancer 1998-2005 identified from the Regional Centre of Oncology national registry. Reporting to this registry is mandatory for all malignant disease and reaches an almost total completeness reducing risk of missing data[113]. By crosschecking with two other data systems (PAX and Melior) the diagnosis and treatment was confirmed. The material was also refined to be population based. The Swedish population registry means that the survival is certain in all patients and no patients were lost to follow up which was extended ensuring reduction of censoring in survival analysis. These precautions were undertaken to ensure the external validity and striving to eliminate selection bias that is a common problem in retrospective patient cohort studies.

Still there are certain methodological pitfalls inherent to patient file data mining; ambiguous definitions of diagnosis, surgical resections and pathology reports demanding interpretations performed at the discretion of an unblinded abstractor leading to possible observer bias. Unfortunately the nature of this research in many cases prohibits, or obstructs, blinding and a certain amount of "enlightened pragmatism" is needed. To reduce the risk of inter-abstractor variability it is important to define the interpretation of variables rigidly *a priori* and adhere to stringency.

A prospective study enables strict control of many factors, reducing the risk of confounding factors and systematic underreporting and may yield a high degree of internal validity. This may on the other hand threaten the external validity by creating an artificial situation not representative of any healthcare system and inducing the risk of the Hawthorne effect, i.e. achieving an effect by the fact that subjects know they are being studied *per se*. Prospective studies usually need longer inclusion time span due to non-eligibility (risk of confounding time span bias as standard of care is changing) or need to be performed as a multicentre study (risk of hospital specific situation bias). With this in mind the chosen study design was considered appropriate for assessing costs and utilities providing an "as is" situation. Clinical data was presented to address the issue of external validity. As a quality control data on surgical outcome, complications and confirmation of N0 status through immunohistochemistry was reported.

Health Related Quality of Life (II and III)

To calculate QALYs a utility score to give weight to achieved life years is needed. One component is HRQL data. In our institution we have used SF-36 in conjunction with other instruments for many years evaluating HRQL of cancer patients. This is one of the most widely used and thoroughly validated PROMs available[114]. The results are however presented as eight scores, one for each included dimension.

Brazier et al. recognized the need for a conversion algorithm for SF-36 to provide a single preference based utility index and consequently developed SF-6D that in short uses 10 of the 36 items to form 6 dimensions[115]. These items yield 18,000 possible health states which, using the SG method on 836 individuals from the general public in Great Britain, were given a preference weight. This provides a theoretical advantage of sensitivity and discrimination as compared to the widely used EQ-5D that includes only 243 health states. Still it is not as extensive as the HUI, thereby tentatively causing a less burden on the patient. It is generally accepted that floor effects (poor differentiation between low scores) occur in SF-6D and ceiling effects (limited differentiation between high scores) occur in EQ-5D[116].

Other important factors that should be considered when choosing a PROM (preference-based or non-preference based) is the outcome of key psychometric testing such as:

1. Responsiveness – Sensitivity of instrument for change over time. An effect size over 0.2 is commonly considered the minimal clinically important change (MCID) (Jan Karlsson, PhD, personal communication)[117].
2. Reliability – The reproducibility of a test and the internal consistency as measured by the correlation between included items (Crohnbach's α a common procedure).
3. Content validity –The adequacy of the instrument for what is measured. Face validity is the subjective assessment of adequacy for what is measured.
4. Criterion validity – Coherence with other instruments or gold standard if available.
5. Construct validity - Items reflecting adequately the domain that is to be assessed.

There is no consensus on what method or instrument should be used in studying HRQL in specific diagnoses. The choice of HRQL questionnaire is a strong determinant of results as utility scores for equivalent states can vary substantially[118]. This means all HRQL data warrants cautious interpretation. Arguably, using more widespread and validated instruments such as SF-36 could increase transferability of results. Also, finding preference for the health states varies depending on the usage of SG, TTO, VAS or other methods, from what community preferences are drawn and on the time frame chosen[81].

Furthermore, the construction of QALYs introduces additional variance in methodology as time frame varies and survival can be drawn from patient cohorts

or extrapolated from the literature. The results can be calculated using various statistics from standard arithmetic to Bayesian Markov modeling. Consequently, a huge variation of methods in several steps is likely to affect the reproducibility and therefore the reliability of the results. To reduce this variance and to aid in interpretation several health economical analysis guidelines from expert panels and national institutions attempts to give direction and, importantly, encourage proper reporting[82,119]. Usually it is stated that information should be available on[119]:

1. Background, purpose and rationale, i.e. current clinical practice and effects of relevant therapies
2. Choice of comparator, i.e. alternative therapeutics
3. Perspective taken, i.e. healthcare, societal or patient
4. Data sources
5. Study design
6. Cost measurement, i.e. subgroups of direct costs, indirect costs or intangible costs
7. Outcome measures, i.e. QALY and the basis for this calculation
8. Time horizon
9. Sensitivity analysis, i.e. provide alternative assumptions for the range of potential values for uncertain parameters

In our study pooling of data from separate samples of the same population was necessary to complete the health economy analysis. This can be done only if likely that samples are representative of the same population. Therefore an assessment of reasons for missing data was carried out to control for confounding and selection bias. The administration of PROM questionnaires may carry an inherent non-random censoring bias due to the fact that very ill patients will not return forms[120]. Indeed, authors have found a strong, and expected, correlation between HRQL and survival[121]. We could however not se such a pattern and the reason for using data from patients receiving personalized palliative care as a proxy for the whole palliative care group was the ready availability together with the assumption that it is a representative population only removing the most desolate cases.

Cost Measures (II and III)

We chose to assume the healthcare perspective. The uncertainty of data outside the healthcare sector and the complexity and increased investigational costs of estimating such data was not considered relevant in the current study population where most patients are retired and the loss of income is a minor factor. Also, estimating and reporting on healthcare costs is valid for health economic evaluation.

Using data from our hospital cost-per-patient registry we could establish estimates of all costs associated with care of pancreatic cancer patients judged close to market values. In paper II only costs raised within the department of surgery was counted as it was assumed most costs were allocated there. In paper III, however, a more thorough estimation was made based on all costs in the department of surgery, the

department of oncology and the primary healthcare sector. It was confirmed that the absolute majority of costs was generated at the department of surgery, mostly because of limited adjuvant therapy at the studied time period but also due to the fact that current standard of care in Sweden involves admission of patients diagnosed with pancreatic cancer to the department of surgery for most reasons. We used data on an individual basis and extracted patients with complete economic reporting to estimate annual and lifetime costs for the various treatment strategies.

To adjust for the time factor and effects of inflation costs were adjusted by the consumer price index. Costs were also subject to discounting at a customary rate of 5% using the formula

$$S_t=S_p/(1+d)^t$$

where t is the time, d the discount rate, S_p the present sum and S_t the sum at time t. In the sensitivity analysis discounting at 3 % and 10 % rates were tested without important effects on cost-effectiveness. This is due to the short time span relevant in the study.

Biological Characterization (Paper IV)

Patient Material (IV)

To avoid selection bias tissue was collected from all patients undergoing surgery with curative aim for pancreatic cancer. The inclusion criterium was consecutively all patients accepting and the exclusion criterium was pathology reports indicating other diagnosis. A theoretical benefit of recruiting patients from institutions in two countries is that the likely geographic variation in CNV is neutralized[122]. The histological report indicated no difference between analyzed groups of short, medium and long survival indicating that traditionally accepted prognostic factors such as tumor stage, nodal involvement or radicality were not predicting cancer specific survival. Failure to detect this could in part be due to limited numbers but as it was not an endpoint it was considered acceptable. Numerically fewer patients with short survival were subject to adjuvant chemotherapy but also this proved not to be a significant prognostic factor.

Genetic Analysis (IV)

A virtual karyotype was established for each patient using a recently developed array combining comparative genomic hybridization (CGH) and single nucleotide polymorphism (SNP) analysis (Agilent Sureprint G3 CGH+SNP Microarray Platform). It is an oligonucleotide array with approximately 120k CGH probes and 60k SNP probes. These yield a resolution of 5-10 Mb for copy neutral loss of heterozygosity (cnLOH). In short array CGH detects CNVs and SNP analysis detects copy neutral LOH and UPD across whole-genomic DNA. Tumor DNA and normal

reference DNA from a Caucasian male are dyed with Cy5 and Cy3 fluorescent dyes, digested by restriction endonucleases digestion (*Alu I* and *Rsa I*) at sites overlapping with known SNP sites and co-hybridized to engineered oligonucleotide sequences attached to the microarray. Fluorescence ratios at arrayed DNA elements provide a locus-by-locus measure of relative DNA copy-number variation and number of uncut alleles compared to known control indicates areas of LOH (Fig. 11).

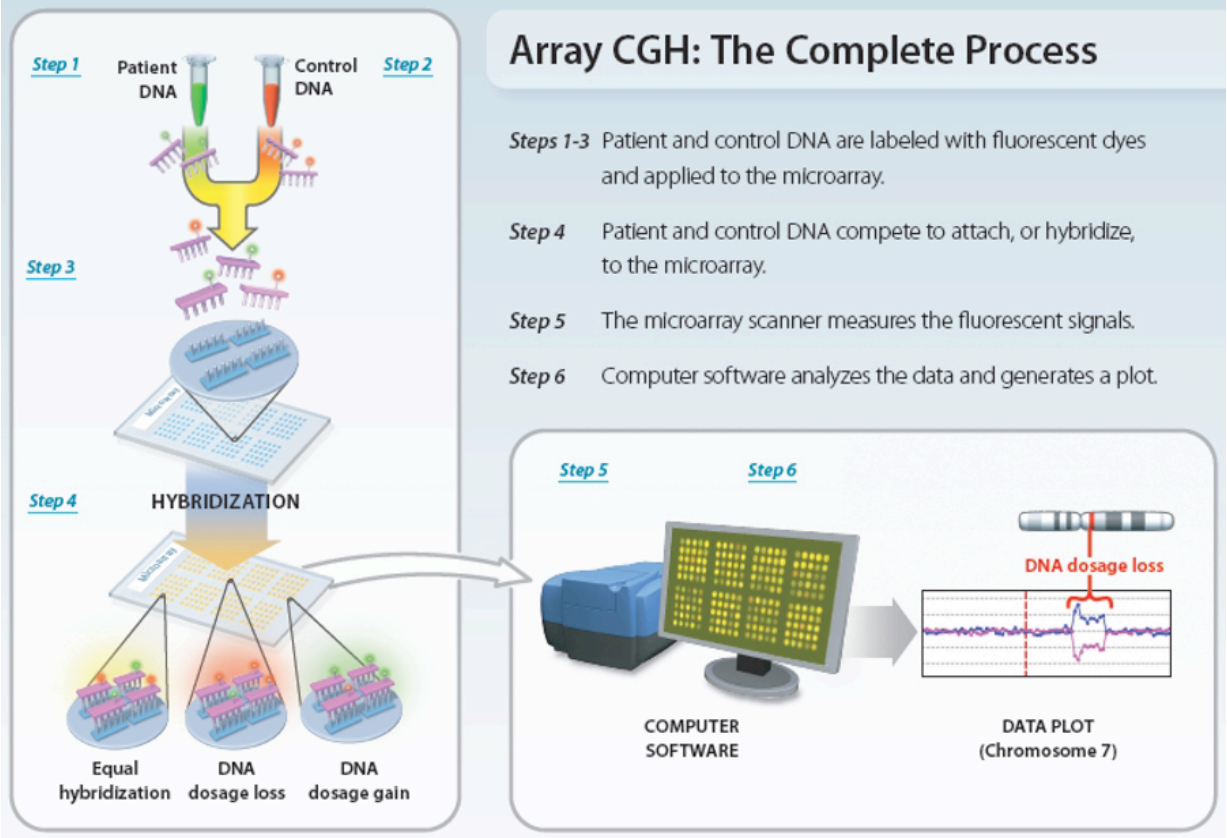


Fig. 11. Thiesen, A. Microarray-based comparative genomic hybridization (aCGH). The addition of SNP array data does not alter the workflow. Reprinted by permission from Nature Publishing Group, Macmillan Publishers Ltd: Nature Education 1(1). © 2008.

High throughput array platform data processing involves several steps, all of which are sensitive and potential sources of error. The following are central aspects of sample preparation:

1. The sample to be analyzed should have DNA that is not degraded. It is still generally held that fresh frozen tissue sections are more reliable than formalin fixed paraffin embedded (FFPE) material. Therefore we used snap frozen tissue from 56 PDAC patients harvested at surgery.

2. Solid tumor material is heterogeneous with a multitude of cell populations, and PDAC is particularly so;
    a. The dense desmoplasia of stroma (fibroblasts such as pancreatic stellate cells, leucocytes and other immune system components, blood vessels and ECM proteins) mixing with epithelial derived tumor cells is abundant[123].
    b. The epithelial derived tumor cells are clonally expanded, these clones display a great variation in mutational profiles presenting great spatial intratumoral variation[88,105].

To remove as much as possible of stroma cells laser capture microdissection has been developed. This method is costly, demanding and carries a risk of yielding insufficient DNA. Whole genomic amplification (WGA) such as PCR based methods is often used to overcome this but is hampered with distortion of genome[124]. However, the microdissection does not account for clonality and mosaichism in tumor tissue. A great advantage to the SNP analysis is the possibility to analyse fractions of multiclonal tumor material, hereby yielding an estimate of purity as determined by the CN (copy number) line fit[125]. This is the rationale for using macrodissected PDAC tissue in our study, where an estimated 94% belonged to the major clone, which we consider support for adequate sample purity. Also, there is an obvious logistic advantage of the ability to use macrodissected tumor tissue in translational research. The advent of fluorescent assisted cell sorting (FACS) in refining tumor cell clones for DNA analysis is however promising[126]. Notably it may for all these methods be that the analyzed clone is not the biologically most important. Therefore gene mutation analysis should ideally be coupled to expression analysis and proteomics for the same cases. At present it is however an almost insurmountable task to ensure adequate and reliable quality in all these analyses.

3. Ideally the control sample is normal tissue from each individual[127] but as this was not available we went with the assumption that pooling patients to a well described standard genome as *Agilent euro male* would ensure good control of CNV and subtraction of irrelevant aberrations. The sex chromosomes were excluded from analysis as samples were hybridized against male genome.

Regarding further processing it is crucial to ensure adequate quality in the following steps:

4. Image analysis – signal quantification and background correction of non-specific hybridization and fluorescence
5. Centralization – making the most common ploidy the zero-point
6. Normalization – accounting for variations in labeling and hybridization efficiencies
7. Log_2 transformation – causes more even spread and variability along intensity range to approach normal distribution
8. Selection of amplification/deletion thresholds
9. Correction for GC bias – the nucleotides guanine and cytosine have three hydrogen bonds and thymine and adenine two, which means DNA is more stable in segments with high GC content. In CGH arrays this causes wavy artifacts that has to be corrected for.

Statistical Methods and Considerations

Paper I

Statistical significance between groups of *in vitro* and *in vivo* tumor models was analyzed by parametric one-way analysis-of-variance (ANOVA) as these groups usually are normally distributed. If significance ($p<0.05$) was found, the location of the significance was determined with a Dunnet's post-test requiring the assumption of homogeneity of variance was met. This test compares each group to a random control. Error bars are displaying standard error of the mean (SE).

Paper II and III

Clinical data were presented as mean with confidence interval (CI) or standard error (SE) if normally distributed and with median and range if skewed. Overall survival functions from the date of diagnosis (or the date of surgery in the resected group) were calculated using the Kaplan–Meier estimator and tested by log rank statistic. The SF-36 v.1 and v.2 to SF-6D conversion algorithm used syntaxes kindly provided by Professor John Brazier, University of Sheffield, UK.

The QALY calculation was performed in line with the concept described by Billingham et al[128]. Hence, the integrated quality-survival product (IQSP) curve was calculated from the survival curve during follow-up multiplied by the value for linear regression of overall HRQL assessed by the SF-6D index, with similar lengths of follow-up:

$$\text{IQSPmean} \pm \text{IQSPSE} = \text{Mean}_{\text{Survival}} \times \text{Mean}_{\text{HRQL}} \pm (\text{Mean}_{\text{Survival}} \times \text{Mean}_{\text{HRQL}}) \sqrt{(\text{SE}_{\text{Survival}}/\text{Mean}_{\text{Survival}})^2 + (\text{SE}_{\text{HRQL}}/\text{Mean}_{\text{HRQL}})^2}$$

To determine the reliability of estimates the SE for the non-parametric Kaplan-Meier data was calculated using the Greenwood formula and then combined with the SE for the parametric HRQL linear regression data to construct propagated confidence intervals:

$$95\%\text{CI} = \text{IQSPmean} \pm \text{IQSPSE} \times 1.96$$

The area under the respective curve was calculated using the trapezoid formula:

$$\text{Area} = \text{Sum of } (\text{Score}_n + \text{Score}_{n+1})/2 \times (\text{Time}_{n+1} - \text{Time}_n)$$

These steps were calculated over 1, 2, 5 and 10 years respectively and constituted the achieved QALYs and confidence limits at different time frames. A p-value of <0.05 was considered significant in two-sided tests.

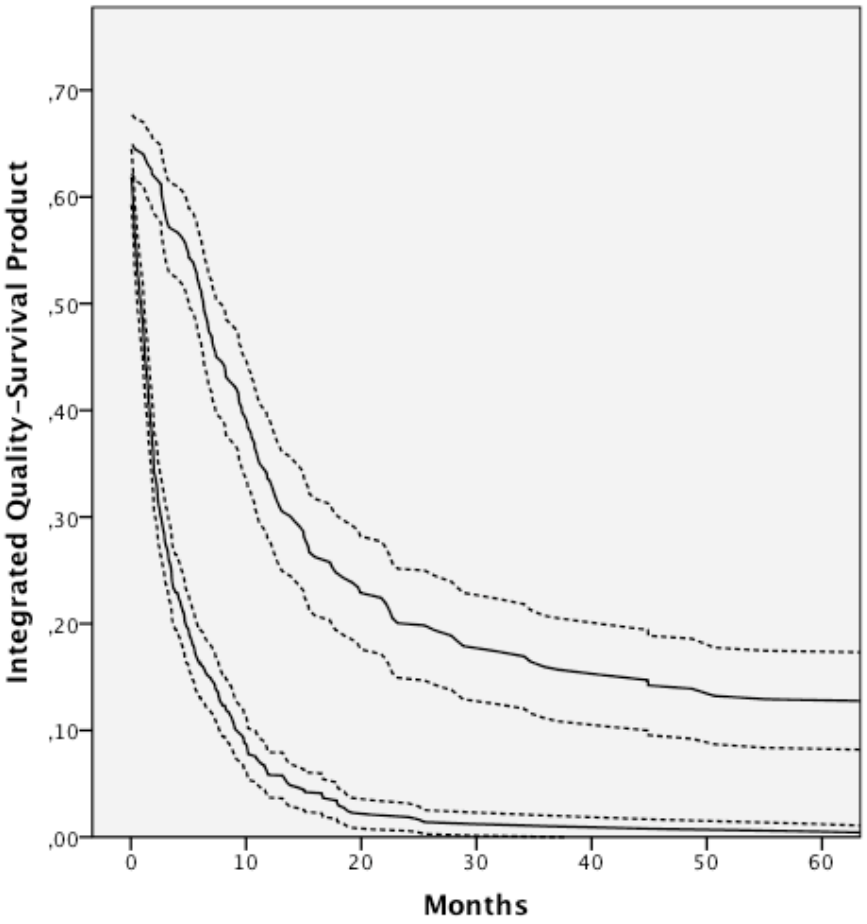


Fig. 12. IQSP ± CI for patients undergoing resection and palliation respectively.

Paper IV

When designing a microarray study one can adopt three different approaches; class comparison, class discovery or class prediction[129,130]. We have performed a class comparison study where genomic alterations of two survival classes were compared. Formal methods of calculating power in array studies do not exist but pragmatic statistic considerations demand at least 50 samples to achieve power[131].

There is so far no consensus statistical approach in analyzing genome wide data. It is a constantly developing field with new algorithms emerging by the day. There is likely a great potential for improvement but with present knowledge some fundamental principles needs to be accounted for. Most obvious is perhaps the issue of significance due to multiple hypotheses testing in explorative genome wide studies. As in the case of SNP analysis in a typical GWAS it is estimated to involve approximately 1 million independent hypotheses[132]. Hence, we work with gigantic data sets with certainty of findings but with questionable meaning. It is therefore of crucial importance to perform hypothesis driven science with *a priori* decisions of analyses instead of falling for the temptation of exploratory data mining yielding statistical significance of limited true value.

When designing the study we therefore decided to focus on CNA, which is vastly

less abundant than SNPs, and linking this information to the two extreme groups of survival; short and long. By pooling samples of polarized data in this way we expected to substantially reduce the inevitable noise. We also decided to use the most robust algorithm available in Agilent's repertoire, ADM-2, and apply conservative thresholds. This algorithm searches for intervals in which a statistical score exceeds the user specified threshold. The score is proportional to the absolute average log ratio of the genomic interval and the square root of the number of probes in the interval[133]. Elevated log ratios are called aberrant. We applied Fuzzy Zero to correct for false positive detection of long aberrations with low log ratio. The statistical confidence interval was set to ±0.2 $\log_2$, as is appropriate according to our experience from earlier studies[134], we have previously also used ±0.1 $\log_2$ corresponding to the 20th and 80th percentile of segment alteration values respectively but considered not enough conservative for this study[135]. All these precautions were taken in order to reduce the risk of type I error, false findings. As we did not find many convincing significant prognostic aberrations in our *a priori* analysis, we allowed ourselves to perform a cluster analysis, which is a class discovery approach, knowing that this increases the risk of type I errors. Even here we did not find any clusters with prognostic significance.

The avoiding of type I usually increases the risk of type II errors, failure to detect. Indeed, this study is subject to a substantial risk of not finding existing relations between DNA structure and prognosis, a type II error. Reasons for this are multiple, one of which is underpower. It is increasingly evident that cancer development is both a deterministic and stochastic process with abundant DNA aberrations[12]. They occur either as a driving force or, as we hypothesize in the conclusion, as mainly passenger mutations, through chromosomal instability a consequence of the development of the complex PDAC phenotype. This increases the likelihood of random patterns, where most aberrations have very low effect sizes. If also taking epigenetic changes and other factors affecting biology and prognosis into account it is plausible that studies to find DNA aberration patterns predictive of longevity takes several thousands of patients. On the other hand the clinical applicability of such findings could be debatable. Rapid gain of ground in bioinformatics and development of shared databases on sequencing material may eventually provide answers in a near future.

Ethical Considerations

In paper I all experiments were done in full compliance with institutional guidelines and with the approval of the Massachusetts General Hospital Institutional Animal Care and Use Committee.
The health economical studies in paper II and III was concluded within the frames of an approval by the regional ethical review board of Nov 17th, 2005 (Dnr 539-05) and the genomic study in paper IV was carried out under the approval of Jan 23rd, 2006 (Dnr 002-06). Living patients or relatives did not need to be informed of the studies.

Results and Discussion

Paper I

The results of the first paper could be condensed to a few insights regarding proteasome inhibition (PI) in pancreatic cancer biology.

- PI was shown to induce apoptosis to a much larger extent than gemcitabine or EGFR inhibition *in vitro*, the latter two adding little to the effect of combination therapy. These findings were reproduced *in vivo* but here the combination therapy was more fruitful. The second generation PI, marizomib, was more efficient than first generation bortezomib in combination with EGFR and VEGF inhibition.

- PI induced phosphorylation of EGFR, ERK, JNK and AKT indicating a lateral and non-specific activation of several key pathways independent of NF-κB. This activation was not cell type specific and blocking EGFR did not reduce degree of activation.

- Inhibition of JNK and AKT did not induce apoptosis as single therapies but augmented apoptotic effects of PI. This indicates a role for these pathways in PI-induced antiapoptotic response. ERK inhibition did not statistically significantly potentiate PI *in vitro*, possibly due to the EGFR inactivation. Inhibition of AKT, ERK and JNK all enhanced antitumoral effects of PI *in vivo*.

- PI inhibited the TNFα-induced NF-κB activity but not the basal NF-κB activity even with the addition of pathway inhibitors, indicating that the apoptotic effects are not primarily NF-κB mediated contrary to expectations.

Altogether there is reason to believe that PI is, at least partly, inducing apoptosis in a NF-κB independent manner and induces an EGFR independent activation of survival response pathways and that the addition of mitogenic inhibitors augments the proapoptotic effects of PI.

There are of course many pitfalls when trying to study the intricate system of tumor biology. By interfering with intracellular signal transduction, however specifically you aim, you reach a multitude of effects. By changing one variable at a time and using different models and cell types one can hope to reveal causal relations. In this paper we present some of the results of a large body of work from the same laboratory where many aspects of PI has been studied. Through this work factors like the appropriate sequence and timing of drug administration, the correct animal model, the appropriate assays and diagnostic tools have been selected, all necessary to reach clear and reproducible findings. Despite this process many things remain unclear and needs further studies.

One example is the reason why combination therapy of PI and EGFRi was working *in vivo* but not *in vitro*. A logical explanation could be the reduced importance of EGFR *in vitro* due to reduced cell-cell interactions leading to up-regulation in kinase cascades. This is supported by further studies of our group showing that PI induces expression of HB-EGF and that this activates EGFR, interestingly HB-EGF and EGFR activation is partly responsible for the profusion of desmoplastic reaction in pancreatic cancer[136]. Another example could be the role of constitutive versus induced NF-κB activity. These questions open new avenues for further research and it is obvious that we have only begun to understand cell signaling and its malignant aberrations. Recently a successful phase I study on Marizomib in combination with the histone deacetylase inhibitor Vorinostat in pancreatic cancer was closed[137].

Paper II and III

In these two papers significant effort was spent to establish reliable estimates of HRQL and costs associated with pancreatic cancer care. The ultimate aim was to illuminate the burden of the disease on the individual patient and the health care system.

First we showed that the sample populations were representative of standard pancreatic cancer populations. Using the generic SF-36 instrument HRQL was shown to be similarly and significantly reduced compared to healthy controls in both groups and all dimensions at baseline. The reasons for this are probably multiple and the psychological effect of receiving the diagnosis is not negligible. The HRQL data for palliative patients were from a selected subgroup with slightly better survival; this is in our opinion simply reflecting non-eligibility of the desolate cases. We therefore considered this group appropriate to compare to patients undergoing resection, which is also a selected group.

The lowered HRQL index was relatively constant over time in both groups, a slight increase in long term follow up particularly in resected patients is in part secondary to censoring bias with healthy survivors remaining and not statistically significant. We also estimated that lifetime direct healthcare costs for patients undergoing resections aimed at cure was about twice as large as for patients receiving palliative treatment. Interestingly, however, this was neutralized by a twice as large utility in the resected group already after 1 year and if extending calculations to lifetime values the palliation was three times more expensive as counted per utility.

The main conclusion to be drawn from this is that the principal determinant of cost-effectiveness is survival. To increase the cost-effectiveness one should strive to add longevity. This is in coherence with the values of the society and, indeed, economic research shows that the single most important good to purchase as we get richer is life years[138]. This is providing a basis for a continuously increased share of overall spending on healthcare. This supports the utilization of the QALY metric that is strongly influenced by the time factor. We did not calculate an ICER for use in

league tables as the groups are selected and, hence, cannot be described as alternative treatments. In fact, it is important to remember the limited possibilities to make meaningful comparisons of costs and achieved utilities between patients undergoing resection and patients receiving palliation since the groups are clinically highly selected. The strength in our analysis is the robust descriptive aggregate data reducing uncertainty in assumptions. It is nevertheless important to underline that all figures are estimates.

The information is mainly possessing relevance as a good foundation for further simulation modeling for decision-making and resource allocation. Indeed, using data from our first paper on this topic a cost-utility analysis has been produced through modeling at MD Anderson[139]. Here ICERs have been estimated using decision tree modeling and in line with our conclusions, surgery with adjuvant treatment is expensive but associated with prolonged survival compared to the non-treatment strategy.

Paper IV

The final paper explores DNA alterations to find prognostic patterns. The findings show in coherence with a growing body of literature that the genetic disease of pancreatic cancer is displaying enormous heterogeneity. This is interpreted as a result of pronounced CIN[140]. Our results indicate a greater genomic degeneration in tumors from patients with short survival; more specifically amplifications were seen to be significantly more common whereas amount of deletions were similar. In our CNA analysis we found a few loci that were significantly more commonly altered in the respective survival groups indicating possible prognostic significance. One notable finding is the deletion in locus 5q13.2 prevalent in one third of cases with short survival but not in reference sample or long-term survivors. Here gene aberrations with possible deleterious influence could be found. Another finding is the amplification in locus 6p21.33 in one third of the long term survivors but not in short term survivors, an observation difficult to interpret.

There is a plethora of literature investigating prognostic biological factors in pancreatic cancer. All levels of the central dogma of molecular biology has been investigated; genomics, transcriptomics and proteomics, i.e. through CGH, SNP or sequencing for DNA, expression profiling for RNA and IHC, PCR and northern blots for protein products. A multitude of publications have been exploring pancreatic cancer genomes proposing a range of key mutations, some of which also reporting prognostic significance and some including confirmative expression analysis and protein detection[100-102,140-148]. So far limited conclusions ca be drawn because of diverging results. Reproducibility is a major concern in all high throughput molecular studies[149] and validation by other groups on other samples and through integration of multidimensional data or using known inter-species conservation is crucial[150]. A recent paper from the Johns Hopkins group explores the prognostic significance of 'the big four'; TP53, KRAS, SMAD4 and CDKN2A through sequencing[151]. It is shown that genomes with 3 or 4 of these mutated have significantly shorter median overall survival, 9 vs. 23 months.

The lasting impression is that an aggressive clinical course is found in tumors with generally degenerated genomes. Importantly, the direction of causality is not clear. It may be that the majority of found aberrations are *passenger* mutations without clinical significance[150] and it is plausible that passenger mutations can achieve significance based on recurrence due to fragile sites in genomically unstable tumors[152]. Investigating these genomes for prognostic singular genes yields significance based on gene penetrance and statistical power rather than finding true causal driver mutations. More likely is the existence of hundreds or thousands of *driver combinations* of coding and non-coding mutations and epigenetic alterations. Indeed, it has been calculated that to achieve a robust short list of prognostic gene alterations thousands of samples are needed[153]. This is particularly true for class prediction studies and lower sample sizes could probably be acceptable in class comparison studies such as ours[131].

Further research is warranted to confirm our results and aid in determination of gene combinations with driving capacity for disease progression. A major technical issue in tissue analysis, in particular in pancreatic cancer, is sample purity. The intermixing with non-epithelial derived cell populations may blur the picture, and the significance of findings. Our work is further evidence that reliable results by necessity stem from not only meticulous sample preparation and characterization, but also that the inter- and intra- individual heterogeneity due to cancer cell clonality demands large study populations for elicitation of significant prognostic and predictive factors. Future technical development in genome sequencing and bioinformatics will likely aid this quest.

Summary

This thesis is intended to integrate vast and disparate research fields concerning pancreatic cancer treatment. The extensiveness is perhaps both its strength and its weakness. The strength is the 'scientific generalist view', a view that arguably is threatened by the sheer volume of exponentially increasing knowledge available, but it is also a view necessary in order to make judicious health care decisions both on an individual level and in society.

Pancreatic cancer is a monumental therapeutic challenge that is costly to treat both in monetary terms and in morbidity. The genomic foundation seems to be extensively varied and the cellular signaling systems complex. Both these aspects need further elucidation for treatment development.

Future Perspectives

The future of pancreatic cancer treatment in the *omics* era is promising. Genomics, transcriptomics, proteomics and metabolomics are all fields that will be central for our understanding of biological processes. Rapid advancements above all in computational abilities enable exponential growth of knowledge. Therefore, the greatest challenge will be structuring and processing this wealth of information within the field of bioinformatics. As speed of whole genome sequencing is picking up and costs are lowered individual genomes will be readily available. Similarly platforms are developed for rapid characterization of other bioinformation. To decipher pancreatic cancer biology the ultimate aim should be making omics data coupled to relevant clinical and histological information globally accessible in online databases. This large scale information processing will combat the complexity of biological systems and possibly provide necessary power. A growing ethical challenge will be the interface between high availability of information with promise of enabling great benefit for mankind and personal integrity.

For cancer treatment in general the knowledge of biological processes and their aberrant versions is crucial when developing early detection tools, creating tumor fingerprinting or designing targeted therapeutics in line with the personalized medicine paradigm. This knowledge will also aid selection of treatments to achieve greatest possible benefit to least cost for the individual and society. The ongoing development of HRQL assessment instruments and health economy methods will be central in ensuring optimal resource allocation, an issue likely to increase in importance along the road ahead.

Conclusions

- Proteasome inhibition exercises multiple effects in pancreatic cancer cells and constitutes a promising therapeutic alternative in multimodal treatment of pancreatic cancer

- Simultaneous inhibition of multiple targets in cancer cell signaling pathways may overcome chemotherapy resistance responses and augments the tumoricidal response

- Pancreatic cancer surgery is initially costly but comparable to other major health care interventions

- Survival is the strongest determinant of cost-effectiveness and the selected group of patients undergoing resection displays good cost-effectiveness compared to patients receiving palliation due to long term survivors

- The pancreatic cancer genome is highly heterogeneous and seems to carry more copy number alterations in patients with short survival where amplifications were more common

- Deletion in 5q13.2 was significantly coupled to short survival and occurred in one third of patients in this group

- It remains uncertain whether genetic alterations are primary determinants of PDAC progression following surgical resection

Summary in Swedish – Sammanfattning på svenska

Bakgrund

Bukspottkörtelcancer har sämst prognos av kända tumörer och utgör den fjärde vanligaste orsaken till cancerdödlighet. Cancern diagnosticeras ofta sent och endast en femtedel kan komma ifråga för kirurgi som utgör den enda potentiellt botande behandlingsmodaliteten. En förutsättning för kirurgi är att tumören är lokaliserad till bukspottkörteln och att patienten har fysiska resurser att genomgå stor kirurgi omfattande delar av bukspottkörteln, tolvfingertarmen, gallgången, regionala lymfkörtlar och ibland en del av magsäcken. Medianöverlevnaden utan behandling är fyra till sex månader och i gruppen som genomgår kirurgi tolv månader. Upp till var femte som blir opererad lever i fem år och uppfattas botad. En marginell tilläggseffekt gör att cytostatika i form av gemcitabin är standardbehandling efter operation eller som bromsande behandling för icke operabla med tillräcklig funktionsnivå. Bukspottkörtelcancer utgör en särskilt aggressiv tumörform och den dystra prognosen gör den till en formidabel klinisk utmaning. I en hårdnande prioriteringsdebatt där bättre underlag för visad nytta och kostnader efterfrågas har bukspottkörtelcancerkirurgi med kort postoperativ överlevnad, hög komplikationsfrekvens och stora kostnader ifrågasatts. Incitamentet att utveckla alternativa och komplementära terapier är starkt och ökad förståelse för bukspottkörtelcancerns biologi och genetik nödvändig för ökad individualisering av terapival.

Frågeställning

Frågor vi sökt besvara är: Vilken börda utgör bukspottkörtelcancer för patienten och samhället? Hur ser det ut avseende hälsorelaterad livskvalitet (HRQL) hos dem som genomgår kurativt syftande resektion respektive dem med inoperabla tumörer? Vilka kostnader medför behandlingen av dessa grupper för svensk sjukvård? Hur påverkar proteasominhibitorer intracellulära signalsystem i bukspottkörtelcancercellinjer? På vilket sätt kan man förstärka dessas tumörbromsande effekt? Finns DNA förändringar som enskilt eller i mönster kan förklara överlevnad? Finns i så fall möjligheter att biologiskt stratifiera bukspottkörtelcancer i mer inerta lokaliserade former respektive dynamiska med större metastaseringsbenägenhet?

Metod

För att kunna bevara ovanstående frågor följer avhandlingen två metodologiska huvudspår; i arbete II och III ligger en patientkohort med diagnos bukspottkörtelcancer till grund för en hälsoekonomisk analys och i arbete I och IV används molekylärbiologiska tekniker för att närmare karaktärisera bukspottkörtelcancerns biologi. Den hälsoekonomiska analysen sammanväger tre huvudfaktorer; kliniska data inkluderande överlevnad, generell HRQL mätt med

hälsoenkäten SF-36 och omräknat till preferensbaserat index med SF-6D och kostnadsdata baserad på sjukvårdens redovisningssystem och tariffer. För att säkerställa extern validitet görs även en genomgång av kliniska variabler såsom komplikationer i den opererade patientgruppen. I det ena molekylärbiologiska arbetet används cellinjer och djurmodeller för att studera effekten av proteasomhämmare på viktiga signalsystem i bukspottkörtelcancercellinjer. Detta görs genom värdering av celldöd med enzymkopplad immunadsorberande metod (ELISA) och flödescytometri samt genom proteindetektion med western blot. I det andra molekylärbiologiska arbetet genomförs en strukturell genanalys med kombinerad komparativ genomisk hybridisering (CGH) och enbaspolymorfi (SNP) analys av tumörvävnad från patienter opererade för bukspottkörtelcancer. Syftet är att identifiera mutationer med koppling till överlevnad.

Resultat och Slutsatser

Arbete I visar att behandling med proteasomhämmare, förutom sin tumördödande effekt, aktiverar intracellulära signalsystem som är mitogena (befrämjar celldelning) och antiapoptotiska (förhindrar programmerad celldöd) och därmed aktiverar en cellulär överlevnadsrespons. Det gäller samtliga studerade komponenter; EGFR, JNK, PI3K/Akt och ERK. Effekterna är inte cellinjespecifika utan föreligger i alla tre prövade cellinjer. Samtidig behandling med EGFR-hämmare påverkar inte proteasomhämmarens aktivering av signalsystemen i cellkultur. Detta tolkas som att proteasomhämmare inte är beroende av EGFR för att utöva effekt på signalsystemen. Däremot förstärks den tumördödande effekten av JNK- och Akt-hämmare i både cellkultur och djurmodell och av ERK-inhibitorer i endast djurmodell. Tillägg av EGFR-hämmare ger förstärkt effekt i djurmodell men inte i cellkultur. Detta antas bero på växelverkan mellan celler (tillgången på parakrint EGF) i djurmodellen. Proteasomhämmare nedreglerar den inducerade NF-κB aktiviteten men inte den konstitutiva (basala). Vid tillägg av selektiva signalsysteminhibitorer nås ingen ytterligare effekt på den basala NF-κB aktiviteten. Det tolkas som att den ökade apoptos som observeras inte är NF-κB medierad.

I arbete II görs en hälsoekonomisk analys utgående från en konsekutiv kohort om 139 patienter med bukspottkörtelcancer som genomgått kirurgi syftande till bot. Överlevnad och kliniska data samt komplikationer bedöms vara i enlighet med litteraturen. Den hälsorelaterade livskvaliteten mätt med SF-36 är signifikant reducerad i samtliga åtta dimensioner talande för global påverkan på hälsan. Innan operation, och fortsatt efter, är livskvalitetsindex nedsatt utan säkerställd återhämtning. Mätt över 5 år från diagnos uppnås 1,13 kvalitetsjusterade levnadsår (QALY) (95 % konfidensintervall [KI] 0,93-1,40). Kirurgins totala kostnader är 37 239 € per person vilket ger en estimerad kostnad per QALY om 34 636 € (95 % KI 28 026 €-41 947 €), vilket är förenligt med andra större hälsointerventioner.

I arbete III görs en analys utgående från de 305 patienter som exkluderas från kirurgi och får palliativ (symtomlindrande) behandling. En undergrupp av dessa får individualiserad avancerad palliativ behandling med insulin, erytropoietin,

indometacin och näringsunderstöd. Dessa bedöms utgöra en selekterad grupp exkluderande dem med extremt kort överlevnad och bidrar med livskvalitetsdata. SF-36 data visar, liksom för dem som genomgått operation, en signifikant global nedsättning i alla dimensionerna vid diagnos. Någon statistisk skillnad föreligger inte mellan opererade och pallierade i någon av dimensionerna. Livskvalitetsindex är även här konstant sänkt under återstående livstid. Mätt över ett år från diagnos uppnås 0,2 (95 % KI 0,17-0,23) QALY i den pallierade gruppen och 0,48 (95 % KI 0,44-0,54) i den opererade. Sjukvårdens totala kostnader baserat på kirurgi, onkologi och primärvård inklusive hospice uppgår till 23 701 € för pallierade och 50 950 € för opererade. Det ger en kostnad på 118 418 € respektive 106 146 € per QALY. Det är uppenbart att de få långtidsöverlevarna i den opererade gruppen väger upp den tvåfaldiga kostnaden för kirurgisk behandling sett över ett år. Ser man över fler år är kostnadseffektiviteten än större i den opererade gruppen. Av detta kan konstateras att förlängande av liv är den helt avgörande faktorn vid kostnadsnyttobedömning av bukspottkörtelcancervård.

I arbete IV analyseras förändringar i genomets kopienummer. 59 tumörprover samhybridiseras med referens-DNA till kända oligonukleotidsekvenser och efter subtraktion av förmodade friska kopienummervarianter fastställs patologiska mutationer på individnivå. Vi finner förvånansvärt få förändringar med våra restriktiva inställningar. För att påvisa gener med prognostisk betydelse delas materialet in i tre grupper; kort, medel och lång överlevnad. Vid jämförelse mellan grupperna kort och lång överlevnad finns enstaka gener som når signifikans men bara hos en del av patienterna. Tolkningen är att mutationsprofilerna är för heterogena för att vi ska kunna detektera prognostiska mönster. Inte heller i konfirmatorisk klusteranalys ses kluster med avseende på överlevnad.

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Appendices

Paper I

Paper II

Editorial on Paper II

Paper III

Paper IV