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Henriksen, Stine Dam

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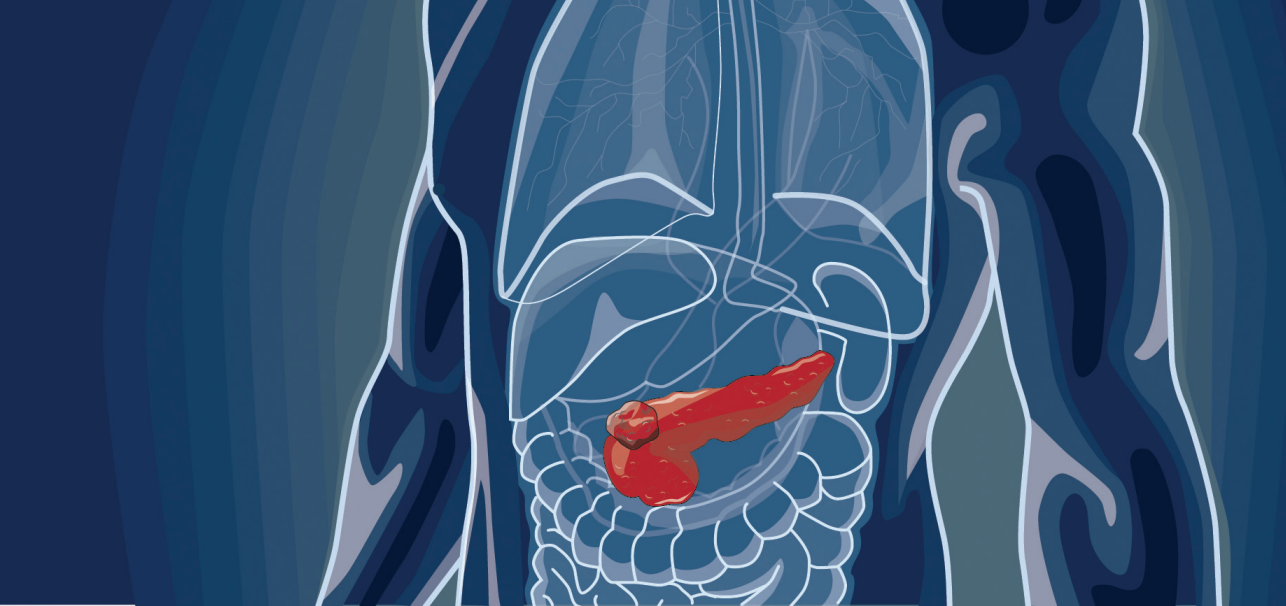
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**CELL-FREE DNA PROMOTER
HYPERMETHYLATION AS BLOOD-BASED
MARKERS FOR PANCREATIC
ADENOCARCINOMA**

**BY
STINE DAM HENRIKSEN**

DISSERTATION SUBMITTED 2017



AALBORG UNIVERSITY
DENMARK

**CELL-FREE DNA PROMOTER
HYPERMETHYLATION AS BLOOD-BASED
MARKERS FOR PANCREATIC
ADENOCARCINOMA.**

PhD dissertation

Stine Dam Henriksen



AALBORG UNIVERSITY
DENMARK

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PhD supervisor: Ole Thorlacius-Ussing
Consultant surgeon, Professor, DMSc
Department of Gastrointestinal Surgery
Aalborg University Hospital, Denmark

Assistant PhD supervisor: Henrik Krarup, Consultant, PhD
Section of Molecular Diagnostics and Clinical Biochemistry
Aalborg University Hospital, Denmark

Poul Madsen, MSc
Section of Molecular Diagnostics and Clinical Biochemistry
Aalborg University Hospital, Denmark

PhD committee: Ursula Falkmer, Consultant, Professor, PhD (chairman)
Aalborg University, Denmark

Jens Hillingsø, Consultant surgeon, Ass. Professor, PhD
University of Copenhagen, Denmark

Mads Thomassen, Ass. Professor, PhD
University of Odense, Denmark

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CV

Stine Dam Henriksen, MD
Born 1981, Aalborg, Denmark
Email: stdh@rn.dk



Education

2016- Second year of specialized training in abdominal surgery.
2013-2016 Ph.D. fellow at Aalborg University
2014-2015 First year of specialized training in abdominal surgery.
2002-2009 Medical graduate (MD), Aarhus University, Denmark
1998-2001 Student (math/physics), Aalborghus Gymnasium, Denmark
1997-1998 Student, Stanhope Elmore High School, Alabama, USA

Previous work

2016- Department of Gastrointestinal Surgery (Second year of specialized training in abdominal surgery), Aalborg University Hospital, Denmark
2015-2016 Department of Gastrointestinal Surgery (Research assistant, Ph.D. fellow), Aalborg University Hospital, Denmark
2014-2015 Department of General Surgery (First year of specialized training in abdominal surgery), Hospital of Vendsyssel, Denmark
2012-2014 Research assistant, Ph.D. fellow, Department of Gastrointestinal Surgery, Aalborg University Hospital, Denmark
2011-2012 Department of General Surgery (One year introductory employment in abdominal surgery), Hospital of Vendsyssel, Denmark
2011 Department of Internal Medicine, Hospital of Vendsyssel, Denmark
2010-2011 General practice, Klarup, Denmark
2009-2010 Department of General Surgery, Hospital of Vendsyssel, Denmark

Scientific work

Cell-free DNA Promoter Hypermethylation in Plasma as a Diagnostic Marker for Pancreatic Adenocarcinoma. *Clinical Epigenetics*, 2016; Vol 8, p 117.
Stine Dam Henriksen, Poul Henning Madsen, Anders Christian Larsen, Martin Berg Johansen, Asbjørn Mohr Drewes, Inge Søkilde Pedersen, Henrik Krarup, Ole Thorlacius-Ussing

Hypermethylated SEPT9 in colorectal cancer compared to pancreatic cancer and benign gastrointestinal disease. Abstract at the annual meeting of the European Society of Coloproctology 2016, Milan, Italy. *Colorectal Disease 2016; Vol 18 (Suppl. 1) 44-125.*

Stine Dam Henriksen, Simon Ladefoged Rasmussen, Mogens Stender, Anders Christian Larsen, Kåre Sunesen, Poul Henning Madsen, Henrik Krarup, Ole Thorlacius-Ussing

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DNA Hypermethylation as a Blood-Based Marker for Pancreatic Cancer: A Literature Review. *Pancreas, 2015; Vol 44, p1036-1045.*

Stine Dam Henriksen, Poul Henning Madsen, Henrik Krarup, Ole Thorlacius-Ussing

DNA Hypermethylation as a Blood-Based Marker for Pancreatic Cancer: A Literature Review. Abstract at the annual meeting of the Danish Surgical Society 2015, Copenhagen, Denmark. *Not published.*

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ENGLISH SUMMARY

Pancreatic cancer is a highly aggressive disease. Over the past decade, the mortality rate of pancreatic cancer has remained stable and the disease continue to have a dismal overall prognosis. One of the main reasons for this poor prognosis is the difficulty of detecting the disease at early stages, emphasizing the need for further research to significantly improve early detection methods and therapeutic options.

This thesis includes four studies. Study I is a review of the literature addressing genes that are aberrantly methylated and detectable in blood from patients with pancreatic cancer, with the aim of gaining knowledge about hypermethylated genes useful as blood-based markers for pancreatic adenocarcinoma. The review revealed that eight studies on cell-free DNA hypermethylation had been published. None of the genes previously examined had the potential to serve as an individual diagnostic marker, suggesting that a panel of several genes was needed to achieve sufficient performance. Based on the literature review, we selected a panel of 28 hypermethylated promoter regions in plasma-derived cell-free DNA.

The aim of study II was to test the selected panel of genes as a diagnostic marker for pancreatic adenocarcinoma. Consecutive patients with pancreatic adenocarcinoma (n = 95) were included prospectively. Three benign control groups were included: patients suspected of but without upper gastrointestinal malignancy (control group 1, n = 27), patients with chronic pancreatitis (control group 2, n = 97), and patients with acute pancreatitis (control group 3, n = 59). In study II we demonstrated that the mean number of hypermethylated genes in the whole gene panel (28 genes) was significantly higher for cancer patients (8.41 (95% confidence interval (CI): 7.62-9.20)) than for the three benign control groups (control group 1 (4.89 (95% CI: 4.07-5.71)), control group 2 (4.34 (95% CI: 3.85-4.83)) and control group 3 (5.34 (95% CI: 4.77-5.91))). Seventeen genes were more frequently hypermethylated in patients with pancreatic adenocarcinoma compared with the combined control group 1+2. We developed a diagnostic prediction model (*BMP3*, *RASSF1A*, *BNC1*, *MEST*_{v2}, *TFPI2*, *APC*, *SFRP1*, *SFRP2*, and the covariate age > 65 years) that enabled the differentiation of pancreatic adenocarcinoma patients and control group 1+2 with 76% sensitivity and 83% specificity (area under the receiver operating characteristic curve (AUC) of 0.86). Furthermore, the diagnostic prediction model was independent of cancer stage.

The aim of study III was to test the selected panel of genes as markers for pancreatic adenocarcinoma staging. We demonstrated in study III that patients with stage IV disease had a significantly higher number of mean hypermethylated genes (10.24 (95% CI: 8.88-11.60)) than patients with stage I, II and III disease (7.09 (95% CI: 5.52-8.67), 7.00 (95% CI: 5.93-8.07) and 6.77 (95% CI: 5.08-8.46)). The

hypermethylation frequencies of seven genes were significantly increased in patients with stage IV disease compared with patients with stage I, II and III disease. We developed a prognostic prediction model (*SEPT9v2*, *SST*, *ALX4*, *CDKN2B*, *HIC1*, *MLH1*, *NEUROG1*, and *BNC1*) that could differentiate stage IV disease from stage I, II and III disease with a sensitivity of 74% and a specificity of 87% (AUC of 0.87). An additional prognostic prediction model (*MLH1*, *SEPT9v2*, *BNC1*, *ALX4*, *CDKN2B*, *NEUROG1*, *WNT5A*, and *TFPI2*) enabled the differentiation of potential resectable disease (stage I and II) from non-resectable pancreatic adenocarcinoma (stage III and IV) with 73% sensitivity and 80% specificity (AUC of 82%).

The aim of study IV was to test the selected panel of genes as markers for survival of pancreatic adenocarcinoma. In an analysis adjusted for cancer stage and age, we found a significant hazard ratio of 2.03 (95% CI: 1.15-3.57) for patients with more than 10 hypermethylated genes compared with patients with less than 10 hypermethylated genes. Several individual genes were associated with survival and varied with cancer stage. Overall, promoter hypermethylation had a negative influence on survival, but hypermethylation of a few specific genes seemed to have a positive effect on survival and could therefore represent less aggressive tumours. Based on the selected panel of 28 genes, we developed prediction models for survival (for the total group of patients and for subgroups (stage I-II and stage IV)), which enabled stratification of patients in risk groups according to survival time.

In conclusion, the findings of our studies indicate that plasma-derived cell-free DNA promoter hypermethylation has potential as blood-based markers for the diagnosis, stage classification and prognosis of pancreatic adenocarcinoma. However, external validation is required to substantiate the results prior to clinical application.

DANSK RESUMÉ

Kræft i bugspytkirtlen er en særdeles aggressiv kræftsygdom forbundet med en yderst dårlig prognose, som ikke er forbedret de seneste årtier. Den høje dødelighed er blandt andet forårsaget af, at diagnosen er vanskelig at stille i de tidlige sygdomsstadier. Ovenstående understreger, at der er behov for yderligere forskning indenfor området, for således at kunne forbedre den tidlige diagnostik og dermed kunne optimere behandlingen.

Denne afhandling omfatter fire studier. Studie I er en gennemgang af den foreliggende litteratur omhandlende kræft i bugspytkirtlen og DNA metyleringer i blodet. Formålet med litteraturgennemgangen var at finde gener, som potentielt kunne være egnet, som blodbaseret markører for kræft i bugspytkirtlen. Der blev fundet otte studier om hypermetyleret cellefrit DNA. Ingen af de tidligere undersøgte gener havde potentiale som individuel diagnostisk markør, hvilket kunne antyde, at der var behov for et større gen panel for derved at øge den diagnostiske evne. Baseret på studie I udvalgte vi et panel af 28 hypermetylerede promoter regioner i cellefrit DNA deriveret fra plasma.

Formålet med studie II var at undersøge det udvalgte genpanel som diagnostisk markør for kræft i bugspytkirtlen. Konsekutive patienter med kræft i bugspytkirtlen (n = 95) blev inkluderet prospektivt. Tre kontrolgrupper uden kræft blev inkluderet: patienter mistænkt for, men uden påviselig kræft i den øverste del af mavetarmsystemet (kontrolgruppe 1 (n = 27)), patienter med kronisk betændelse i bugspytkirtlen (kontrolgruppe 2 (n = 97)) og patienter med akut betændelse i bugspytkirtlen (kontrolgruppe 3 (n = 59)). I studie II demonstrerede vi, at det gennemsnitlige antal hypermetylerede gener i genpanelet var signifikant højere hos kræftpatienterne (8.41 (95% CI: 7.62-9.20)) sammenlignet med de tre kontrolgrupper (kontrolgruppe 1 (4.89 (95% CI: 4.07-5.71)), kontrolgruppe 2 (4.34 (95% CI: 3.85-4.83)) and kontrolgruppe 3 (5.34 (95% CI: 4.77-5.91))). Sytten gener var signifikant hyppigere hypermetylerede ved kræft i bugspytkirtlen sammenlignet med kontrolgruppe 1+2. Vi udviklede en diagnostisk prædiktionsmodel (*BMP3*, *RASSF1A*, *BNC1*, *MESTv2*, *TFPI2*, *APC*, *SFRP1*, *SFRP2* og kovariaten alder > 65 år), som muliggjorde differentiering mellem patienter med kræft i bugspytkirtlen uafhængig af stadie, og patienter i kontrolgruppe 1+2 med en sensitivitet på 76% og en specificitet på 83% (AUC = 0.86).

Formålet med studie III var at undersøge det udvalgte genpanel som markør for stadieinddeling af kræft i bugspytkirtlen. I studie III fandt vi, at patienter med stadie IV sygdom havde signifikant flere hypermetylerede gener (10.24 (95% CI: 8.88-11.60)) sammenlignet med patienter med stadie I, II og III sygdom (7.09 (95% CI: 5.52-8.67), 7.00 (95% CI: 5.93-8.07) og 6.77 (95% CI: 5.08-8.46)). Syv gener var

signifikant hyppigere hypermetylerede hos patienter med stadie IV sygdom sammenlignet med stadie I, II og III sygdom. Vi udviklede herefter en prognostisk prædiktionsmodel (*SEPT9v2*, *SST*, *ALX4*, *CDKN2B*, *HIC1*, *MLH1*, *NEUROG1*, og *BNC1*), som kunne skelne patienter med stadie IV sygdom fra patienter med stadie I, II og III sygdom med en sensitivitet på 74% og en specificitet på 87% (AUC = 0.87). En anden prognostisk prædiktionsmodel (*MLH1*, *SEPT9v2*, *BNC1*, *ALX4*, *CDKN2B*, *NEUROG1*, *WNT5A*, og *TFPI2*) gjorde det muligt at differentiere mellem potentiel resektabel sygdom (stadie I og II) og ikke resektabel sygdom (stadie III og IV) med en sensitivitet på 73% og en specificitet på 80% (AUC = 0.82)

Formålet med studie IV var at undersøge det udvalgte genpanel som markør for overlevelse af kræft i bugspytkirtlen. Vi fandt i en analyse justeret for kræftstadium og alder, at patienter med mere end 10 hypermetylerede gener havde en hazard ratio på 2.03 (95% CI: 1.15-3.57) sammenlignet med patienter med mindre end 10 hypermetylerede gener. Desuden var flere individuelle gener associeret med overlevelse. Hypermetylering havde oftest en negativ indvirkning på overlevelsen og dermed associeret med en dårligere prognose. Vi fandt dog, at hypermetylering af få specifikke gener påvirkede overlevelsen i en positiv retning og derved kunne repræsentere en gruppe af mindre aggressive tumorer. Baseret på det udvalgte genpanel udviklede vi prædiktionsmodeller for overlevelse (for den samlede gruppe af patienter med kræft i bugspytkirtlen uafhængig af stadium og for undergrupper (stadie I-II og stadie IV)), som gjorde det muligt at opdele patienterne i risikogrupper i forhold til overlevelsestid.

Baseret på resultaterne fra vores studier er promotor hypermetylering i plasma derivet celle-frit DNA potentielt brugbar som blodbaseret markør for diagnosticering, stadiuminddeling og prognostisering af kræft i bugspytkirtlen. Ekstern validering er dog påkrævet for at verificere vores resultater, og ligeledes en nødvendighed for at markørerne kan blive klinisk anvendelige.

This PhD thesis is based on the following four papers:

- I. DNA Hypermethylation as a Blood-Based Marker for Pancreatic Cancer: A Literature Review. *Pancreas*, 2015, Vol 44, p1036-1045.

Stine Dam Henriksen, Poul Henning Madsen, Henrik Krarup, Ole Thorlacius-Ussing

- II. Cell-free DNA Promoter Hypermethylation in Plasma as a Diagnostic Marker for Pancreatic Adenocarcinoma. *Clinical Epigenetics*, 2016, Vol 8, p 117.

Stine Dam Henriksen, Poul Henning Madsen, Anders Christian Larsen, Martin Berg Johansen, Asbjørn Mohr Drewes, Inge Søkilde Pedersen, Henrik Krarup, Ole Thorlacius-Ussing

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- IV. Cell-Free DNA Promoter Hypermethylation in Plasma as a Predictive Marker for Survival of Patients with Pancreatic Adenocarcinoma. *Submitted for publication, Oncotarget, December 2016.*

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Stine Dam Henriksen

TABLE OF CONTENTS

CV

English summary

Danish summary/Dansk resumé

Acknowledgements

Table of contents

Funding

Abbreviations

List of genes

Table of figures

1. Introduction.....	23
1.1. Pancreatic cancer.....	23
1.1.1. Anatomy and function of the pancreas.....	23
1.1.2. Pathology of pancreatic cancer.....	24
1.1.3. Incidence.....	24
1.1.4. Risk factors.....	25
1.1.5. Diagnosing and staging.....	28
1.1.6. Treatment and Prognosis.....	29
1.2. Development of pancreatic cancer.....	31
1.3. Epigenetics.....	33
1.3.1. DNA Hypermethylation.....	35
1.4. Cell-free DNA.....	36
1.5. Methods to investigate DNA Methylation.....	38
1.5.1. Bisulfite treatment.....	38
2. Objectives.....	41
3. Materials and Methods.....	43
3.1. Study design.....	43
3.2. Method study I.....	43
3.3. Method study II, III and IV.....	43

3.3.1.	Patients with suspected or biopsy-verified pancreatic adenocarcinoma	43
3.3.2.	Patients with chronic pancreatitis	44
3.3.3.	Patients with acute pancreatitis	45
3.4.	Blood sampling.....	45
3.4.1	Patients with pancreatic adenocarcinoma and patients suspected of but without upper gastrointestinal malignancy.....	45
3.4.2	Patients with chronic pancreatitis	45
3.4.3	Patients with acute pancreatitis	46
3.5	Analytical methods	46
3.5.1	Extraction of cell-free DNA.....	46
3.5.2	Bisulfite treatment and deamination	46
3.5.3	First-round PCR	47
3.5.4	Second-round PCR.....	47
3.5.5	Gene panel	48
3.5.6	Primer design – probe design.....	48
3.5.7	Dilution series	48
3.6	Ethical issues	49
3.7	Statistics	49
3.7.1	Paper II	50
3.7.2	Paper III.....	51
3.7.3	Paper IV.....	52
4.	Summary of results	55
4.1.	Study I/Paper I.....	57
4.2.	Study II/Paper II	59
4.3.	Study III/Paper III.....	65
4.4.	Study IV/Paper IV	69
5.	Discussion.....	77
5.1.	Limitations of the studies	77
5.2.	Strengths of the studies	78
5.3.	Discussion of the findings in relation to the published literature.....	79
6.	Conclusions	89

6.1.	Study I/Paper I	89
6.2.	Study II/Paper II.....	89
6.3.	Study III/Paper III	89
6.4.	Study IV/Paper IV.....	89
6.5.	Final conclusion	90
7.	Perspectives	91
8.	Future research.....	93
9.	Literature list.....	95

Appendix A: DNA sequences for probes and primers

Appendix B: Characteristics of genes used in the gene panel

Appendix C: Hypermethylation of each gene by patient group

Appendix D: Hypermethylation of each gene by cancer stage

Appendix E: Hazard ratio for each gene based on univariate Cox regression analysis

Published papers and submitted manuscripts

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ABBREVIATIONS

AUC	Area under the receiver operating characteristic curve
AJCC	American Joint Committee on Cancer stage classification
ASA	American Society of Anesthesiologists
Bp	Base pair
CA-19-9	Carbohydrate antigen-19-9
CH3	Methyl
CI	Confidence interval
Ct	Threshold cycle
CT	Computed tomography
DNMT	DNA methyltransferase
EUS	Endoscopic ultrasound
ERCP	Endoscopic retrograde cholangiopancreatography
FDR	First-degree relative
FPC	Familial pancreatic cancer
HAT	Histone acetylase
HDAC	Histone deacetylases
HDACI	Histone deacetylases inhibitor
HR	Hazard ratio
IPMC	Intraductal papillary mucinous carcinoma
IPMN	Intraductal papillary mucinous neoplasm
LUS	Laparoscopic ultrasound
N	Lymph node
M	Distant metastasis
MCN	Mucinous cystic neoplasms
MethDet 56	Microarray-mediated methylation analysis of 56 fragments
MiRNA	MicroRNA
MOB	Methylation on beads
MSP	Methylation-specific PCR
OR	Odds ratio
PanIN	Pancreatic intraepithelial neoplasia
PET	Positron-emissions-tomography
PS	WHO performance status
QMSP	Quantitative methylation-specific PCR
RR	Relative risk
SD	Standard deviation
T	Primary tumour

LIST OF GENES

<i>ADAMTS1</i>	<i>A Disintegrin-like Metalloproteinase Thrombospondin Type 1 Motif 1</i>
<i>APC</i>	<i>Adenomatous Polyposis Coli</i>
<i>ALX4</i>	<i>Aristaless-like Homeobox 4</i>
<i>BNC1</i>	<i>Basonuclin Zinc Finger Protein 1</i>
<i>BMP3</i>	<i>Bone Morphogenetic Protein 3</i>
<i>BRAF</i>	<i>B-Raf Proto-Oncogene, Serine/Threonine Kinase</i>
<i>BRCA1</i>	<i>Breast Cancer 1</i>
<i>BRCA2</i>	<i>Breast Cancer 2</i>
<i>CDKN2A</i>	<i>Cyclin-Dependent Kinase Inhibitor 2A (P16/P14ARF)</i>
<i>CDKN2B</i>	<i>Cyclin-Dependent Kinase Inhibitor 2B (P15)</i>
<i>CHFR</i>	<i>Checkpoint with Forkhead and Ring Finger Domains</i>
<i>CFTR</i>	<i>Cystic Fibrosis Transmembrane Conductance Regulator</i>
<i>CTRC</i>	<i>Chymotrypsin C</i>
<i>DCC</i>	<i>Deleted in Colorectal Carcinoma</i>
<i>ESR1</i>	<i>Estrogen Receptor 1</i>
<i>EYA2</i>	<i>EYA Transcriptional Coactivator and Phosphatase 2</i>
<i>GSTP1</i>	<i>Glutathione S-transferase Pi 1</i>
<i>HIC1</i>	<i>Hypermethylated in Cancer 1</i>
<i>HLTF</i>	<i>Helicase-like Transcription Factor</i>
<i>HPP1</i>	<i>Hyperpigmentation, Progressive, 1</i>
<i>KRAS</i>	<i>Kirsten Rat Sarcoma Viral Oncogene Homolog</i>
<i>MESTv1</i>	<i>Mesoderm Specific Transcript Variant 1</i>
<i>MESTv2</i>	<i>Mesoderm Specific Transcript Variant 2</i>
<i>MGMT</i>	<i>O-6-Methylguanine-DNA Methyltransferase</i>
<i>MLH1</i>	<i>MutL Homolog 1</i>
<i>MSH2</i>	<i>MutS Homolog 2</i>
<i>MSH6</i>	<i>MutS Homolog 6</i>
<i>NEUROG1</i>	<i>Neurogenin 1</i>
<i>NPTX2</i>	<i>Neuronal Pentraxin 2</i>
<i>PENK</i>	<i>Preproenkephalin</i>
<i>PALB2</i>	<i>Partner and Localizer of BRCA2</i>
<i>PMS2</i>	<i>PMS1 Homolog 2, Mismatch Repair System Component</i>
<i>PRSS1</i>	<i>Protease, Serine 1</i>
<i>PRSS2</i>	<i>Protease, Serine 2</i>
<i>PTEN</i>	<i>Phosphatase and Tensin Homolog</i>
<i>RARB</i>	<i>Retinoic Acid Receptor Beta</i>
<i>RASSF1A</i>	<i>Ras Associated Domain Family Member 1</i>
<i>RNF43</i>	<i>Ring Finger Protein 43</i>
<i>SEPT9v2</i>	<i>Septin 9 Transcript Variant 2</i>
<i>SFRP1</i>	<i>Secreted Frizzled-Related Protein 1</i>
<i>SFRP2</i>	<i>Secreted Frizzled-Related Protein 2</i>
<i>SMAD4</i>	<i>Mother Against Decapentaplegic Homolog 4</i>
<i>SPINK1</i>	<i>Serine Peptidase Inhibitor, Kazal Type 1</i>

<i>SST</i>	<i>Somatostatin</i>
<i>STK11</i>	<i>Serine/Threonine Kinase 11</i>
<i>TAC1</i>	<i>Tachykinin, Precursor 1 (Substance P)</i>
<i>TFPI2</i>	<i>Tissue Factor Pathway Inhibitor 2</i>
<i>TP53</i>	<i>Tumour Protein P53</i>
<i>UCHL1</i>	<i>Ubiquitin Carboxy-terminal Hydrolase L1</i>
<i>VIM</i>	<i>Vimentin</i>
<i>WNT5A</i>	<i>Wingless-Type MMTV Integration Site Family, Member 5A</i>

TABLE OF FIGURES

- Figure 1. The pancreas
- Figure 2. The Whipple procedure
- Figure 3. The neoplastic development of PanIN
- Figure 4. The chromatin structure with epigenetic marks
- Figure 5. Methylation of cytosine
- Figure 6. The release of cell-free DNA into the blood
- Figure 7. Bisulfite treatment
- Figure 8. Flow diagram of the inclusion of patients
- Figure 9. Review of the literature
- Figure 10. Diagnostic prediction model: Stepwise backwards elimination
- Figure 11. Performance of diagnostic prediction Model 13
- Figure 12. Prognostic prediction model stage I, II and III vs IV: Stepwise backwards elimination
- Figure 13. Performance of prognostic prediction Model 10: Stage I, II and III vs IV
- Figure 14. Prognostic prediction model stage I and II vs III and IV: Stepwise backwards elimination
- Figure 15. Performance of prognostic prediction Model 7: Stage I and II vs III and IV
- Figure 16. Survival according to the total number of hypermethylated genes
- Figure 17. Survival analysis for the total group of patients with pancreatic adenocarcinoma prior to staging
- Figure 18. Survival analysis for stage I and II pancreatic adenocarcinoma
- Figure 19. Survival analysis for stage IV pancreatic adenocarcinoma

Illustrations by Katrine Dam Henriksen

1. INTRODUCTION

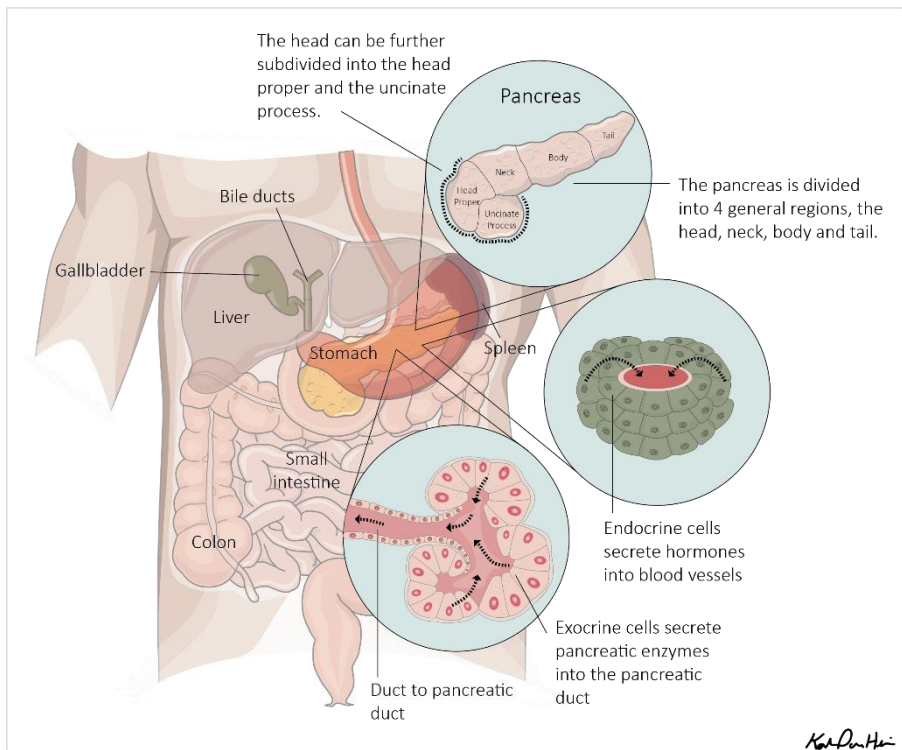
1.1. PANCREATIC CANCER

Pancreatic cancer is one of the most challenging tumours worldwide. It is characterized as a highly aggressive disease that is usually diagnosed at advanced stages and is resistant to therapy, resulting in a dismal overall prognosis. Over the past decade, a downward trend in mortality has been observed for most other major cancer sites. However, the mortality rate for pancreatic cancer has remained stable.¹ The poor prognosis emphasizes the need to understand its pathogenesis to significantly improve early detection methods and therapeutic options.

1.1.1. ANATOMY AND FUNCTION OF THE PANCREAS

The pancreas is j-shaped, approximately 15 cm long and has a weight of 70-100 grams (Figure 1).²

Figure 1. The pancreas



The pancreas is located in the deep part of the upper abdomen, behind the stomach and the peritoneum on the ventral side of the first and second lumbar vertebra. The head of the pancreas is surrounded by the curve of the duodenum, overlying the vena cava. The aorta and the superior mesenteric vessels lie behind the neck of the pancreas. The tail of the pancreas extends up to the spleen. Furthermore, the pancreas is located near the liver, the gallbladder and the bile duct (Figure 1).²

The pancreas is a glandular organ of the digestive system and consists of exocrine and endocrine functions (Figure 1). The exocrine pancreas represents 80-90% of the organ and comprises both acinar and ductal cells, where the acinar cells (or acini) are organized into lobules; the acinar cells are responsible for the synthesis, storage and secretion of enzymes such as amylase, lipase and trypsinogen. The acinar cells are located around a central lumen, which communicate with the duct system.² The exocrine cells produce 1500-2000 ml of pancreatic juice daily, consisting of alkaline fluid and digestive enzymes, which is secreted through the pancreatic duct to the duodenum.² The pancreatic ducts are lined by epithelial cells. The pancreatic secretion is maintained by a complex interaction between neural, hormonal and mucosal factors.³ The main function of the endocrine cells is to secrete multiple hormones, including insulin and glucagon, into the bloodstream to regulate glucose homeostasis. The endocrine cells are distributed in clusters called islets of Langerhans, which are located between the exocrine cells.²

1.1.2. PATHOLOGY OF PANCREATIC CANCER

Pancreatic cancer can arise from all cells of the pancreatic tissue, resulting in tumours from exocrine cells and tumours originating from endocrine cells. However, the most common type of pancreatic cancer is pancreatic adenocarcinoma arising from the pancreatic ductal epithelium. Pancreatic adenocarcinoma accounts for approximately 80-90% of all pancreatic cancer cases.^{2,4}

This PhD thesis focuses solely on pancreatic adenocarcinoma.

1.1.3. INCIDENCE

The incidence of pancreatic cancer in the general population is low (life-time risk of 1.3%).⁵ In 2014, 954 patients were diagnosed with pancreatic cancer in Denmark.⁶ However, world-wide, approximately 337000 patients are diagnosed with pancreatic cancer annually.⁷ In total, pancreatic cancer accounts for 2-3% of all adult cancer cases.^{1,8}

1.1.4. RISK FACTORS

Age and Gender

According to worldwide data, pancreatic cancer is slightly more common in men than in women^{1,8}; however the incidence in Denmark has been identical between genders for the past couple of years.⁶ Advanced age is one of the most important risk factors,^{6,8} with a very low risk until the age of 50. The risk subsequently increases, with a median patient age of 71 years at the time of diagnosis.⁹

Smoking and Alcohol

Smoking is the most important modifiable risk factor for pancreatic cancer. Smoking is estimated to be responsible for approximately 20-30% of pancreatic cancer cases.¹⁰ Smokers have a 74% higher risk for pancreatic cancer than non-smokers.¹¹ In addition, smokers with a family history of pancreatic cancer have an even greater risk.¹² Data regarding alcohol and the risk of developing pancreatic cancer are conflicting. However, high alcohol consumption tends to be associated with an increased risk of pancreatic cancer.^{10,11,13,14}

Obesity and Overweight

Obesity and overweight have been linked to an increased risk of pancreatic cancer.^{10,15} Obese individuals have a 20% higher risk of developing pancreatic cancer than normal weight individuals.¹⁵

Diabetes

Diabetes is a risk factor for pancreatic cancer.¹¹ Patients with long-term type two diabetes have a 50% increased risk of pancreatic cancer compared with non-diabetic individuals. Patients with type one diabetes also have an increased risk.¹⁶ Furthermore, new-onset diabetes is a potential sign of disease.¹⁷ Approximately 25% of patients suffer from diabetes at diagnosis.¹⁸

Pancreatitis

There is strong evidence for an association between long-standing chronic pancreatitis and pancreatic cancer.¹⁹ Chronic pancreatitis is an inflammatory disease involving the pancreatic parenchyma, which is progressively destroyed and replaced by fibrotic tissue. The risk correlates with the duration of recurrent pancreatitis and chronic inflammation.¹⁹ Four percent of patients with chronic pancreatitis develop pancreatic cancer within 20 years of diagnosis.^{11,19} Patients with a rare type of pancreatitis, hereditary pancreatitis, have an even higher risk of pancreatic cancer, with an assessed life-time risk of 25-55%.¹⁹⁻²¹

Genetic risk

The majority of pancreatic cancer appears to be sporadic, and only 5-10% of pancreatic cancer cases are caused by inherited genetic factors. The genetic basis of

much of the inherited susceptibility to pancreatic cancer remains unexplained⁵. However, there are a number of tumour predisposition syndromes, that entail an increased risk of pancreatic cancer (Table 1).^{4,5,11,21} In addition, hereditary pancreatitis and cystic fibrosis also have an increased risk of pancreatic cancer due to a genetically determined early change in the pancreas tissue.^{4,11,21}

Familial pancreatic cancer (FPC) refers to families with two or more first-degree relatives (FDRs) diagnosed with pancreatic cancer without a known genetic defect. Individuals with two FDRs with pancreatic cancer have an estimated life-time risk of developing pancreatic cancer of 6-12%, whereas individuals with three or more FDRs have a life-time risk of 30-40%.^{4,20,21}

Table 1. Tumour predisposition syndromes entailing an increased risk of pancreatic cancer

<i>Syndromes</i>	<i>Genetic mutation</i>	<i>Risk of pancreatic cancer</i>
Hereditary breast and ovarian cancer ^{5,11,21,22}		
	<i>BRCA2</i>	3-10 fold increased risk. RR: 3.5 (95% CI: 1.87-6.58) Accounts for the highest percentage (15%) of known causes of inherited pancreatic cancer cases.
	<i>PALB2</i>	Similar increased risk as <i>BRCA2</i> mutation. Accounts for 3% of known causes of inherited pancreatic cancer cases. ^{5,22}
	<i>BRCA1</i>	2-3 fold increased risk. RR: 2.3-2.55
Peutz-Jeghers Syndrome ^{5,11,21,22}		
	<i>STK11</i>	132 fold increased risk. Life-time risk: 11-36% up to age 65-70. RR: 76 (95% CI: 36-160)
Hereditary non-polyposis colorectal cancer (HNPCC or Lynch syndrome) ^{5,11,21}		
	<i>MLH1</i> <i>MSH2</i> <i>MSH6</i> <i>PMS2</i>	8.6 fold increased risk. Life-time risk: 3.7
Familial-atypical multiple mole melanoma (FAMMM) ^{5,11,21}		
	<i>CDKN2A</i>	13-22 fold increased risk. Life-time risk: 17% by age 75 years.
Familial adenomatous polyposis (FAP) ¹¹		
	<i>APC</i>	RR: 4.46 (95% CI: 1.2-11.4)
Li- Fraumeni ¹¹		
	<i>TP53</i>	RR: 7.3
Cystic fibrosis ¹¹		
	<i>CFTR</i>	2 fold increased risk before the age of 60 year. RR: 5.3 (95% CI: 2.4-10.1)
Hereditary pancreatitis ^{5,11,21,22}		
	<i>PRSS1</i> – autosomal dominant <i>SPINK1</i> – autosomal recessive <i>PRSS2</i> <i>CTRC</i>	26-70 fold increased risk. Life-time risk: 25-55% by age 70.

RR: Relative risk.

CI: Confidence interval.

1.1.5. DIAGNOSING AND STAGING

Diagnosing early-stage pancreatic cancer is challenged by the lack of symptoms in the early stages of the disease. If patients present with symptoms, it is likely to be unspecific symptoms such as abdominal pains, weight loss, fatigue and jaundice⁴. Such symptoms are also related to chronic pancreatitis, an essential differential diagnosis and a known risk factor for pancreatic cancer.^{19,23}

Several different imaging modalities are used in the diagnostic work-up, such as positron emission tomography (PET) scan, computed tomography (CT) scan, endoscopic (EUS) or laparoscopic ultrasound (LUS) and endoscopic retrograde cholangiopancreatography (ERCP).^{4,5} Some of these methods are invasive and entail a risk of complications. However, histological evaluation is often necessary. Despite the use of these techniques, diagnosis may remain difficult. In extreme cases, surgery may be needed to establish a definite diagnosis, which also implies a risk of overtreatment.

The only clinical available biomarker for pancreatic cancer is carbohydrate antigen-19-9 (CA-19-9). However, CA-19-9 lacks sufficient sensitivity and specificity for use as a diagnostic marker.²⁴⁻²⁷ In addition, 10% of the population lacks the ability to produce CA-19-9 due to Le^{a-b-} blood group status, which makes its utility less apparent.^{24,25,28} It would be a major advance for patients if additional minimal invasive markers were available to facilitate the detection of the disease at an early stage. A blood-based diagnostic marker for pancreatic cancer would be ideal for screening high-risk individuals and patients with an intermediate risk of pancreatic cancer, such as patients with chronic pancreatitis and late-onset diabetes. Furthermore, such a marker could serve as a supplement to existing clinical tools in the diagnostic work-up of patients suspected of pancreatic cancer.

Pancreatic cancer is staged according to the extent of disease, as defined by the primary tumour (T), lymph node (N) and distant metastasis (M) system (Table 2).²⁹ Only 20% of patients have localized cancer at time of diagnosis. The remaining patients either have locally advanced or metastatic disease.³⁰ Correct staging is very important because treatment and prognosis are stage-specific.^{4,31}

Table 2. Pancreatic cancer AJCC staging 7th edition²⁹

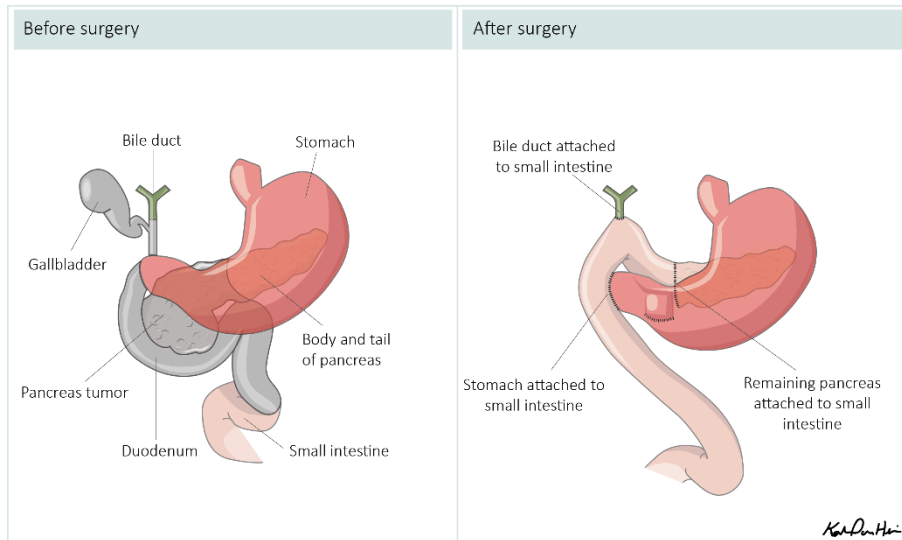
	T	N	M	5-year survival rate
Stage 0	Tis	N0	M0	-
Stage IA	T1	N0	M0	14%
Stage IB	T2	N0	M0	12%
Stage IIA	T3	N0	M0	7%
Stage IIB	T1/T2/T3	N1	M0	5%
Stage III	T4	Any N	M0	3%
Stage IV	Any T	Any N	M1	1%
Primary tumour (T)				
Tis	Carcinoma in situ (also includes the PanIN-3)			
T1	Tumour limited to the pancreas, 2 cm or less in greatest dimension			
T2	Tumour limited to the pancreas, more than 2 cm in greatest dimension			
T3	Tumour extends beyond the pancreas but without involvement of the celiac axis or the superior mesenteric artery			
T4	Tumour involves the celiac axis or the superior mesenteric artery (unresectable primary tumour)			
Regional Lymph Nodes (N)				
N0	No regional lymph node metastases			
N1	Regional lymph node metastases			
Distant Metastases (M)				
M0	No distant metastases			
M1	Distant metastases			

AJCC: American Joint Committee on Cancer stage classification.

1.1.6. TREATMENT AND PROGNOSIS

The only curative treatment for pancreatic cancer is complete tumour resection. Only stage I and II pancreatic cancer are potentially resectable.^{4,31} The most commonly used procedure is pancreatoduodenectomy, also known as the Whipple procedure (Figure 2).² The Whipple procedure involves complex and extensive surgery, including the removal of a portion (the caput/head) of the pancreas involving the tumour, the duodenum, the gallbladder and part of the bile duct. The remaining organs are reattached to permit digestion of food (Figure 2).^{2,4,31}

Figure 2. The Whipple procedure



Unfortunately, only 10-20% of patients receive curatively intended treatment. Despite surgery, 50% of patients experience recurrence.^{4,30} For a small subgroup of patients with resectable tumours and no co-morbidity, a 5-year survival rate of up to 54% has been demonstrated.³² Patients who are ineligible for curative treatment due to more advanced pancreatic cancer are offered palliative treatment with chemotherapy or chemo-radio-therapy.³⁰ The median survival time of patients who do not undergo surgery is only 3 to 6 months.^{30,31}

Difficulties in detecting the disease at an early stage, aggressive malignant behaviour and a largely radio-/chemotherapy-resistant phenotype result in very high mortality (Table 2). Pancreatic cancer is one of the leading causes of cancer death worldwide, with an overall 5-year survival rate of only 5-7%.^{7,9}

Minimally invasive markers for pancreatic cancer prognosis and survival are lacking. However, CA-19-9 has prognostic properties, as elevated levels are more common in advanced cancer stages. In addition, a preoperative increased level of CA-19-9 is associated with decreased survival and a low resectability rate.^{25,33}

Additional prognostic markers would be highly beneficial and could facilitate the initial identification of patients with more aggressive tumour biology, help direct patient expectations, optimize therapeutic decision making and promote individualized therapy.

1.2. DEVELOPMENT OF PANCREATIC CANCER

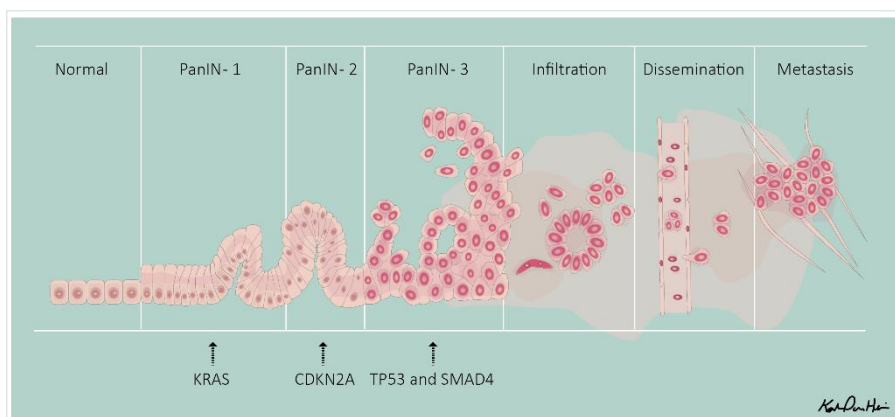
The development of pancreatic cancer occurs over several years. The carcinogenesis involves multiple biological alterations, including an accumulation of both inherited and acquired genetic and epigenetic modifications.^{34,35}

There are three known types of precursor lesions, which represent alternate routes to pancreatic cancer formation.

Pancreatic intraepithelial neoplasia (PanIN)

The most common type of precursor is PanIN (Figure 3), microscopic lesions arising from the pancreatic ducts. PanINs are classified into three grades depending on the degree of architectural and cytological atypia.³⁴ Low-grade PanIN-1 is common, whereas high-grade PanIN-3 (carcinoma in situ) is more rare and is usually found together with invasive pancreatic carcinoma.^{4,36} The overall risk of PanINs developing into cancer is one percent, with the highest risk for PanIN-3.³⁴

Figure 3. The neoplastic development of PanIN



Intraductal papillary mucinous neoplasm (IPMN)

IPMNs are far less common than PanINs.³⁴ They are radiographically detectable cystic tumours that communicate with the pancreatic duct and are present in approximately 2% of adults and 10% of individuals above 70 years of age.⁴ They are divided into adenoma, borderline and intraductal papillary mucinous carcinoma (IPMC) according to the degree of dysplasia.³⁴ IPMNs are associated with an overall risk of invasive cancer of 20-50%, with those arising from the main pancreatic duct having a considerably higher risk than those originating from the branch duct.^{4,34}

Mucinous cystic neoplasms (MCN)

MCNs are large mucin-secreting neoplasms with a size of 1-3 cm and are associated with an ovarian type stroma. MCNs are very rare; however, the incidence is much higher in women than in men (20:1). Approximately 20% of MCNs are associated with pancreatic cancer, and all MCNs have potential to progress into carcinoma in situ.³⁴

Genetic mutations in precursor lesions and pancreatic cancer

The most common type of somatic mutation in pancreatic cancer is mutation of the *KRAS* gene (a single point mutation involving a single amino acid substitution from G to D at codon 12).^{22,34} Oncogenic *KRAS* activates the MAP kinase and/or the PI3K pathways, leading to increased cell proliferation, cell division and cell survival.^{4,22,34,36} Furthermore, oncogenic *KRAS* stimulates the desmoplastic stroma. *KRAS* mutation is present in the majority of pancreatic cancers, including in more than 90% of PanINs of all grades,^{4,34,36} and approximately 50% of IPMNs and MCNs, and the prevalence increases with the degree of dysplasia.^{4,34}

Mutation in *BRAF*, which is also involved in the MAP kinase pathway, is observed in 7-15% of pancreatic cancer cases³⁴ and in a small number of PanINs.^{4,36}

Mutation in the *GNAS* gene (encoding the G-protein subunit alpha-s, which activates adenylate cyclase leading to cyclic AMP production) is present in 40-80% of IPMNs and is commonly observed in pancreatic cancer arising from IPMNs.^{4,37}

CDKN2A is a tumour suppressor gene encoding two tumour suppressor proteins: P16 and P14. P16 is an inhibitor of the cyclin D-dependent kinases CDK4 and CDK6, which indirectly prevents phosphorylation of the retinoblastoma protein and consequently arrests the cell cycle. Loss of P16 function leads to cell proliferation by entry into the cell cycle. P16 inactivation is observed in 95% of pancreatic cancer cases and is the most frequently inactivated tumour suppressor gene in pancreatic cancer.³⁴ However, the inactivation is caused by a variety of mechanisms, including homozygous deletion, intragenic mutation and promoter methylation.³⁴ *CDKN2A* mutation is also observed in all precursors (PanINs, IPMNs and MCNs), with increasing incidence with increasing lesion grade.^{4,34,36}

The tumour suppressor gene *SMAD4* is involved in the TGF beta pathway and in activation of P21 transcription. P21 is a cell cycle inhibitor, and loss of function results in uncontrolled proliferation. *SMAD4* mutation generally appears late in the neoplastic progression (PanIN-3, IPMC and cancer arising from MCNs) and is present in approximately 55% of pancreatic cancer cases.^{4,34,36}

Mutation of the tumour suppressor gene *TP53* (encoding Tumour protein 53) is also a late event in neoplastic development. Tumour protein 53 regulates the G1-S cell cycle checkpoint, maintaining G2-M arrest and inducing apoptosis.³⁷ Loss of Tumour protein 53 enables cellular survival and division in the presence of DNA damage³⁷. Inactivation of the *TP53* gene is present in 75% of pancreatic cancer cases, including 12% of PanIN-3, 30% of IPMN adenoma/-borderline, and 50-60% of IPMCs.^{4,34,36}

Inactivating mutations in the *RNF43* gene (which encodes a ubiquitin ligase and acts as a tumour suppressor inhibiting the Wnt pathway) are frequently detected in MCNs and in approximately 50% of IPMNs.^{4,37}

MicroRNAs (miRNAs) in pancreatic cancer

MiRNAs, which are small non-coding RNAs (20-22nt), have also been linked to cancer initiation and progression. Alterations in the expression of miRNAs can occur in early to late precursor lesions towards pancreatic cancer and can be caused by several different mechanisms. MiRNAs are involved in the negative regulation of mRNA translation. More than 130 miRNAs have been documented as deregulated in pancreatic cancer.^{34,38,39}

Telomere length

Telomeres are DNA-protein complexes that contain repetitive nucleotide sequences at the ends of the chromosome arms. Telomeres prevent chromosome fusion and help maintain genomic stability. Telomere length is shortened in pancreatic cancer and it is detectable even in low-grade PanINs and IPMNs.³⁷

Acinar-to-ductal metaplasia

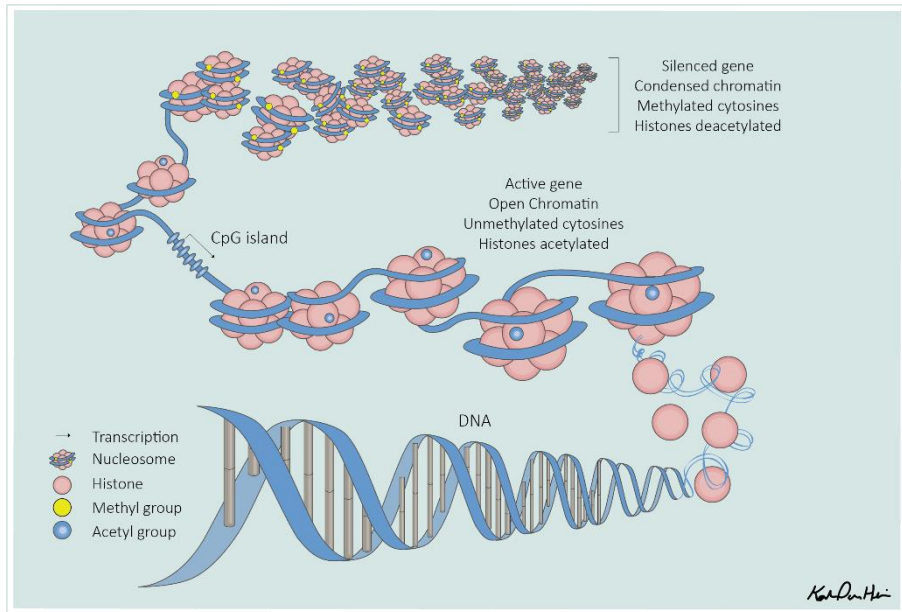
Ductal cells may be intuitively considered the cell of origin for ductal adenocarcinoma. However, several studies have suggested multiple cell types as potential cells of origin in pancreatic adenocarcinoma. Acinar cells usually have a strong ability to undergo regeneration and renewal in response to tissue injury, but loss of acinar cell identity due to pancreatic injury, may lead to acinar-ductal metaplasia.⁴⁰ Acinar cells expressing *KRAS* mutation can be reprogrammed into ductal cells and subsequently form PanIN.³⁷ Additionally, centroacinar cells, which are situated at the terminal ends of the pancreatic ducts, have also been suggested as the cell of origin for pancreatic adenocarcinoma. Inactivation of the tumour suppressor gene *PTEN* in centroacinar cells in mice activates the Akt pathway, leading to ductal metaplasia and malignant transformation.³⁷

1.3. EPIGENETICS

In the context of molecular biology, Art Riggs et al. (1996) defined epigenetics as “The study of mitotically heritable changes in gene expression that occur without changes in the DNA sequence”.⁴¹ Mitotic heritability is a phenomenon related to cell division and causes identical expression of genes in the mother and daughter cells, resulting in identical phenotypes of the two cells. The central aspect of epigenetics involves chromatin dynamics. Condensed chromatin (heterochromatin) is associated with gene silencing and inactivation. An open, lightly packed chromatin structure (euchromatin) is associated with gene transcription and activation (Figure 4). Epigenetic modifications change the chromatin structure and, consequently the gene

expression change. The main epigenetic modifications include histone modification/chromatin remodelling and DNA methylation.^{35,38,42} The epigenetic modifications are reversible and therefore potential therapeutic targets in cancer treatment.^{38,42}

Figure 4. The chromatin structure with epigenetic marks



Histone modification/chromatin remodelling

Histone proteins (Figure 4) are the foundation of chromatin and modified by various posttranslational modifications to alter chromatin structure and the compaction of DNA. Acetylation and deacetylation of lysine residues within the histone tails are epigenetic mechanisms that regulate gene expression. Acetylation of histone 3 and/or histone 4 lysine residues is mediated by histone acetylases (HATs), and results in chromatin relaxation, gene transcription and activation. Deacetylation is mediated by histone deacetylases (HDACs) and induces a tightly packed chromatin structure and gene silencing.^{35,38,43} HDAC activity is increased in various type of cancers, including pancreatic cancer. HDAC inhibitors (HDACIs) have been developed. Certain HDACIs induce the death of cultured pancreatic cells, and are promising as epigenetic drugs in cancer treatment.^{42,43}

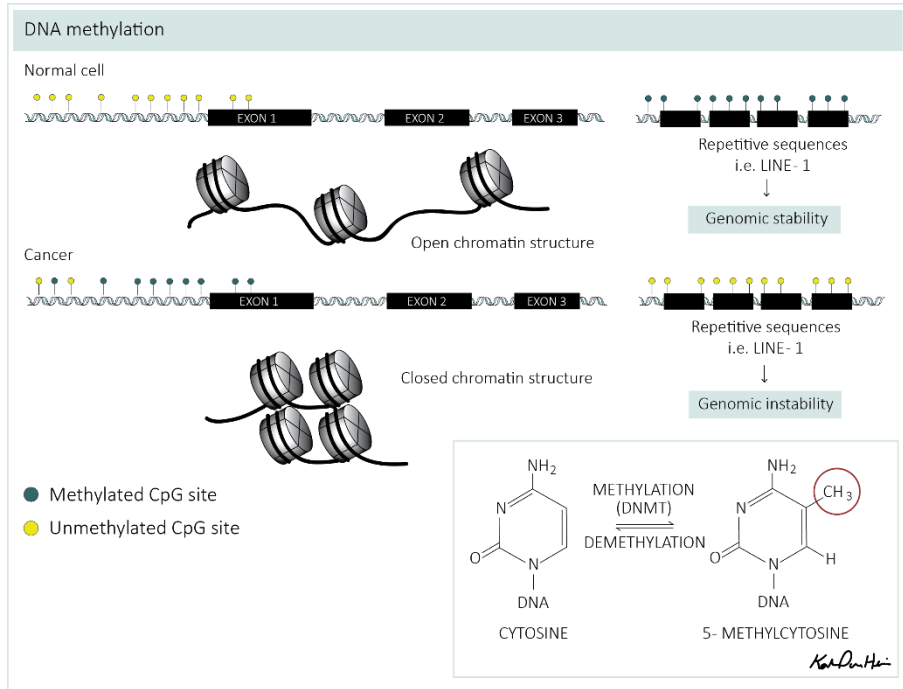
Methylation of lysine on histone 3 is another epigenetic mechanism regulating gene expression. Polycomb complexes and heterochromatin protein 1 both mediate gene silencing by methylation of specific lysine residues on histone 3.^{35,38,43}

1.3.1 DNA HYPERMETHYLATION

DNA methylation consists of the addition of a methyl (CH₃) residue to a cytosine preceding a guanosine, known as a CpG dinucleotide (Figure 5). The methyl group is added to the number five carbon of the cytosine pyrimidine ring. The reaction is catalysed by a family of enzymes known as DNA methyltransferases (DNMTs).^{34,35,38,43} CpG dinucleotides are located in CpG-rich regions known as CpG islands. In the entire human genome, approximately 50-70% of CpG dinucleotides are methylated. The majority of methylated CpG dinucleotides are located in repetitive intragenomic sequences. In addition, 60% of genes in the human genome contain one or more CpG islands in the promoter region. However, only 5% of these promoter sequences are methylated under normal conditions.^{34,38} Methylated DNA results in a tightly packed chromatin structure (heterochromatin), and unmethylated DNA is associated with lightly packed chromatin (euchromatin) (Figure 4 and Figure 5). Healthy cells regulate cellular differentiation, X-chromosome inactivation, genomic imprinting, intragenomic elements and genome stability by DNA methylation.^{34,43,44}

Aberrant DNA methylation (hypo- and hypermethylation) is a fundamental part of carcinogenesis (Figure 5). Global DNA hypomethylation of repetitive sequences is a part of early carcinogenesis and causes chromosomal instability when large parts of the genome are affected. DNA hypermethylation often occurs in the CpG islands of the promoter sequences of genes. Hypermethylation in the promoter regions of tumour suppressor genes results in downregulation or silencing of tumour suppressor function. Hypomethylation in promoter regions of oncogenes may result in increased gene expression.^{34,38,42} Carcinogenesis and DNA hypermethylation is associated with the overexpression of DNMT.^{34,38} Three types of DNMTs exist. DNMT1 is involved in the maintenance of methylation and preserving the methylation pattern from the mother cell to the daughter cell. DNMT3A and DNMT3B are involved in de novo methylation.^{22,43} The epigenetic modifications and the mechanism by which promoter hypermethylation results in gene silencing are currently not fully understood. However, it has been suggested that methylation induces gene repression by inhibiting the access of transcription factors to their binding sites and by recruiting methyl-CpG-binding proteins and histone-modifying enzymes. DNA methylation, like other epigenetic mechanisms, is a reversible process. The DNMT inhibitor 5-aza-2-deoxycytidine enables demethylation and is approved for the treatment of myelodysplastic syndrome.^{35,38,43}

Figure 5. Methylation of cytosine



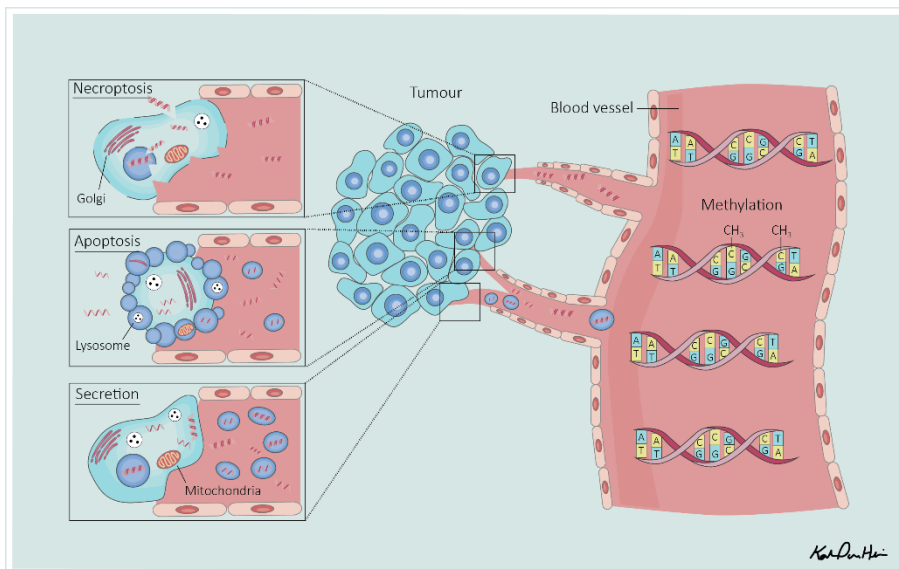
1.4. CELL-FREE DNA

The presence of cell-free nuclear acids in peripheral blood has been known for decades.^{45,46} Cell-free DNA in the serum of patients with cancer was first described in 1977, in a study that showed that patients with cancer had a larger amount of cell-free DNA (range between 0 and > 1000 ng per ml of blood) than healthy individuals.⁴⁷ In 1983, similar results were described for pancreatic disease: Patients with pancreatic cancer had significantly higher levels of cell-free DNA compared to patients with chronic or acute pancreatitis.⁴⁸ It was later shown that the amount of cell-free DNA varies with cancer type and stage of the disease.⁴⁹ In recent years, free circulating or cell-free DNA have become of major interest as tools for minimal invasive diagnostics, i.e., “liquid biopsy”. It is an alternative approach to cancer tissue biopsy for analysing genetic and epigenetic aberrations, and several studies have shown that circulating tumour DNA fragments contain genetic and epigenetic alterations identical to those in the primary tumour.^{46,49–51}

The biology of circulating tumour DNA remains unclear.^{46,52} However, the release of nucleic acids into the blood is thought to be related to the apoptosis and necrosis of cancer cells or secretion by cancer cells (Figure 6).^{46,53} Furthermore, it has been suggested that a part of the cell-free DNA may origin from circulating tumour cells undergoing cell death or acting as micrometastases.^{46,53} Nuclear acids are cleared from the blood by the liver and the kidney.⁴⁶ The half-life of cell-free DNA is only 15 minutes to a few hours,^{46,54,55} suggesting its potential utility for monitoring tumour burden to assess response to treatment, minimal residual disease and relapse.

Cell-free tumour-derived DNA has a length of 70 to 200 base pairs (bp),⁴⁶ with a peak of approximately 166 bp.⁵² A fragment size of 166 bp is the length of the DNA wrapped around a nucleosome and its linker and may result from the action of a caspase-dependent endonuclease that cleaves the DNA after a core histone.⁵² The irregular distribution of nucleosomes along the genome may contribute to the varying fragment lengths. Furthermore, studies have shown that the sizes of the fragments vary with type and stage of cancer.⁵² In addition, circulating tumour-derived DNA in plasma is shorter than wild-type cell-free DNA.⁵²

Figure 6. The release of cell-free DNA into the blood



Tumours are usually heterogenic, with a mixture of different cancer cell clones and normal cell types, resulting in the release of both tumour-derived and wild-type cell-free nuclear acids into the blood during tumour progression.⁴⁶ One of the major

challenges in working with cell-free DNA is differentiating circulating tumour DNA from circulating non-tumour DNA.⁵² This challenge is enhanced by the fact that several benign conditions, such as inflammatory disease, acute coronary syndrome, trauma and sepsis, also are associated with an increased level of cell-free DNA due to the shedding of nucleic acids into the blood by apoptotic and necrotic cells.⁵⁶

1.5. METHODS TO INVESTIGATE DNA METHYLATION

Various methods are available to determine the methylation status of specific genomic sequences.⁵⁷ There are methods based on restriction endonucleases, whose activity is influenced by methylation of the recognition site, and methods that use proteins with different affinities for methylated and non-methylated DNA. Furthermore, chemical reactions that modify either cytosine or 5-methylcytosine, such as bisulfite treatment, are widely used.⁵⁸ Bisulfite treatment followed by either microarray or sequencing are suitable and commonly used methods for studies of unknown candidate genes.⁵⁷ Digestion-based assays followed by PCR or bisulfite treatment followed by PCR and sequencing are suitable methods for studies of known candidate genes.⁵⁷

We performed bisulfite treatment for methylation analysis followed by real-time PCR. Bisulfite treatment will be described in detail below.

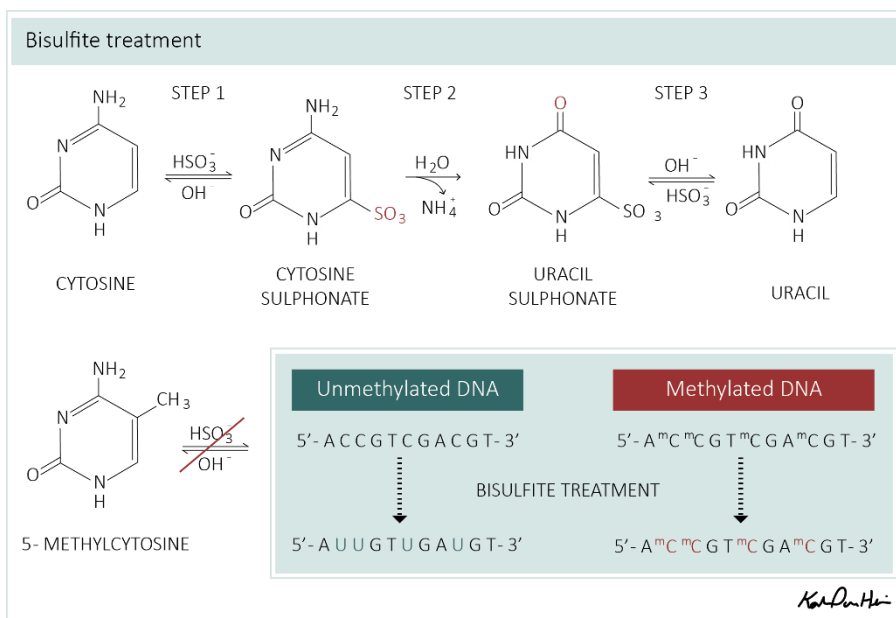
1.5.1. BISULFITE TREATMENT

Bisulfite treatment is a method frequently used for methylation analysis. Hayatsu et al. (1970) examined the addition of bisulfite to uracil and cytosine. When cytosine was treated with bisulfite, 5,6-dihydrouracil-6-sulfonate was formed via two steps (Figure 7).⁵⁹ Step 2 in Figure 7 was later shown to be the rate-determining step.⁶⁰ In addition, when uracil was treated with bisulfite, a rapid reaction occurred, forming 5,6-dihydrouracil-6-sulfonate (Figure 7).⁵⁹

Hayatsu et al. also demonstrated that 5-methylcytosine reacts with bisulfite to form thymine. The reaction of 5-methylcytosine and bisulfite, however, was much weaker than the reaction between cytosine and bisulfite. This discovery by Hayatsu et al. formed the basis for the discrimination between cytosine and 5-methylcytosine by bisulfite treatment.⁵⁹ Non-methylated cytosine treated with sodium bisulfite was deaminated to form 5,6-dihydrouracil-6-sulfonate, which was converted to uracil on treatment with mild alkali (Figure 7). In addition, bisulfite treatment converted 5-methylcytosine to thymine. However, the reaction was very weak, as the methyl-substitution at position five of cytosine made the amino group at position four almost

resistant to bisulfite deamination; thus, 5-methylcytosine remained largely intact during bisulfite treatment (Figure 7).⁵⁹

Figure 7. Bisulfite treatment



Previously there were several disadvantages to methods based on bisulfite conversion. First, the method was a time-consuming procedure, requiring several hours to achieve complete conversion of cytosine to uracil. Second, the recovery of the bisulfite-converted DNA was very poor (approximately 5%).⁶¹ Previous methods described deamination using a sodium bisulfite solution of 3-5 M with an incubation period of 12-16 hours at 50°C.⁶² In 2004 Hayatsu and Shiraishi described a rapid bisulfite-treatment protocol.^{58,60} They demonstrated that the rate of deamination was approximately proportional to the bisulfite concentration and, furthermore, that higher temperature increased the deamination rate without affecting the deamination of 5-methyl-2'-deoxycytidine.⁶⁰ Treatment with 9 M bisulfite at 90°C for 10 minutes resulted in 99.6% conversion of 2'-deoxycytidine into 2'-deoxyuridine and less than 10% deamination of methylcytosine, while the other bases were unaffected.⁶⁰ Later the same year, similar results were described for human genomic DNA: A bisulfite concentration of 10 M at 90°C resulted in complete conversion of cytosine to uracil within 20 minutes, without significantly influencing 5-methylcytosine.⁵⁸ In addition, the high temperature and concentration of bisulfite did not cause more extensive DNA degradation than conventional treatment.⁵⁸

Pedersen et al. (2012) published a protocol on high recovery of cell-free methylated DNA.⁶³ The method was based on the rapid bisulfite-treatment protocol published by Hayatsu and Shiraishi in 2004.^{58,60} Previous methods, including the protocol by Hayatsu and Shiraishi, were not suitable for analysing sample material containing only sparse amounts of DNA due to degradation of DNA and inappropriate conversion of 5-methylcytosine as a result of prolonged bisulfite treatment. Using standard procedures, a starting material of < 200 ng DNA led to a loss of more than 95% of the bisulfite-treated DNA during desulfonation and purification.⁶¹ Pedersen et al. managed to extensively optimize the method, resulting in a recovery of approximately 60% of the deaminated DNA. The major improvement of the method was achieved by alterations in the purification procedure after deamination. Lysis and extraction buffers were replaced by ethanol, leading to great increase in the recovery. The optimized method by Pedersen et al. enabled analysis of samples only containing sparse amounts of DNA, as in methylation analysis of plasma cell-free DNA.⁶³

The extraction and deamination procedures used in the studies presented in this PhD thesis are based on the method described by Pedersen et al.⁶³

2. OBJECTIVES

The hypothesis:

DNA promoter hypermethylation occurs during the development and progression of pancreatic adenocarcinoma. The alterations are detectable in cell-free DNA and usable as blood-based markers for pancreatic adenocarcinoma.

The aims:

1. To perform a systematic review of the literature primarily concerning DNA-hypermethylation as blood-based markers for pancreatic adenocarcinoma (Study I/Paper I)
2. To determine if plasma-derived cell-free DNA promoter hypermethylation can be used as a diagnostic marker for pancreatic adenocarcinoma (Study II/Paper II)
3. To determine if plasma-derived cell-free DNA promoter hypermethylation can be used as markers for pancreatic adenocarcinoma staging (Study III/Paper III)
4. To determine if plasma-derived cell-free DNA promoter hypermethylation can be used as markers for survival of pancreatic adenocarcinoma (Study IV/Paper IV).

3. MATERIALS AND METHODS

3.1. STUDY DESIGN

Study I was a review of the literature on pancreatic adenocarcinoma and DNA hypermethylation analysed in blood samples.

Study II was conducted as a cross-sectional observational study of patients with pancreatic adenocarcinoma and patients with benign disease (patients with acute or chronic pancreatitis and patients suspected of but without upper gastrointestinal malignancy) at the time of diagnosis, to evaluate the diagnostic value of a selected panel of hypermethylated promoter regions in plasma-derived cell-free DNA.

Study III was conducted as a cross-sectional observational study of patients with pancreatic adenocarcinoma at the time of diagnosis, to evaluate a selected panel of hypermethylated promoter regions in plasma-derived cell-free DNA as markers for pancreatic adenocarcinoma staging.

Study IV was conducted as an observational cohort study of patients with pancreatic adenocarcinoma, to evaluate a selected panel of hypermethylated promoter regions in plasma-derived cell-free DNA as markers for survival.

3.2. METHOD STUDY I

A systematic search of the literature was performed in June 2014 using the PubMed and Embase databases. The following MeSH terms/thesaurus terms and free text were used: pancreatic disease, pancreatic cancer, pancreatic neoplasm, methylation, DNA hypermethylation, CG rich sequence, CpG island, cell-free DNA, blood, plasma, serum, fluids and secretions. To identify additional studies within the field, the reference lists of all relevant review articles were reviewed.

3.3. METHOD STUDY II, III AND IV

3.3.1. PATIENTS WITH SUSPECTED OR BIOPSY-VERIFIED PANCREATIC ADENOCARCINOMA

Patients with suspected or biopsy-verified upper gastrointestinal cancer who were admitted to the Department of Gastrointestinal Surgery, Aalborg University Hospital, between February 2008 and February 2011 were considered for inclusion in a

previous study of upper gastrointestinal malignancy and thromboembolism⁶⁴. Consecutive patients were included prospectively before diagnostic work-up and treatment.

Exclusion criteria were previous or concomitant cancer, known congenital thrombophilia, previous venous thromboembolism, connective tissue disease, or ongoing anticoagulant therapy.⁶⁴

After diagnostic work-up (gastroscopy, EUS, LUS, magnetic resonance imaging (MRI) scan, CT scan or PET scan), the subjects were divided into subgroups based on the final diagnosis. In study II, the subgroups of patients diagnosed with pancreatic adenocarcinoma and patients suspected of but without evidence of upper gastrointestinal malignancy were included. For study III and IV, only patients with pancreatic adenocarcinoma were included.

Patients diagnosed with pancreatic adenocarcinoma were staged according to TNM classification 7th Edition.²⁹ CT and PET scans of the thorax and abdomen were performed in the diagnostic work-up of all patients. Histopathological analysis of biopsy specimens obtained by either EUS or LUS confirmed the cancer diagnosis. The T and N categories were determined by histopathological analysis for patients who underwent intended curative surgery. If surgery was not performed, the final clinical decision determined the T and N categories. All patients were discussed at a multidisciplinary team conference, where consensus was reached on staging and treatment.⁶⁴

WHO performance status (PS) and the American Society of Anesthesiologists (ASA) score were registered at the time of inclusion.

3.3.2. PATIENTS WITH CHRONIC PANCREATITIS

Patients diagnosed with chronic pancreatitis who were hospitalized or had a scheduled appointment in the outpatient clinic at Aalborg University Hospital from August 2013 to August 2014 were considered for inclusion in study II.

The diagnosis of chronic pancreatitis was based on the Lüneburg criteria, and chronic pancreatitis was defined as a score ≥ 4 .⁶⁵

Exclusion criteria were previous cancer history, known immunological connective tissue disorder or ongoing anticoagulant therapy. Patients with autoimmune pancreatitis were not excluded.

3.3.3. PATIENTS WITH ACUTE PANCREATITIS

Patients diagnosed with acute pancreatitis at the Department of Surgical Gastroenterology, Aalborg University Hospital, or Department of General Surgery, Hospital of Vendsyssel, from November 2013 until May 2015 were considered for inclusion in study II.

Inclusion criteria were acute pancreatitis defined as upper abdominal pain and increased serum amylase or acute pancreatitis verified by ultrasound, CT- or MRI scan. Exclusion criteria were previous cancer history.

3.4. BLOOD SAMPLING

All blood samples were obtained by skilled technicians using venipuncture according to the procedure recommended by the European Concerted Action on Thrombosis. Routine analysis was performed immediately afterwards. EDTA plasma for methylation analysis was centrifuged 20 min. (4000 rpm) at 4 C° and stored at -80 C° within two hours after sampling until further methylation analysis.

3.4.1 PATIENTS WITH PANCREATIC ADENOCARCINOMA AND PATIENTS SUSPECTED OF BUT WITHOUT UPPER GASTROINTESTINAL MALIGNANCY

Blood samples were collected on admission before diagnostic work-up and treatment. Patients with pancreatic adenocarcinoma had blood samples drawn every 3 months for a two-year period. Patients who were offered surgical treatment had additional blood samples obtained postoperatively on day 3-5 and day 8-10.

3.4.2 PATIENTS WITH CHRONIC PANCREATITIS

Routine blood samples and EDTA-plasma for methylation analysis were obtained at enrolment and every 6 months for two years.

3.4.3 PATIENTS WITH ACUTE PANCREATITIS

Routine blood sample analysis was performed on a near daily basis according to the department's standard practice. EDTA-plasma for methylation analysis was obtained every second day for the first week of hospitalization and once a week during the remaining hospital stay, as well as one and six months after discharge.

3.5 ANALYTICAL METHODS

The purification of cell-free DNA and bisulfite treatment were based on the protocol published by Pedersen et al. in 2012 mentioned above.⁶³

All methylation analyses were performed by a single scientist. The analyses were performed non-blinded for study II and blinded for study III and study IV.

3.5.1 EXTRACTION OF CELL-FREE DNA

Cell-free DNA was extracted using the easyMAGTM platform (NucliSens® [bioMérieux SA, France]) according to the manufacturer's recommended protocol for plasma.

Approximately 500 µl EDTA plasma was used for the extraction of cell-free DNA. The purified DNA was eluted in 35 µl elution buffer (NucliSens® [bioMérieux SA, France]). Five µl were used for DNA quantitation and the rest was deaminated.⁶³

3.5.2 BISULFITE TREATMENT AND DEAMINATION

Thirty µl of DNA extract was mixed with 60 µl of deamination solution (10 M (NH₄) HSO₃-NaHSO₃) and deaminated for 10 minutes at 90 C° and subsequently cooled at room temperature. The solution containing the DNA-bisulfite adducts was afterwards purified using the easy-MAG platform (NucliSens® [bioMérieux SA, France]) according to manufacturer's instructions, except for changes made to the lysis buffer, the extraction buffers A and B, and the elution buffer:

- 2 ml easyMAG lysis buffer (NucliSens® [bioMérieux SA, France]) was replaced by 1 ml 50% ethanol.

- The extraction buffer A and B (NucliSens® [bioMérieux SA, France]) were both replaced by 33% ethanol in H₂O.

The desulfonation was performed by eluding the DNA in 25 µl 10 mM KOH.⁶³

3.5.3 FIRST-ROUND PCR

A first round of PCR was necessary in order to amplify the amount of deaminated DNA of interest. The first round of PCR was conducted using a mix of outer methylation-specific primers (Appendix A: List of primer and probe sequences).

The reaction buffer for each sample consisted of 25 µl containing PCR stock, 13 µM MgCl₂, 0.6 mM dNTP, 250 nM of each outer methylation-specific primer, 1.5 U Taq polymerase (MyTaq™ [Bioline, Singapore]), and 0.3 U UNG (Invitrogen). The reaction mix was distributed to individual 200 µl PCR tubes and incubated for 5 minutes at 37 °C (UNG activity), followed by incubation at 95 °C for 5 minutes and cooling to room temperature.

To each PCR tube, containing the first-round reaction mix, 25 µl of purified deamination product were added.

PCR was performed for 20 cycles at 92 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds.

3.5.4 SECOND-ROUND PCR

Each gene was analyzed separately in the second-round PCR, using inner methylation-specific primers and methylation-specific (HEX or FAM) probes for each gene in the panel.

Ten µl of mix containing 0.4 µM inner methylation-specific primers and methylation-specific probes were distributed in 30 individual wells in a 96-well PCR plate. Ten µl of first-round PCR product were added to 710 µl of reaction mix containing PCR stock, 250 µM dNTP, 10 µM MgCl₂, and 15 U Taq polymerase (MyTaq™ [Bioline, Singapore]). Twenty µl of the reaction mix were added to each of the 30 wells containing the inner methylations-specific primers and methylation-specific probes.

Real-time PCR was carried out for 45 cycles at 94 °C for 15 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds.

3.5.5 GENE PANEL

A panel of 28 genes was selected for methylation analysis (Appendix B: List of genes in the panel). The genes were primarily selected based on the literature review in study I. All genes selected for methylation analysis had previously been detected as hypermethylated in either plasma- or serum-derived cell-free DNA, pancreatic juice or tumour tissue from patients with pancreatic adenocarcinoma. Additional genes were selected based on a pilot study performed by our group on colorectal cancer (unpublished data), which determined that these genes were of particular interest for adenocarcinoma.

The hemimethylated *MESTv1* gene was used as the reference gene in both the first and second rounds of PCR.

3.5.6 PRIMER DESIGN – PROBE DESIGN

The software Beacon Designer® [PREMIER Biosoft International, Palo Alto, CA] was used to design primers and probes for the selected genes. Methylation of the primers and probes were evaluated by MethPrimer® [The Li Lab, Peking, China].⁶⁶ The primers were designed to be rich on CpGs and to be located in the promoter region, which was interpreted as the region up-stream of exon one. The aim was to design primers resulting in PCR products with a length less than 140-150 bp, as cell-free DNA fragments most likely have a length of 160 bp. The methylation-specific primers and probes were designed and optimized for this present study. However, effort was made to design primers for previously tested promoter sequences (Appendix A: Primer and probe sequences).

3.5.7 DILUTION SERIES

To certify the sensitivity, global methylated DNA was used to ensure that each gene promoter was detected with comparable sensitivity. A first-round PCR was performed with 1, 10, 100, 1000 and 10000 copies of deaminated DNA. Each gene was always detected when using 100 and more copies. Furthermore, there was a 90% detection rate when using 10 copies. To guarantee specificity, we used unmethylated *MESTv1*, which never was detectable in global methylated DNA.

3.6 ETHICAL ISSUES

The study was approved by the Research Ethics Committee for the North Denmark Region (N-2013037) and registered at ClinicalTrials.gov (NCT02079363). The database was approved by The Agency of Danish Data Protection (2008-58-0028). Oral and written informed consent were obtained from patients with acute and chronic pancreatitis.

Patients with pancreatic adenocarcinoma and patients suspected of but without upper gastrointestinal malignancy had all provided oral and written informed consent for the previously mentioned study on thromboembolism.⁶⁴ Blood samples from these patients had been stored in a biobank. It was not possible to obtain new informed consent from this patient group as more than 90% of the patients with pancreatic adenocarcinoma had died. The Research Ethics Committee for the North Denmark Region granted exemption for consent regarding the subjects with pancreatic adenocarcinoma and control group 1, as knowledge about the methylation profile would not have any consequences for these patients.

3.7 STATISTICS

The studies were characterized as exploratory pilot studies, and thus no power calculation was performed prior to the studies. The studies were performed based on the sample material available from the biobank.

Level of cell-free DNA

The median level (ng/ml) of cell-free DNA for each group was calculated. The nonparametric Wilcoxon rank sum test was used to compare the cancer group with the benign control groups.

Hypermethylated genes

Each gene in the gene panel was analysed as a binary variable. A threshold cycle (Ct) of 0 was interpreted as a non-methylated gene and $Ct > 0$ was interpreted as a hypermethylated gene.

The total number of hypermethylated genes was calculated for each patient. The mean numbers of hypermethylated genes were compared as numerical data using the nonparametric Wilcoxon rank sum test due to statistically significant differences in the standard deviation (SD) among the groups. A p-value below 0.05 was considered statistically significant unless otherwise stated. Kendall's rank test was used for correlation analysis of the total number of hypermethylated genes and the level of cell-free DNA.

Validation of dichotomous data

Dichotomous data was validated by calculating Δ Ct, which we defined as the difference between the Ct value of the hemimethylated reference gene *MESTv1* and the Ct value of each gene for which Ct > 0. To assess the amount of information lost in study II due to dichotomization, histograms of Δ Ct for the cancer group and control group 1 combined with control group 2 were produced (data not shown). A similar approach was used for study III; histograms of Δ Ct for stage I, II and III vs IV, and stage I and II vs III and IV were produced (data not shown).

All data were analysed using STATA 14.0 software [StataCorp LP, Texas].

3.7.1 PAPER II

Pancreatic adenocarcinoma was the primary outcome of the prediction model in study II.

The hypermethylation frequency of each gene and the (exact) 95% confidence interval (CI) were calculated for each patient group. The mean number of hypermethylated genes in each patient group and the 95% CI were calculated.

Development of the diagnostic prediction model

1. *Screening of each individual variable as a diagnostic marker for pancreatic adenocarcinoma:* Logistic regression was performed separately for each gene in the gene panel and for the covariates smoking status, gender and age > 65 years. The p-value and the area under the receiver operating characteristic curve (AUC) were calculated.
2. *The selection of variables:* Variables having a p-value less than 0.2 were selected for further analysis.
3. *Model selection:* Stepwise backwards elimination in logistic regression models was performed to select the relevant variables using 0.05 as the significance level for removal from the model. For each intermediate model, the AUC value was calculated.
4. *Determination of the best model:* The decision was based on the model complexity combined with the model performance according to the AUC.
5. *Interactions between the variables:* The significance of interactions between all pairs of variables was assessed in the final model. Interactions with a p-value less than 0.01 were considered statistically significant.

6. *Validation:* To account for optimism in the internal validation of discriminative model performance (measured by the AUC) leave pair out cross validation was used.⁶⁷ For the calibration performance, Hosmer-Lemeshow test was performed.
7. *Probability score:* For each patient, a probability score was calculated.

3.7.2 PAPER III

The primary outcome of study III was stage according to AJCC staging of pancreatic adenocarcinoma.²⁹ Prediction models to differentiate (stage I, II and III vs IV) and (stage I and II vs III and IV) were developed.

Patients were divided into groups according to AJCC²⁹ staging based on the TNM classification. The mean number of hypermethylated genes and the (exact) 95% CI were calculated for each group according to stage.

Development of the prognostic prediction model

1. *Screening of each individual variable as a prognostic marker for pancreatic adenocarcinoma staging:* Logistic regression was performed separately for each gene in the gene panel and for age > 65, gender, ASA score and PS. The p-value and the AUC were calculated.
2. *The selection of variables:* Variables having a p-value less than 0.3 were selected for further analysis.
3. *Model selection:* To select the relevant variables stepwise backwards elimination in logistic regression models was performed using 0.10 as the significance level for removal from the model. For each intermediate model, the AUC value was calculated.
4. *Determination of the best model:* Model performance according to the AUC combined with model complexity determined the best model.
5. *Interactions between the variables:* The significance of interactions between all pairs of variables were assessed in the final model. Interactions with a p-value less than 0.01 were considered statistically significant.

6. *Validation:* Leave pair out cross validation⁶⁷ was used to account for optimism in the internal validation of discriminative model performance (measured by the AUC). Hosmer-Lemeshow test was performed for calibration performance.
7. *Probability score:* For each patient a probability score was calculated.

3.7.3 PAPER IV

The primary outcome of study IV was overall survival of pancreatic adenocarcinoma patients. Survival time was calculated as the difference between date of inclusion in the study (the date the patient was referred to the hospital suspected of or with symptoms of upper gastrointestinal malignancy) and the date of censoring/date of death. The date of death was available in the medical records.

Patients were divided into quartiles based on the total number of hypermethylated genes and Kaplan-Meier survival curves were used to evaluate the survival according to the total number of hypermethylated genes.

As described in details below, survival analysis was performed using Cox proportional hazards regression for the total patient group and for subgroups according to cancer stage ((I and II) and (IV)).

Survival prediction model development

1. *Screening of each individual variable as a predictor of survival:* Regression was performed for each gene in the gene panel and for age > 65, gender, ASA score and PS. The hazard ratios (HR) and p-values were calculated. Variables with a p-value less than 0.3 were considered as potential predictors and selected for further analysis
2. *Variable selection:* Stepwise backwards elimination in Cox regression models was performed to select the relevant variables using 0.05 as the significance level for removal from the model. For each intermediate model Harrell's overall concordance (*c*) statistic was calculated.⁶⁸
3. *Determination of the best model:* The model with the best performance measure according to Harrell's *c* was determined as the final model.

4. *Interactions between the variables:* The interaction between all variables was checked in the final models. Interactions with a p-value less than 0.01 were considered statistically significant.
5. *Validation:* The May-Hosmer goodness of fit test was performed for calibration performance.

Subsequently, the patients were divided into risk groups according to the final survival prediction models. Kaplan-Meier survival curves were used to illustrate the survival of the risk groups.

4. SUMMARY OF RESULTS

Patients

Ninety-five patients with pancreatic adenocarcinoma were included (Figure 8), and 35 patients suspected of but without upper gastrointestinal malignancy were included as a control group containing patients with symptoms mimicking those of pancreatic cancer (control group 1) (Figure 8). We subsequently excluded seven patients from control group 1 as a review of the medical records revealed that one patient had developed duodenal cancer shortly after inclusion and that four patients were diagnosed with different types of cancer (pulmonary cancer, pancreatic cancer, neuroendocrine tumour and cancer vocalis) more than 1½ years after inclusion. An additional two patients were excluded due to a lack of sample material.

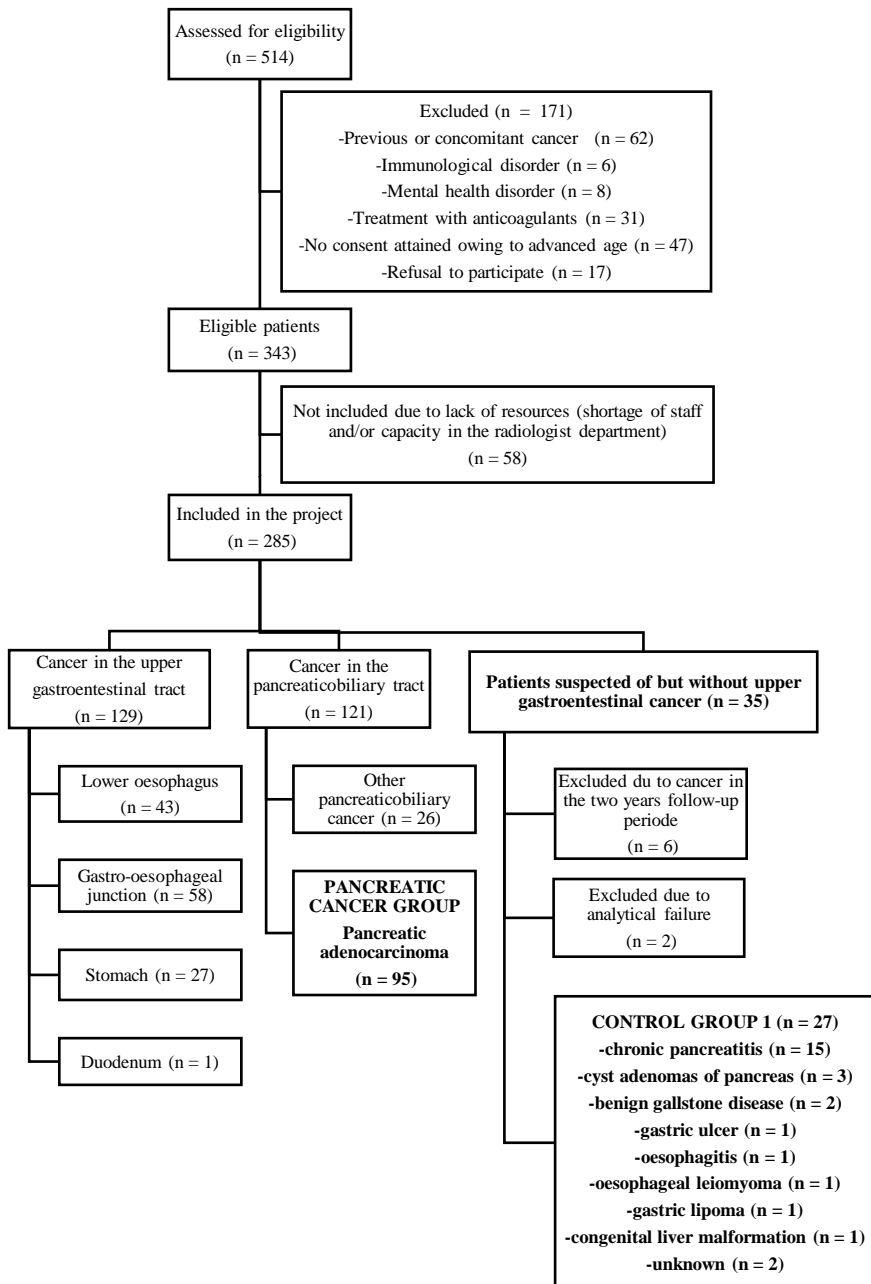
A total of 103 patients with chronic pancreatitis (control group 2) were enrolled in the study. In collaboration with the Department of Medical Gastroenterology, Aalborg University Hospital, 88 patients who had a scheduled time in the outpatient clinic were enrolled. The remaining 15 patients were enrolled during hospitalization at either the Department of Medical Gastroenterology or Department of Gastrointestinal Surgery, Aalborg University Hospital. Patients in control group 2 were followed for at least two years. We subsequently excluded five patients due to a cancer diagnosis in the follow-up period. Two patients had pulmonary cancer, one patient had oral cancer, one patient had corpus uteri cancer, and one patient was diagnosed with pancreatic cancer. Unfortunately, we had to exclude one additional patient due analytical failure.

A total of 62 patients with acute pancreatitis (control group 3) were enrolled in the study. Forty-nine patients were enrolled from Aalborg University Hospital, and 13 patients were enrolled from the Hospital of Vendsyssel. We subsequently excluded three patients: one due to a lack of sample material and two due to analytical failure.

Validation of dichotomous data

No difference was observed in the distribution of Δ Ct in study II and study III, which indicated that no significant amount of information was lost by dichotomizing the genes as hypermethylated or non-methylated regardless of the observed Ct value. In addition, we stratified the distribution of Ct values (0, 0-25, 25-30, and > 30) for each gene within each patient group (data not shown). A slight difference in Ct values was observed between the groups, with a tendency towards lower Ct values in the cancer group than in the benign control groups. However, the limited study power did not allow the evaluation of this difference in the multivariable analyses.

Figure 8. Flow diagram of the inclusion of patients

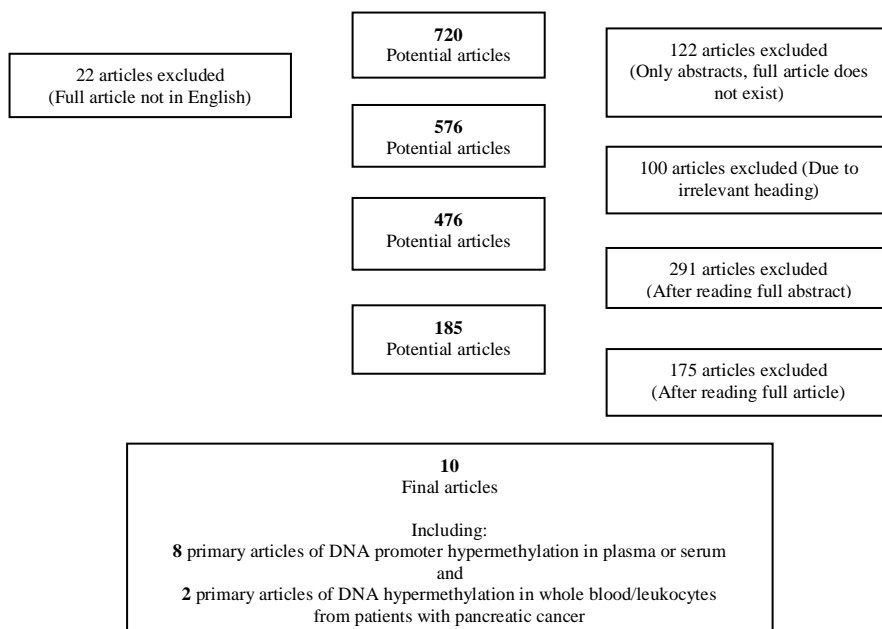


4.1. STUDY I/PAPER I

DNA Hypermethylation as a Blood-Based Marker for Pancreatic Cancer: A Literature Review

The literature search yielded 720 potential articles. Only full-text studies in English addressing pancreatic adenocarcinoma and methylated genes in blood samples were included. The subsequent review of the literature is illustrated in Figure 9. Eight primary studies of cell-free DNA promoter hypermethylation in plasma or serum (Table 3) and two studies of DNA hypermethylation in whole blood/leukocytes were identified. When analysing whole blood, it is essential to consider that the majority of DNA is derived from leukocytes and that the effects of circulating cancer cells and potentially cancer-specific cell-free DNA are minimal.⁶⁹

Figure 9. Review of the literature



A systematic search of the literature was performed in June 2014.

A total of 461 potential publications were found in PubMed.

A total of 501 potential publications were found in Embase.

After elimination of duplicates, the literature search yielded 720 potential articles.

Table 3. Studies on pancreatic adenocarcinoma and cell-free DNA methylation in plasma/serum

<i>Reference</i>	<i>Genes examined</i>	<i>Method</i>	<i>Pancreatic cancer</i>	<i>Chronic pancreatitis</i>	<i>Gallstone disease</i>	<i>Healthy controls</i>
Joo Mi Yi, 2013 ⁷⁰	<i>BNC1</i>	MOB	42	---	---	26
Melson, 2013 ⁷¹	<i>ADAMTS1</i>	Microarray	30	---	---	30
Kawasaki, 2013 ⁷²	<i>APC</i> <i>DCC</i> <i>CDKN2A</i> <i>P14</i> <i>RASSF1A</i>	MSP	47	---	---	---
Park, Ryu, 2012 ⁷³	<i>NPTX2</i>	QMSP	104	60	5	---
Park, Baek, 2012 ²³	<i>NPTX2</i> <i>UCHL1</i> <i>SFRP1</i> <i>PENK</i> <i>CDKN2A</i> <i>RASSF1A</i>	MSP	16	13	---	29
Melnikov, 2009 ⁷⁴		Microarray	30	30	---	30
Liggett, 2007 ⁷⁵		Microarray	30	30	---	30
Jiao Li, 2007 ⁷⁶	<i>PENK</i> <i>CDKN2A</i>	MSP	83	---	---	---

MOB: Methylation on beads.

QMSP: Quantitative methylation-specific PCR.

MSP: Methylation-specific PCR.

The studies based on plasma or serum-derived cell-free DNA are listed in Table 3. The majority of the studies included a limited number of patients, and most of the genes were only examined in a single published study, without further validation (Table 3 and Table 4). Most of the studies lacked well-defined control groups of patients with benign pancreatic disease to enable differentiation of pancreatic cancer-specific hypermethylations and hypermethylation in response to unspecific pancreatic disease. Apart from the studies based on microarray, only methylation status of a single gene or a small gene panel was analysed. The hypermethylation frequency of each individual gene according to patient group is listed in Table 4. No single gene was identified as an individual diagnostic marker, which may suggest that a panel of several genes is needed to achieve sufficient performance.

Table 4. Frequency of cell-free DNA hypermethylation.

<i>Gene</i>	<i>Pancreatic cancer</i>	<i>Chronic pancreatitis</i>	<i>Gallstone disease</i>	<i>Healthy controls</i>
<i>BNC1</i> ⁷⁰	79% (33/42)	-	-	11,5% (3/26)
<i>ADAMTS1</i> ⁷⁰	48% (20/42)	-	-	7,7% (2/26)
<i>NPTX2</i> ⁷³	84% (87/104)	33% (20/60)	0% (0/5)	-
<i>NPTX2</i> ²³	37,5% (6/16)	30,8% (4/13)	-	0% (0/29)
<i>PENK</i> ²³	31,3% (5/16)	15,4% (2/13)	-	0% (0/29)
<i>PENK</i> ⁷⁶	29,3% (22/(83-8))	-	-	-
<i>CDKN2A</i> ²³	25% (4/16)	15,4% (2/13)	-	3,4% (1/29)
<i>CDKN2A</i> ⁷²	17% (8/47)	-	-	-
<i>CDKN2A</i> ⁷⁶	24,6% (14/(83-26))	-	-	-
<i>RASSF1A</i> ²³	6,3% (1/16)	7,7% (1/13)	-	0% (0/29)
<i>RASSF1A</i> ⁷²	34% (16/47)	-	-	-
<i>UCHL1</i> ²³	25% (4/16)	15,4% (2/13)	-	0% (0/29)
<i>SFRP1</i> ²³	31,3% (5/16)	23,1% (3/13)	-	0% (0/29)
<i>APC</i> ⁷²	23,4% (11/47)	-	-	-
<i>DCC</i> ⁷²	6,4% (3/47)	-	-	-
<i>P14</i> ⁷²	14,9% (7/47)	-	-	-

4.2. STUDY II/PAPER II

Cell-free DNA Promoter Hypermethylation in Plasma as a Diagnostic Marker for Pancreatic Adenocarcinoma

Baseline characteristics of the patients

Overall, 95 patients with pancreatic adenocarcinoma were included in study II (Figure 8). As a benign control group, 27 patients suspected of but without evidence of upper gastrointestinal malignancy were included (control group 1) (Figure 8). In addition, 97 patients with chronic pancreatitis (control group 2) and 59 patients with acute pancreatitis (control group 3) were included. The baseline data for the four groups are shown in Table 5. The mean age of the patients with pancreatic adenocarcinoma was 66 years, significantly older than patients in the control groups.

Table 5. Baseline characteristics of all patients

	<i>Pancreatic cancer</i>		<i>Control group 1</i>		<i>Control group 2</i>		<i>Control group 3</i>		
	<i>N</i>	<i>%</i>	<i>N</i>	<i>%</i>	<i>N</i>	<i>%</i>	<i>N</i>	<i>%</i>	
N	95		27		97		59		
Mean age, years (range)	66 (45-85)		60 (37-82)		57 (22-87)		56 (22-87)		
Sex, male (%)	57	60	12	44	67	69	32	54	
Smoking status	currently	30	32	11	41	64	66	23	39
	previous	33	35	7	26	24	25	11	19
	never	30	32	9	33	9	9	23	39
	unknown status	2	2	0	0	0	0	2	3
Stage	I (IA and IB)	11	12						
	II (IIA and IIB)	29	30						
	III	13	14						
	IV	42	44						
Tumour location	Caput	61	64						
	Corpus	6	6						
	Cauda	12	13						
	Unknown	16	17						

Control group 1: Patients suspected of but without upper gastrointestinal malignancy.

Control group 2: Patients with chronic pancreatitis.

Control group 3: Patients with acute pancreatitis.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Level of cell-free DNA

The median level of cell-free DNA was significantly higher in the cancer group (11.60 ng/ml (range: 0.60-957.17)) compared with control group 1 (6.17 ng/ml (range: 1.06-48.43)), control group 2 (2.18 ng/ml (range: 0.11-115.44)) and control group 3 (4.09 ng/ml (range: 0.65-62.42)). Furthermore, the correlation between the number of hypermethylated genes and the level of cell-free DNA was statistically significant (p-value < 0.0001), with a Kendall's τ of 0.34.

Hypermethylated genes

The hypermethylation frequency of each gene in each patient group is presented in Appendix C. The mean number of hypermethylated genes in the whole gene panel (28 genes) was significantly higher for cancer patients (8.41 (95% CI: 7.62-9.20)) compared with the three benign control groups (Table 6).

Table 6. Mean number of hypermethylated genes in each patient group in study II

<i>Group</i>	<i>N</i>	<i>Mean number of methylated genes</i>	<i>95% CI</i>	<i>P-value</i>
Pancreatic cancer	95	8.41	(7.62-9.20)	
Control group 1: Suspected of but without cancer	27	4.89	(4.07-5.71)	
Control group 2: Chronic pancreatitis	97	4.34	(3.85-4.83)	
Control group 3: Acute pancreatitis	59	5.34	(4.77-5.91)	
Control group 1+2	124	4.46	(4.04-4.88)	<0.0001*
Control group 1+2+3	183	4.74	(4.40-5.08)	<0.0001**

The means were compared as numerical data with the nonparametric Wilcoxon rank sum test. P-values less than 0.05 were considered statistically significant.

CI: Confidence interval.

* Significant difference between patients with pancreatic cancer and control group 1+2.

** Significant difference between patients with pancreatic cancer and control group 1+2+3.

Development of the diagnostic prediction model

To develop a diagnostic prediction model, we chose to combine the control group of patients suspected of but without upper gastrointestinal malignancy and the control group of patients with chronic pancreatitis because these patients were likely to have had symptoms or clinical presentations resembling those of pancreatic adenocarcinoma. Therefore, we considered the development of a biomarker to differentiate these patients from patients with pancreatic adenocarcinoma of utmost clinical relevance. Patients with acute pancreatitis were not included in this part of the analysis because a clinical presentation of severe acute inflammation is rarely observed in pancreatic cancer.

The hypermethylation frequencies of seventeen genes (*APC*, *ALX4*, *BMP3*, *BNCl*, *ESR1*, *HIC1*, *MESTv2*, *NPTX2*, *RARB*, *RASSF1A*, *SFRP1*, *SFRP2*, *SEPT9v2*, *SST*, *TFPI2*, *TAC1*, and *WNT5A*) (Table 7 and Appendix C) were significantly higher in the cancer group compared with the combined control group 1+2. There was no significant difference in gender, and thus this variable was excluded from the subsequent analysis. In our study, smoking was a protective factor for cancer, which strongly contradicts with the view of smoking as a well-known risk factor for pancreatic cancer, as previously mentioned. This finding likely reflects our control group, which mainly included patients with chronic pancreatitis, who have a substantially greater use of tobacco compared with the general population.^{77,78} We chose to exclude smoking from the model because it is a risk factor for cancer. There was a significant difference in age between the cancer group and the control group. Age was incorporated as a covariate in the diagnostic prediction model because the incidence of pancreatic cancer increases with age and hypermethylation of certain genes can be an age-related phenomenon.⁷⁹

Table 7. Variables in the study II

	<i>OR</i>	<i>95% CI</i>	<i>P-value</i>	<i>AUC</i>
<i>ALX4</i>	4.29	(1.62-11.35)	0.0034	0.57
<i>APC</i>	4.16	(2.21-7.84)	9.67 x 10⁻⁶	0.65
<i>BMP3</i>	7.37	(3.20-16.95)	2.64 x 10⁻⁶	0.64
<i>BNC1</i>	9.32	(3.90-22.25)	5.02 x 10⁻⁷	0.65
<i>BRCA1</i>	1.21	(0.49-2.98)	0.6804	0.51
<i>CDKN2A</i>	2.27	(0.66-11.17)	0.1652	0.52
<i>CDKN2B</i>	2.42	(0.91-6.40)	0.0757	0.53
<i>CHFR</i>	0.43	(0.04-4.19)	0.4668	0.51
<i>ESR1</i>	2.23	(1.22-4.07)	0.0095	0.58
<i>EYA2</i>	2.30	(0.91-5.80)	0.0778	0.54
<i>GSTP1</i>	4.01	(0.41-39.18)	0.2323	0.51
<i>HIC1</i>	3.69	(1.37-9.91)	0.0097	0.55
<i>MESTv2</i>	2.99	(1.63-5.49)	0.0004	0.62
<i>MGMT</i>	2.24	(0.52-9.62)	0.2778	0.51
<i>MLH1</i>	1.48	(0.66-3.31)	0.3448	0.52
<i>NPTX2</i>	3.37	(1.88-6.02)	4.34 x 10⁻⁵	0.64
<i>NEUROG1</i>	1.50	(0.59-3.86)	0.3969	0.52
<i>RARB</i>	1.81	(1.04-3.15)	0.0348	0.57
<i>RASSF1A</i>	5.28	(2.69-10.39)	1.4 x 10⁻⁶	0.65
<i>SFRP1</i>	3.30	(1.81-6.03)	0.0001	0.62
<i>SFRP2</i>	2.00	(1.12-3.58)	0.0197	0.57
<i>SEPT9v2</i>	6.97	(1.94-25.03)	0.0029	0.56
<i>SST</i>	3.04	(1.75-5.30)	8.69 x 10⁻⁵	0.64
<i>TFPI2</i>	12.16	(3.51-42.04)	7.96 x 10⁻⁵	0.60
<i>TAC1</i>	3.25	(1.86-5.69)	3.63 x 10⁻⁵	0.64
<i>VIM</i>	-	-	*	-
<i>WNT5A</i>	11.31	(1.39-92.08)	0.0234	0.54
<i>PENK</i>	-	-	*	-
sex	0.85	(0.49-1.48)	0.5750	0.52
age60	3.88	(2.17-6.92)	4.58 x 10 ⁻⁶	0.66
age65	4.14	(2.33-7.33)	1.14 x 10⁻⁶	0.67
age70	4.05	(2.04-8.02)	6.06 x 10 ⁻⁵	0.62

All variables were analyzed by simple logistic regression comparing the pancreatic cancer group and control groups 1+2.

Bold marks the genes, where there is significant difference ($p < 0.05$) in hypermethylation frequency between the cancer group and control groups 1+2.

**VIM* and *PENK* could not be evaluated by logistic regression as none of the patients in the control group had hypermethylation of the two genes, however chi-square test found significant difference between the cancer group and the control group 1+2. Despite that, *VIM* and *PENK* were excluded from the following analysis because only few cancer patients had *VIM* or *PENK* hypermethylation.

Control group 1: Patients suspected of but without upper gastrointestinal malignancy.

Control group 2: Patients with chronic pancreatitis.

OR: Odds ratio.

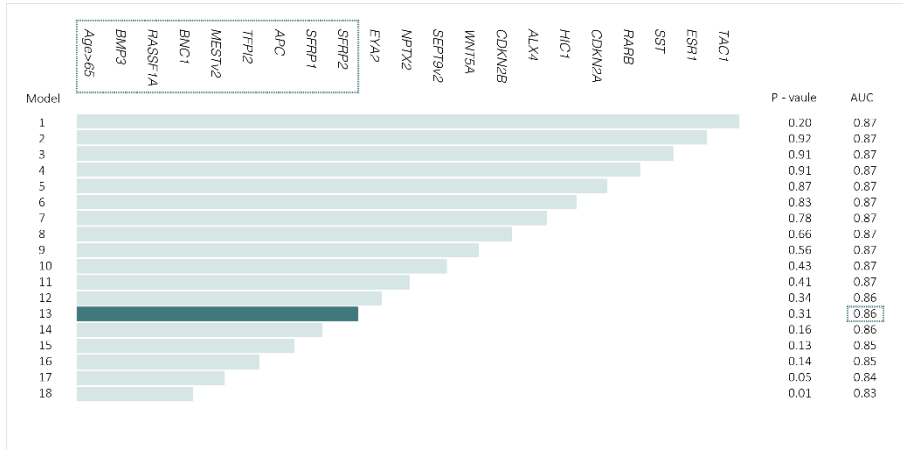
CI: Confidence interval.

AUC: Area under the receiver operating characteristic curve.

The hypermethylation of twenty genes was determined as potential predictors and included, together with the covariate age > 65, in multivariable logistic regression analysis. Stepwise backwards elimination was performed (Figure 10), and a diagnostic prediction model was developed containing the eight most significant genes (Model 13: *BMP3*, *RASSF1A*, *BNC1*, *MESTv2*, *TFPI2*, *APC*, *SFRP1*, and *SFRP2*) and the covariate age > 65 years. The model had an AUC of 0.86 (95% CI: 0.81-0.91) (Figure 10 and Figure 11a). Model 1, containing the twenty most significant genes, had the most superior AUC of 0.87. However, model 13 was determined as the final model because it contained a limited number of variables and because leaving out the 12 least significant genes only resulted in a minimal loss of predictive power. The mean probability of having pancreatic adenocarcinoma was 0.67 (95% CI: 0.61-0.72) for the cancer group and 0.26 (95% CI: 0.22-0.29) for control groups 1+2. With a probability cut-point of 0.50, the diagnostic prediction model 13 had a sensitivity of 76% and a specificity of 83%. In addition, the performance of the model was independent of cancer location. The model contained no significant variable interactions, was well calibrated and had an estimated optimism in AUC of 0.03. The model was developed based on the total group of patients with pancreatic adenocarcinoma representing all cancer stages. To ensure performance for early-stage disease, the model was tested on the subgroup of patients with stage I and II tumours. Similar high performance was observed with an AUC of 0.86 (95% CI: 0.79-0.93) (Figure 11b), (probability cut-point of 0.50: sensitivity 73% and specificity 83%), and an optimism in AUC of 0.06.

To exclude pancreatic cancer in the control group, a three-phase CT scan was performed in patients (n = 6) with a probability score of 0.6 or above. Unfortunately, in one patient with chronic pancreatitis (probability score of 0.9), the CT scan was suspicious of malignancy in the head of the pancreas with partial constriction of the superior mesenteric vein. In addition, ERCP was performed with fine needle biopsy, revealing IPMN in the head of pancreas invading the common bile duct. Although the histological evaluation failed to detect malignancy, the disease was considered malignant due to the invasive nature. The patient was evaluated as ineligible for surgery due to poor general health status and died a couple of months later.

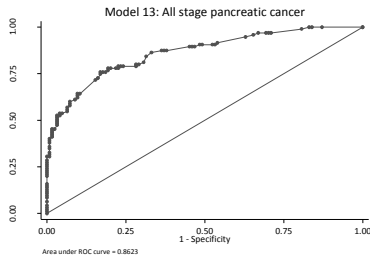
Figure 10. Diagnostic prediction model: Stepwise backwards elimination



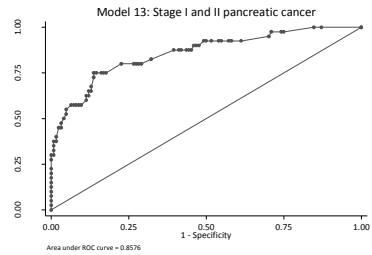
Stepwise backwards elimination of genes with the corresponding p-value and the area under the receiver operating characteristic curve (AUC). Model 13 was determined as the final model.

Figure 11. Performance of diagnostic prediction Model 13

a)



b)



Model 13: *BMP3*, *RASSF1A*, *BNC1*, *MESTv2*, *TFPI2*, *APC*, *SFRP1*, *SFRP2* and the covariate age > 65 years.

a) Model performance on the total patients group.

AUC=0.86 (95% CI: 0.81-0.91) (probability cut-point of 0.50: sensitivity of 76% and a specificity of 83%).

b) Model performance for the subgroup of patients with stage I and II disease.

AUC = 0.86 (95% CI: 0.79-0.93) (probability cut-point of 0.50: sensitivity 73% and specificity 83%).

AUC: Area under the receiver operating characteristic curve.

CI: Confidence interval.

4.3. STUDY III/PAPER III

DNA Promoter Hypermethylation in Plasma-Derived Cell-Free DNA as a Prognostic Marker for of Pancreatic Adenocarcinoma Staging.

Baseline characteristics of the patients

In study III, 95 patients with pancreatic adenocarcinoma were included. The baseline characteristics of the patients are listed in Table 5 and Table 8.

Table 8. Baseline characteristics of patients with pancreatic adenocarcinoma (N = 95)

Stage	I (Ia+Ib)		II (IIa+IIb)		III		IV	
N	11		29		13		42	
Age (mean) (SD)	70	(10.81)	67	(8.21)	65	(8.25)	65	(9.21)
Sex (men:women)	6:5		19:10		10:3		22:20	
ASA 1	4	36%	14	48%	8	62%	12	29%
ASA 2	4	36%	11	38%	3	23%	18	43%
ASA 3	3	27%	4	14%	2	15%	12	29%

ASA: American Society of Anesthesiologists score.

SD: Standard deviation.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Level of cell-free DNA according to cancer stage

There were no significant differences in the median level of cell-free DNA among the different stages of the disease.

Hypermethylated genes

The hypermethylation frequencies of each gene according to cancer stage are listed in Appendix D. The mean number of methylated genes was significantly higher for patients with stage IV pancreatic adenocarcinoma compared with stage I, II and III disease (Table 9).

Table 9. Mean number of hypermethylated genes according to stage

Stage	N	Mean	95% CI	P-value
I (IA and IB)	11	7.09	(5.52-8.67)	
II (IIA and IIB)	29	7.00	(5.93-8.07)	
III	13	6.77	(5.08-8.46)	
IV	42	10.24	(8.88-11.60)	
I and II	40	7.03	(6.17-7.88)	
III and IV	55	9.42	(8.26-10.58)	0,0078*
I, II and III	53	6.96	(6.23-7.70)	0,0002**

CI: Confidence interval.

*Significant difference between stages I and II vs III and IV.

**Significant difference between stages I, II and III vs IV.

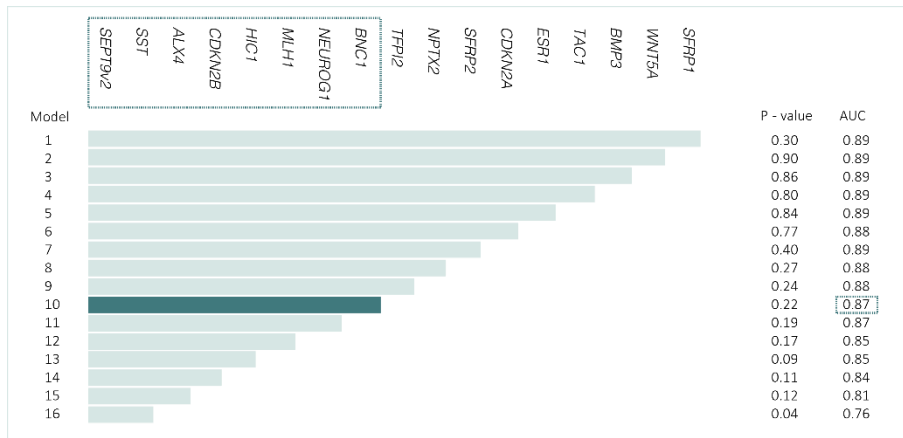
Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Stage I, II and III vs stage IV

We compared patients with stage I, II and III pancreatic adenocarcinoma with patients with stage IV disease and found that the hypermethylation frequency of seven genes (*ALX4*, *BNC1*, *HIC1*, *SEPT9v2*, *SST*, *TFPI2*, and *TAC1*) was significantly higher in stage IV disease compared with stage I, II and III disease.

A prognostic prediction model was developed to differentiate patients diagnosed with pancreatic adenocarcinoma with distant metastases (stage IV) from patients without metastases (stage I, II and III). No significant differences in gender, age, ASA score or PS were observed between the groups, and thus these variables were not analysed further. Seventeen of the 28 examined genes were included in the multivariable logistic regression analysis because these variables had individual p-values of less than 0.3. Stepwise backwards elimination was performed (Figure 12). Model 10 (*SEPT9v2*, *SST*, *ALX4*, *CDKN2B*, *HIC1*, *MLH1*, *NEUROG1*, and *BNC1*) was determined as the final model with an AUC of 0.87 (95% CI: 0.80-0.95) (Figure 12 and Figure 13). With a probability cut-point of 0.55, prognostic prediction model 10 had a sensitivity of 74% and a specificity of 87% for stage IV disease. The mean probability score for patients with stage I, II and III was 0.26 (95% CI: 0.20-0.31), compared with a mean probability score of 0.67 (95% CI: 0.59-0.76) for patients with stage IV disease. Model 10 contained no significant interactions between variables, was well calibrated and had an estimated optimism in AUC of 0.05.

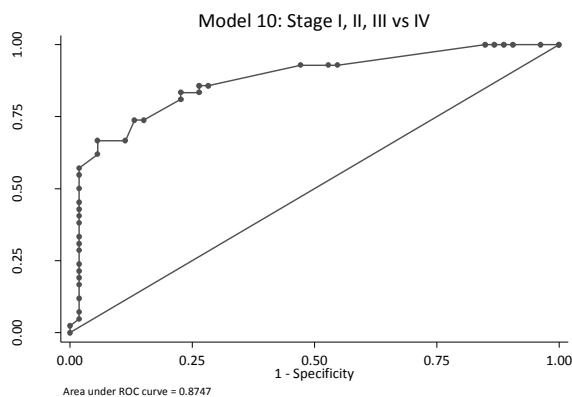
Figure 12. Prognostic prediction model stage I, II and III vs IV: Stepwise backwards elimination



Stepwise backwards elimination of genes with the corresponding p-value and the area under the receiver operating characteristic curve (AUC). Model 10 was determined as the final model to differentiate stage I, II and III vs stage IV disease.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Figure 13. Performance of prognostic prediction Model 10:
Stage I, II and III vs IV



Model 10: *SEPT9v2*, *SST*, *ALX4*, *CDKN2B*, *HIC1*, *MLH1*, *NEUROG1*, and *BNC1*.

AUC: Area under the receiver operating characteristic curve.

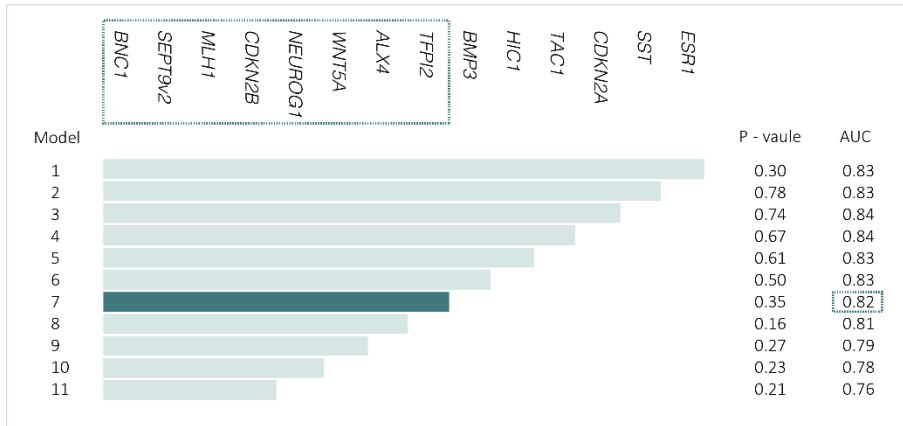
AUC = 0.87 (95% CI: 0.80-0.95) (probability score cut-point of 0.55: sensitivity 74% and specificity 87%).

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Stage I and II vs stage III and IV

We also compared patients with pancreatic adenocarcinoma stage I and II to patients with stage III and IV disease, to determine if the gene panel could distinguish potential resectable disease from non-resectable disease. The hypermethylation frequency of four genes (*ALX4*, *BNC1*, *SEPT9v2*, and *SST*) was significantly higher (p-value < 0.05) in patients with stage III and IV pancreatic adenocarcinoma compared with patients with stage I and II pancreatic adenocarcinoma. A prognostic prediction model to differentiate potentially resectable disease (stage I or II) from non-resectable disease (stage III or IV) was developed. No statistically significant differences in the covariates of gender, age, ASA score and PS were observed between the groups. Genes with a p-value < 0.3 in the univariate screening (14 of 28 examined genes) were included in the multivariable logistic regression analysis using stepwise backwards elimination (Figure 14). Model 7 (*MLH1*, *SEPT9v2*, *BNC1*, *ALX4*, *CDKN2B*, *NEUROG1*, *WNT5A*, and *TFPI2*) was determined as the final model, with an AUC of 0.82 (95% CI: 0.74-0.90) (sensitivity of 73% and specificity of 80% with a probability cut-point of 0.66) (Figure 14 and Figure 15). There were no significant variable interactions in the model. The model was well calibrated and had an estimated optimism in AUC of 0.06.

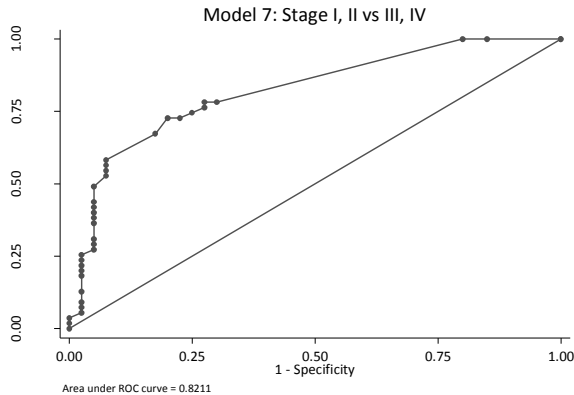
Figure 14. Prognostic prediction model stage I and II vs III and IV:
Stepwise backwards elimination



Stepwise backwards elimination of genes with the corresponding p-value and the area under the receiver operating characteristic curve (AUC). Model 7 was determined as the final model to differentiate stage I and II vs stage III and IV disease.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Figure 15. Performance of prognostic prediction Model 7:
Stage I and II vs III and IV



Model 7: *MLH1*, *SEPT9v2*, *BNC1*, *ALX4*, *CDKN2B*, *NEUROG1*, *WNT5A*, and *TFPI2*.

AUC: Area under the receiver operating characteristic curve.

AUC = 0.82 (95% CI: 0.74-0.90) (probability cut-point of 0.66: sensitivity 73% and specificity 80%).

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

4.4. STUDY IV/PAPER IV

Cell-Free DNA Promoter Hypermethylation in Plasma as a Predictive Marker for Survival of Patients with Pancreatic Adenocarcinoma

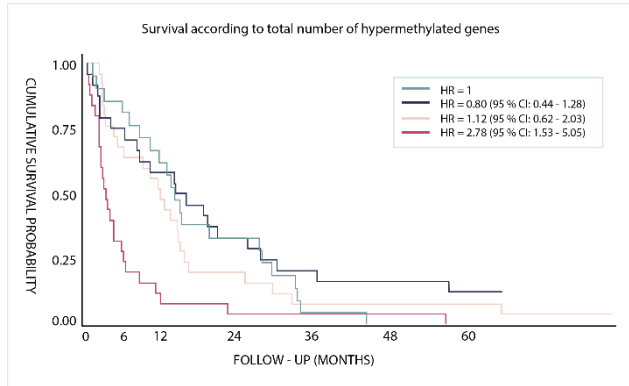
The baseline characteristics of the patients in study IV were identical to those of the patients in study III and are listed in Table 5 and Table 8.

Survival analyses according to the total number of hypermethylated genes

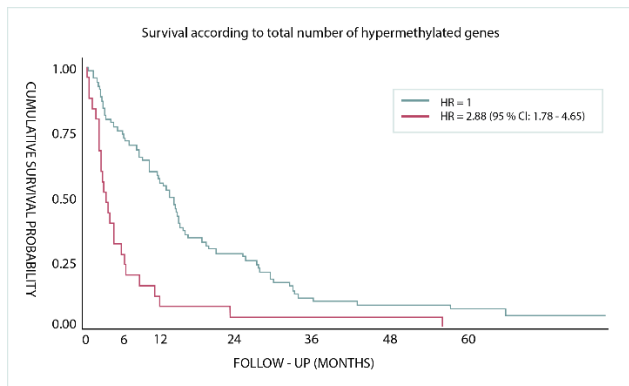
Patients were divided into quartiles based on the total number of hypermethylated genes. There were no significant differences in HR among the 1st, 2nd and 3rd quartiles. However, the 4th quartile had a HR of 2.74 (95% CI: 1.51-4.98), which was highly significantly different (p-value < 0.001) from the 1st quartile (Figure 16a). We combined the 1st, 2nd and 3rd quartiles (1-10 methylated genes) and compared them to the 4th quartile (more than 10 methylated genes) (Figure 16b). We adjusted the analysis for cancer stage and age and found a significant HR of 2.03 (95% CI: 1.15-3.57) for patients with more than 10 hypermethylated genes. Six-month, one-year and two-year survival were superior for patients with 0-10 hypermethylated genes (73% (95% CI: 61%-82%), 56% (95% CI: 43%-66%), and 28% (95% CI: 19%-39%), respectively) compared with patients with more than 10 hypermethylated genes (28% (95% CI: 12%-46%), 12% (95% CI: 3%-28%) and 4% (95% CI: 0.3%-17%), respectively) (Figure 16b).

Figure 16. Survival according to the total number of hypermethylated genes

a)



b)



For each patient the total number of hypermethylated genes was calculated. Based on that calculation, patients were divided into quartiles. The Kaplan-Meier curves illustrate the survival estimates according to the total number of hypermethylated genes in plasma-derived cell-free DNA.

a) Light blue line: 1st quartile (1-5 hypermethylated genes).

Dark blue line: 2nd quartile (6-7 hypermethylated genes).

Pink line: 3rd quartile (8-10 hypermethylated genes).

Red line: 4th quartile (>10 hypermethylated genes).

There were no significant differences in the HR among the 1st, 2nd and 3rd quartiles. However, the 4th quartile had a HR of 2.78 (95% CI: 1.53-5.05).

b) Light blue line: 1st quartile, 2nd quartile and 3rd quartile (1-10 hypermethylated genes) were combined as survival estimates were identical for the first three quartiles.

Red line: 4th quartile (>10 hypermethylated genes)

The 4th quartile had a HR of 2.88 (95% CI: 1.78-4.65) compared with the combined group of the 1st, 2nd and 3rd quartiles.

HR: Hazard ratio.

CI: Confidence interval.

Development of prediction models for survival of pancreatic adenocarcinoma

The total group of patients with pancreatic adenocarcinoma

We first analysed the total group of cancer patients (n = 95) without considering the subsequent stage classification. The purpose was to develop a prediction model for the survival of patients diagnosed with pancreatic adenocarcinoma, for use prior to staging. Eight genes (*BNC1*, *GSTP1*, *MLH1*, *SFRP1*, *SEPT9v2*, *SST*, *TFPI2*, and *WNT5A*) yielded a statistically significant HR by univariate screening (Appendix E). Furthermore, patients with an ASA score of three compared with an ASA score of one had a HR of 2.63 (95% CI: 1.49-4.63) and PS > 0 compared with PS = 0 resulted in a HR of 2.49 (95% CI: 1.61-3.84). The HRs for age and gender were not significant.

Fourteen genes were determined as potential predictors. These variables were used to develop a prediction model for survival together with an ASA score of three and PS > 0. The model including ASA score of three, *GSTP1*, *SFRP2*, *BNC1*, *SFRP1* and *TFPI2* was determined as the final model with the best performance (Harrell's c of 0.73) (Table 10). PS was eliminated in the stepwise selection. *SFRP2* hypermethylation was a protective factor, rendering an individual HR of 0.45 (95% CI: 0.27-0.73). There were no significant interactions between variables in the model, and the model was well calibrated (p-value = 0.9956). Patients were divided into four risk groups based on the prediction model. Figure 17 illustrates the survival curves of the groups and the gene combination together with the corresponding HRs.

Table 10. Survival prediction model for the total patient group

Model	ASA = 3	<i>BNC1</i>	<i>GSTP1</i>	<i>SFRP1</i>	<i>SFRP2</i>	<i>TFPI2</i>
HR	3.34	2.00	9.55	1.94	0.45	2.52
95% CI	(1.91-5.84)	(1.26-3.18)	(2.70-33.82)	(1.24-3.02)	(0.27-0.73)	(1.42-4.47)

Harrell's c = 0.73

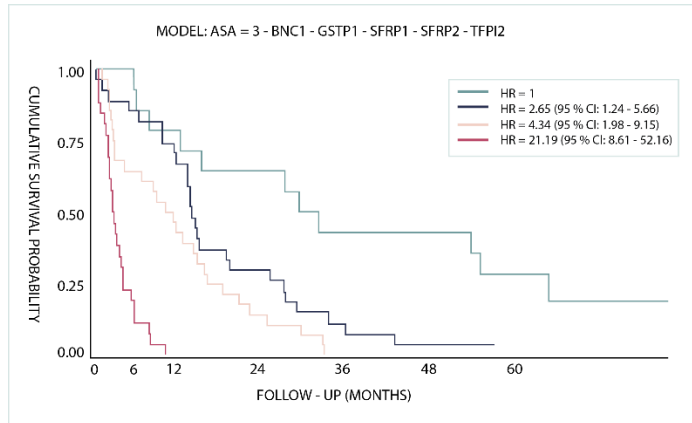
HR: Hazard ratio.

CI: Confidence interval.

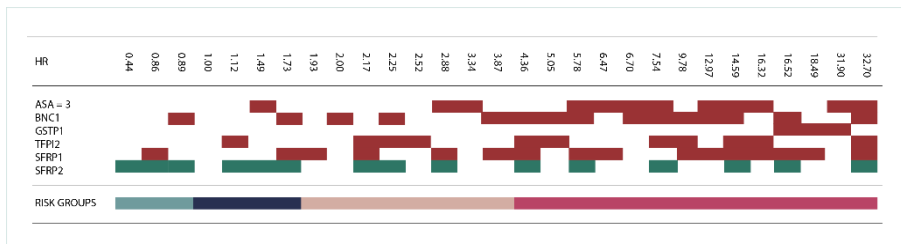
ASA: American Society of Anesthesiologists score.

Figure 17. Survival analysis for the total group of patients with pancreatic adenocarcinoma prior to staging

a)



b)



a) Survival prediction model for the total group of patients prior to stage classification, developed by multivariable Cox regression analysis using stepwise backwards elimination.

The patients in risk group 2, risk group 3 and risk group 4 had a HR of 2.65 (95% CI: 1.24-5.66), 4.34 (95% CI: 1.98-9.51) and 21.19 (95% CI: 8.61-52.15), respectively, compared with patients in risk group 1.

b) The gene combinations together with the corresponding HRs are illustrated for the survival prediction model (ASA = 3, BNC1, GSTP1, TFPI2, SFRP1, and SFRP2).

HR: Hazard ratio. CI: Confidence interval. ASA: American Society of Anesthesiologists score.

Light blue: Risk group 1.

Dark blue: Risk group 2.

Pink: Risk group 3.

Red: Risk group 4.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Subgroup analysis for stage I and II pancreatic adenocarcinoma (n = 40)

First, we analysed patients with potentially resectable pancreatic adenocarcinoma (stage I and II). The aim was to develop a model to predict survival of this specific subgroup of patients at time of diagnosis and prior to any treatment. In the univariate Cox regression analysis, we found that hypermethylation of two genes (*SFRP2* and *CDKN2A*) (Appendix E) was significantly associated with overall survival. The covariates gender and age were not significantly associated with overall survival and were therefore excluded from further analysis. An ASA score equal to three compared to an ASA score of one yielded a HR of 4.85 (95% CI: 1.85-12.76). Furthermore, PS > 0 was associated with an increased HR of 3.39 (95% CI: 1.64-7.02) compared with PS = 0. However, surgeons routinely use the ASA score in the evaluation of patient operability. We therefore chose to exclude PS from further analysis and solely include the ASA score in the multivariable analysis regarding stage I and II disease. Based on the univariate screening, hypermethylation of nine genes were potential predictors for survival. These genes, together with an ASA score of three were evaluated by multivariable Cox regression analysis. The final prediction model for survival of stage I and stage II pancreatic adenocarcinoma included an ASA score of three and hypermethylation of *SFRP2* and *MESTv2* (Harrell's *c* of 0.75) (Table 11). There were no significant interactions between any of the variables in the model. The variable with the greatest impact on survival in this subgroup was an ASA score of three (Table 11). Once again, *SFRP2* hypermethylation proved to be a protective factor with a HR of 0.18 (95% CI: 0.07-0.45), whereas *MESTv2* hypermethylation had a negative impact on survival (HR of 2.39 (95% CI: 0.97-5.94)). Based on the survival prediction model, patients were divided into four risk groups. Figure 18 illustrates the survival of the risk groups according to the final model. Patients in risk group 1 had two-year survival of 80% (95% CI: 50%-93%) and three-year survival of 47% (95% CI: 21%-69%) compared with patients in risk group 2 with two year survival of only 22% (95% CI: 7%-43%) no patients alive after three years (Figure 18). Three patients were alive without residual disease or recurrence after five years of follow-up. All three patients had an ASA score of less than three and *SFRP2* hypermethylation at the time of diagnosis. An ASA score of three (group 3 and 4) resulted in poor survival independent of hypermethylation status (Figure 18).

Table 11. Survival prediction model for stage I and stage II pancreatic adenocarcinoma

Model	ASA = 3	<i>MESTv2</i>	<i>SFRP2</i>
HR	14.13	2.39	0.18
95% CI	(4.46-43.81)	(0.97-5.94)	(0.07-0.45)

Harrell's *c* = 0.75

HR: Hazard ratio.

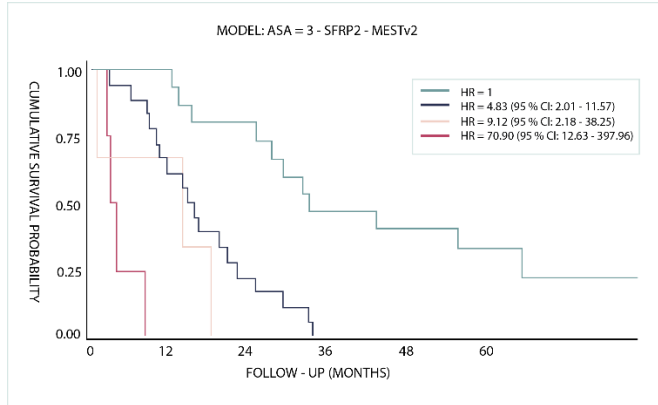
CI: Confidence interval.

ASA: American Society of Anesthesiologists score.

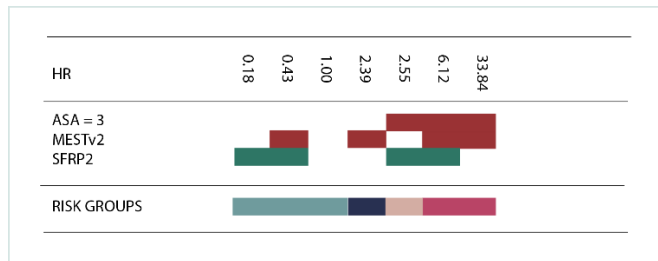
Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Figure 18. Survival analysis for stage I and II pancreatic adenocarcinoma

a)



b)



a) Survival prediction model for the stage I and II patients, developed by multivariable Cox regression analysis using stepwise backwards elimination.

The patients in risk group 2, risk group 3 and risk group 4 had a HR of 4.83 (95% CI: 2.01-11.57), 9.12 (95% CI: 2.18-38.25) and 70.90 (95% CI: 12.63-397.96), respectively, compared with patients in risk group 1.

b) The gene combinations together with the corresponding HRs are illustrated for the survival prediction model (ASA = 3, MESTv2, and SFRP2).

HR: Hazard ratio. CI: Confidence interval. ASA: American Society of Anesthesiologists score.

Light blue: Risk group 1.

Dark blue: Risk group 2.

Pink: Risk group 3.

Red: Risk group 4.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Subgroup analysis for stage IV pancreatic adenocarcinoma (n = 42)

The purpose of the subgroup analysis of stage IV disease was to develop a prediction model for the survival of pancreatic adenocarcinoma patients with distant metastases. We used an approach similar to that described above for stage I and stage II disease. In the univariate screening three genes (*BMP3*, *SFRP1* and *TFPI2*) yielded a significant HR (Appendix E). The HRs for age, gender and ASA score were insignificant and therefore excluded from the multivariable analysis. PS is routinely used by oncologist in the determination of treatment for stage IV patients. However, PS was excluded from further analysis because it was not significantly associated with survival of stage IV disease (p-value = 0.074). A prediction model was developed based on hypermethylation of eleven potential predictor genes. The variables *BMP3*, *MGMT*, *NPTX2*, and *SFRP1* were included in the final model, which reached a Harrell's *c* of 0.71 (Table 12) and was well calibrated (p-value = 0.5494). *NPTX2* hypermethylation was the only variable with a HR of less than one (Table 12). Based on the prediction model for stage IV pancreatic adenocarcinoma, patients were divided into two risk groups (Figure 19). Patients in risk group 2 had a HR of 5.23 (95% CI: 2.13-12.82) compared with patients in risk group 1. The 6-month and one-year survival (64% (95% CI: 38%-82%) and 59% (95% CI: 33%-78%)) of patients in risk group 1 were significantly superior to those of patients in risk group 2, with 6-month survival of 14% (95% CI: 3%-30%) and no patients in risk group 2 alive after one year (Figure 19).

Table 12. Survival prediction model for stage IV pancreatic adenocarcinoma

Model	<i>BMP3</i>	<i>MGMT</i>	<i>NPTX2</i>	<i>SFRP1</i>
HR	2.65	2.11	0.45	2.77
95% CI	(1.11-6.29)	(0.57-7.87)	(0.17-1.18)	(1.15-6.67)

Harrel's *c* = 0.71

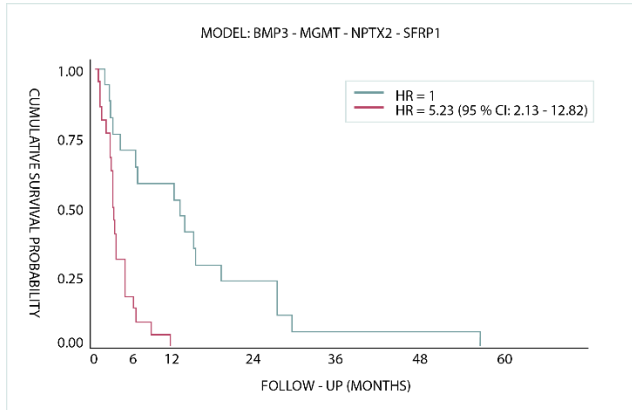
HR: Hazard ratio.

CI: Confidence interval.

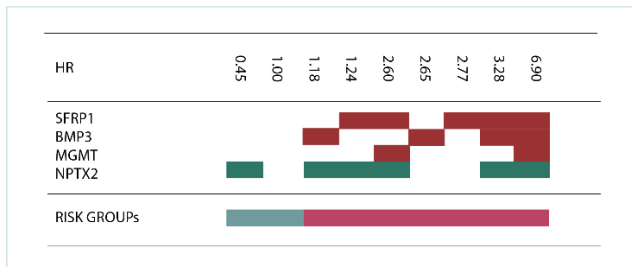
Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Figure 19. Survival analysis for stage IV pancreatic adenocarcinoma

a)



b)



a) Survival prediction model for the stage IV patients, developed by multivariable Cox regression analysis using stepwise backwards elimination.

The patients in risk group 2 had a HR of 5.23 (95% CI: 2.13-12.82) compared with patients in risk group 1.

b) The gene combinations together with the corresponding HRs are illustrated for the survival prediction model (*BMP3*, *MGMT*, *NPTX2*, and *SFRP1*).

HR: Hazard ratio.

CI: Confidence interval.

Light blue: Risk group 1.

Red: Risk group 2.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

5. DISCUSSION

5.1. LIMITATIONS OF THE STUDIES

General limitations of the studies

In addition to the literature review, we conducted three studies to evaluate hypermethylation of plasma-derived cell-free DNA as blood-based diagnostic and prognostic markers for pancreatic adenocarcinoma. Our studies were all exploratory and analysed the gene panel in a single group of patients with pancreatic adenocarcinoma or benign disease. The evaluation of each prediction model in an independent cohort is considered the gold standard for biomarker validation to substantiate the results because prediction models built on a single data set can produce an overestimation of test performance due to overfitting. It was, however, impossible for us to reach this standard during the development phase, as pancreatic adenocarcinoma is a relatively rare disease.

The pancreatic adenocarcinoma patients and the patients suspected of but without upper gastrointestinal malignancy were primarily included as part of a study of upper gastrointestinal malignancy and thromboembolism, which may have caused selection bias due to the exclusion criteria of the primary study.

In addition, only a limited amount of sample material was available from patients with pancreatic adenocarcinoma, which made it impossible to conduct replicate analysis. Approximately 500 µl of EDTA plasma was used for DNA extraction. Although we used an optimized method with a high recovery of cell-free methylated DNA,⁶³ more sample material would most likely lead to improved sensitivity.

We performed bisulfite treatment for methylation analysis followed by first- and second-round methylation-specific PCR. This method is quantitative when using hemimethylated *MESTv1* as a reference gene.⁶³ However, due to limited power, the effect of this difference could not be evaluated in multivariable analyses. Consequently, we analysed hypermethylation as a binary variable, which resulted in a loss of quantitative information.

Furthermore, the method we used for methylation analysis did not provide information regarding the numbers or proportion of CpGs methylated in the investigated part of the promoter sequence. Detailed information about CpG methylation could have been obtained by DNA sequencing of the PCR products.

Inter-study comparison is difficult when studying DNA hypermethylation because several methods have been described for methylation analysis. In addition, the use of different primer sequences for the same gene may lead to conflicting results, which is a general limitation of studies within this field.⁵⁷

At the end of the analyses, we discovered that UNG (Invitrogen) tended to decrease the sensitivity compared with COD UNG (ArcticZymes). We determined that heating did not completely inactivate UNG (Invitrogen), which potentially could result in DNA degradation, whereas COD UNG (ArcticZymes) was completely thermolabile. We analysed all samples using UNG (Invitrogen), as it was impossible to repeat all analyses using COD UNG (ArcticZymes) due to the lack of sample material.

Limitations of study II

The methylation analyses in study II were performed non-blinded. Furthermore, patients with pancreatic adenocarcinoma and the patients in the control groups were not matched according to age. This can be a potential disadvantage because epigenetic changes are a part of ageing.⁷⁹ To address this problem and to avoid variable selection driven by possible differences in general methylation status between patients of different ages, we incorporated age as a covariate in the diagnostic prediction model.

It would have been relevant to compare the performance of the diagnostic prediction model with that of CA-19-9. Unfortunately, CA-19-9 was only available for one third of the patients, since this test was first implemented in our department during the study period.

Patient compliance was a major challenge in the subgroup of patients with alcoholic chronic pancreatitis. Many patients failed to attend the primary visit or follow-up appointments, despite several reminders both by phone and mail. This may have caused an underrepresentation of patients with current alcohol abuse in the control group. However, our analysis revealed no difference in methylation profiles between patients with chronic alcoholic pancreatitis and patients with chronic pancreatitis of another aetiology.

Limitations of study III and IV

In study III and IV, some of the subgroups contained a limited number of patients, which may be responsible for the lack of differences in methylation profile between stage I and IIa vs IIb and stage I and II vs III in study III. Similar to study IV, the subgroup of stage III patients contained only 13 patients, making it impossible to develop a survival prediction model for stage III disease.

5.2. STRENGTHS OF THE STUDIES

We tested promoter hypermethylation in plasma-derived cell-free DNA using a broad gene panel in a large group of consecutive patients with pancreatic adenocarcinoma included prospectively before diagnostic work up and before treatment. The study was conducted as a single-center study, and only a few health professionals were

responsible for patient inclusion, enabling a uniform and consistent comprehensive diagnostic work-up of all patients to ensure correct diagnosis and stage classification.

Study II was designed to compare the methylation status of malignant and benign pancreatic disease. We consecutively included a large and extremely relevant control group consisting of patients with benign disease, which is clinically difficult to differentiate from pancreatic cancer. We developed a diagnostic prediction model for pancreatic adenocarcinoma with high performance, independent of cancer stage. As external validation was not possible, we performed internal validation using leave pair out cross validation, which revealed only a modest optimism in performance.

Diagnostic and prognostic biomarkers for pancreatic cancer are lacking. We developed both a diagnostic test and prognostic tests for stage classification and survival of pancreatic adenocarcinoma, which all are blood-based markers and therefore have several advantages compared to tissue-based markers. Furthermore, biomarkers based on hypermethylated cell-free DNA do not appear to depend on blood group status, which is an essential advantage compared with CA-19-9.

The analyses for study III and IV were performed blinded. The methylation analysis for all three studies was based on an optimized method of bisulfite treatment. This method enables high recovery of cell-free methylated DNA from samples with minute amounts of DNA (< 0.01 ng/ml) and thus has improved sensitivity compared with previous methods. In addition, the method results in deamination of DNA in less than two hours.⁶³

5.3. DISCUSSION OF THE FINDINGS IN RELATION TO THE PUBLISHED LITERATURE

The gene panel

We designed a panel of 28 genes primarily based on study I (the literature review), which addressed genes aberrantly methylated in pancreatic adenocarcinoma. Several approaches exist for designing a gene panel. We used this strategy to evaluate the overall performance of genes that previously had been examined separately as markers for pancreatic cancer. Based on the selected panel of hypermethylated genes in cell-free DNA, we developed both diagnostic and prognostic models for pancreatic adenocarcinoma.

Because we solely analysed plasma, we were unable to determine if the hypermethylated cell-free DNA originated from the tumour. If our objective had included an assurance of tumour specificity, we should have used an approach analysing both hypermethylation of tumour tissue and plasma. Genes hypermethylated in both tumour tissue and plasma-derived cell-free DNA improve

the confidence of tumour origin, provided that the same genes are non-methylated in samples from healthy individuals.⁵⁷

Regardless, all genes in our panel, with the exception of *ALX4*, *MESTv2*, *SEPT9v2*, and *SST*, had in previous literature been detected as hypermethylated in primary tumour tissue and, in addition, in either pancreatic juice, plasma or serum from patients with pancreatic adenocarcinoma.

Number of hypermethylated genes in plasma-derived cell-free DNA

Previous studies have shown that healthy individuals only have sparse amounts of cell-free DNA^{47,48} as well as only very few detectable hypermethylated genes in cell-free DNA.^{23,73,80} Similar to other studies, we demonstrated that hypermethylated cell-free DNA is detectable in all stages of pancreatic adenocarcinoma,^{23,81} even in stage I disease, making it a potential marker for early-stage diagnostics.⁴⁹ In addition, we detected hypermethylated cell-free DNA in patients with chronic pancreatitis and in patients with symptoms mimicking upper gastrointestinal cancer, albeit to a much lesser extent. Furthermore, we analysed the hypermethylation profiles of patients with acute pancreatitis. A previous study described increased levels of cell-free DNA during acute pancreatitis.⁸² In addition, we demonstrated that DNA hypermethylation can be detected in patients with acute pancreatitis at less pronounced levels compared with patients with pancreatic adenocarcinoma but slightly higher levels compared with patients with chronic inflammation of the pancreas.

Furthermore, we discovered that patients with distant metastases had an even higher number of hypermethylated genes compared with patients with localized disease. We were unable to demonstrate that the number of hypermethylated genes in plasma-derived cell-free DNA also increased from stage I to stage III disease. This might be due to a lack of power in our study. Distant metastasis has been reported to result in a larger amount of cell-free DNA.⁴⁹ However, the level of cell-free DNA was not associated with cancer stage in our study. The association between metastatic pancreatic adenocarcinoma and a higher number of hypermethylated genes in cell-free DNA has not been described previously. Two small studies were not able to show this association, which could be due to a lack of power or differences in genes analysed.^{23,83} However, our results for hypermethylated cell-free DNA are consistent with those of a study on pancreatic cancer tumour tissue that observed DNA hypermethylation in early precursor lesions (PanIN-1) and an increase in the number of hypermethylated genes from PanIN-1 to PanIN-3.⁸⁴ Together with our results, these observations suggest that hypermethylated promoter regions accumulate during the course of pancreatic adenocarcinoma development and progression.

We also demonstrated that the number of hypermethylated genes in cell-free DNA influenced survival. Patients with more than ten hypermethylated genes in cell-free DNA were more likely to die during the first year after diagnosis than patients with fewer hypermethylated genes. Similarly, in head and neck squamous cell carcinoma

tissue, hypermethylation of more than six of eleven examined genes was associated with poor overall survival and decreased disease-free survival.⁸⁵

Diagnostic value of plasma-derived cell-free DNA promoter hypermethylation

In our study, 17 of the 28 promoter regions in the gene panel were more frequently hypermethylated in patients with pancreatic adenocarcinoma than in patients in the control groups. Furthermore, hypermethylation of *BMP3*, *MESTv2*, *SST*, *TFPI2*, *TAC1*, *ALX4*, *HIC1*, *SFRP2*, *SEPT9v2*, and *WNT5A* has not previously been analysed in cell-free DNA of patients with pancreatic adenocarcinoma.

BNC1 hypermethylation in cell-free DNA was described by Yi et al. as having a sensitivity of 79% and a specificity of 89% when comparing pancreatic cancer with healthy individuals.⁷⁰ In our study, hypermethylated *BNC1* had a sensitivity of only 36% and a specificity of 94%. Previous studies of *NPTX2* hypermethylation in cell-free DNA have yielded conflicting results. A small study evaluating a panel of six genes (*NPTX2*, *UCHL1*, *SFRP1*, *PENK*, *CDKN2A* and *RASSF1A*) described *NPTX2* hypermethylation as having 38% sensitivity and 83% specificity,⁸⁶ whereas another study demonstrated a sensitivity of 84% with a specificity of 69%.⁷³ We found *NPTX2* hypermethylation to have 75% sensitivity but a specificity of only 53%. Hypermethylation of *SFRP1* in cell-free DNA was previously demonstrated to have 31% sensitivity and 86% specificity,⁸⁶ similar to our findings. In our study, *RASSF1A* hypermethylation had a sensitivity of 42% and a specificity of 88%. The reported sensitivities of *RASSF1A* in cell-free DNA range from 6%⁸⁶ to 34%⁷² with a specificity of approximately 90%.⁸⁶ Furthermore, our study only managed to detect limited hypermethylation of *PENK* and *CDKN2A*, with sensitivities of 2% and 6%, respectively, in contrast to previous studies of cell-free DNA that have described *PENK* hypermethylation as having a sensitivity of approximately 30% and 88% specificity.^{76,86} Similarly, *CDKN2A* hypermethylation in cell-free DNA has previously been detected with 17%⁷² to 25%^{76,86} sensitivity and 86% specificity.⁸⁶ The inconsistency between our findings and previous results may be due to the use of non-identical primer sequences and different analytical methods. The uneven distribution of cancer stages between the studies and differences in the compositions of the control groups also contributed to the different results.

Consistent with previous studies of DNA hypermethylation as blood-based markers for pancreatic cancer, none of the examined genes in our panel had the potential to function as an individual diagnostic marker, suggesting that a panel of genes is needed to achieve sufficient performance. Only a few studies, have analysed pancreatic cancer and a panel of hypermethylated genes in cell-free DNA. Park et al. published a small study analysing a panel of six genes using methylation-specific PCR, which enabled discrimination between pancreatic cancer and healthy controls. However, the panel was unable to differentiate malignant from benign pancreatic disease,⁸⁶ which could be due to a lack of power. Melnikov et al. (2009) analysed plasma DNA using microarray-mediated methylation analysis of 56 fragments

(MethDet 56). A panel of five hypomethylated promoter regions had 76% sensitivity and 59% specificity for pancreatic cancer compared with healthy individuals.⁸⁷ Liggett et al. (2010) described the most promising results using MethDet 56. A panel of 14 gene promoters (both hypo- and hypermethylation) enabled the differentiation of pancreatic cancer from chronic pancreatitis with a sensitivity of 91% and a specificity of 91%.⁸⁸ However, no further validation of the results has been published.

We analysed hypermethylation in a 28-gene panel by methylation-specific PCR and developed a diagnostic prediction model for pancreatic adenocarcinoma. The model contained eight promoter sequences (*BMP3*, *RASSF1A*, *BNC1*, *MESTv2*, *TFPI2*, *APC*, *SFRP1*, and *SFRP2*) and the covariate age > 65 years. Our test enabled the differentiation of patients with pancreatic adenocarcinoma and a benign control group (patients with chronic pancreatitis and patients suspected of but without upper gastrointestinal malignancy) with 76% sensitivity and a specificity of 83%. Our control group was highly clinically relevant as these patients had symptoms or diagnostic imaging mimicking those of pancreatic cancer, which is a well-known clinical challenge in the diagnostic work-up of patients suspected of pancreatic cancer. In addition, the diagnostic value of our test was independent of cancer stage, which is of utmost clinical importance because only early-stage pancreatic adenocarcinoma (stage I-II) is potentially curable. Our diagnostic test performed well and was superior to CA-19-9, which currently is the only clinically implemented blood-based test for pancreatic cancer. Although the performance of our diagnostic test and previously described gene panels do not allow any of them to be used as a stand-alone test for pancreatic cancer diagnosis, validation of our test may enable its application in combination with other modalities in the work-up of patients suspected of pancreatic cancer. In addition, there is potential for further improvement of our diagnostic test by expanding the gene panel with other relevant genes; however, additional studies will be warranted. Furthermore, it would be interesting and of high clinical relevance to evaluate the performance of our diagnostic test in patients with pancreatic cancer precursor lesions.

Plasma-derived cell-free DNA promoter hypermethylation with regard to pancreatic adenocarcinoma staging

Our studies showed that cell-free DNA hypermethylation of seven individual genes (*ALX4*, *BNC1*, *HIC1*, *SEPT9v2*, *SST*, *TFPI2*, and *TAC1*) was associated with distant metastasis. In general, studies of the prognostic value of hypermethylated cell-free DNA are lacking. However, a few studies of pancreatic cancer tissue have indicated prognostic value of hypermethylated DNA. *HIC1* hypermethylation has been detected more frequently in pancreatic cancer tissue from stage III-IV disease compared with stage I-II disease, consistent with our findings in plasma.⁸⁹ Similarly, cell-free DNA hypermethylation of *TFPI2* in colorectal cancer patients was also associated with stage IV disease.⁹⁰ Furthermore, hypermethylated *ALX4* has been detected in colorectal cancer tissue, albeit at the same frequencies in all stages of the

disease,⁹¹ in contrast to the findings of our study. Similar, *SEPT9v2* hypermethylation has been detected in cancer tissue and cell-free DNA from colorectal cancer patients at the same frequency in all stages of the disease.^{91,92} This indicates that hypermethylation of *SEPT9v2* occurs earlier in the development of colorectal cancer than in the development of pancreatic cancer.⁹³ In addition, there is a commercialized blood-based diagnostic test for colorectal cancer available, that is based on *SEPT9* hypermethylation.⁹⁴

Because none of the genes in our panel had the potential for use as a single marker for staging patients with pancreatic adenocarcinoma, we developed two prediction models for this purpose. A panel based on the hypermethylation status of eight genes (*SEPT9v2*, *SST*, *ALX4*, *CDKN2B*, *HIC1*, *MLH1*, *NEUROG1*, and *BNC1*) enabled with high performance (AUC of 0.87) the distinction of pancreatic adenocarcinoma patients with distant metastasis (stage IV) from patients without distant metastasis (stage I-III). Another panel (*MLH1*, *SEPT9v2*, *BNC1*, *ALX4*, *CDKN2B*, *NEUROG1*, *WNT5A*, and *TFPI2*) enabled the differentiation of potentially resectable disease (stage I and II) from non-resectable disease (stage III and IV), albeit with lower performance (AUC of 0.82). To our knowledge, we are the first to develop prediction models based on hypermethylated cell-free DNA, for pancreatic adenocarcinoma staging. Both tests are of high clinical relevance and may supplement existing tools for stage classification and aid the difficult evaluation of tumour resectability.

As previously mentioned, our studies indicate that hypermethylated promoter sequences in cell-free DNA accumulate during the development and progression of pancreatic adenocarcinoma. Furthermore, our studies indicate that promoter hypermethylation changes during the course of the disease, as illustrated by the varying composition of the gene panels developed for the various applications. Only *BNC1* recurred in the diagnostic and prognostic gene panels, and six out of eight genes in the two panels for stage classification overlap. Taken together, these observations indicate, that hypermethylation of certain genes occurs at different stages of neoplastic development.

Promoter hypermethylation of cell-free DNA may represent different subtypes of pancreatic adenocarcinoma

We also investigated the association between cell-free DNA hypermethylation and survival of patients with pancreatic adenocarcinoma. Our findings showed that hypermethylation of several individual genes was associated with survival. Overall, promoter hypermethylation had a negative impact on survival, whereas hypermethylation of a few specific genes seemed to have a positive effect on survival.

We observed that *CDKN2A* hypermethylation was significantly associated with decreased survival in patients with early-stage pancreatic adenocarcinoma. However, this finding is subject to great uncertainty as only one patient with stage I-II disease

had *CDKN2A* hypermethylation. *CDKN2A* hypermethylation has previously been observed in pancreatic cancer tumour tissue,⁹⁵ in other solid tumours,^{96–98} and in various medias related to pancreatic cancer,^{99–101} including cell-free DNA.⁸⁶ Previous studies have reported low expression of *CDKN2A* in pancreatic adenocarcinoma tissue to be associated with decreased survival in early-stage disease, in line with our results.^{95,102}

Furthermore, our study showed that hypermethylation of *SFRP2* had a positive impact on survival of stage I and II pancreatic adenocarcinoma. The *SFRP2* gene encodes the secreted frizzled-related protein 2, which modulates the Wnt signalling pathway (both as an antagonist and an agonist).¹⁰³ Hypermethylation of *SFRP2* has previously been associated with the development of colorectal cancer,^{104–106} gastric cancer,¹⁰⁷ and pancreatic cancer.^{108,109} However, *SFRP2* hypermethylation in cell-free DNA has not previously been associated with improved prognosis of stage I and II pancreatic adenocarcinoma.

In our study hypermethylation of three individual genes (*SFRP1*, *BMP3*, and *TFPI2*) was significantly associated with decreased survival in stage IV disease. *SFRP1* encodes secreted frizzled-related protein 1, which similar to *SFRP2*, acts as a modulator (however, only antagonistic) of the Wnt signalling pathway to affect cell proliferation, differentiation and apoptosis.¹⁰³ Upregulation of the Wnt pathway due to promoter hypermethylation of *SFRP1* genes has previously been associated with cancer formation. Promoter hypermethylation of *SFRP1* has previously been detected in tumour tissue,¹⁰⁸ pancreatic juice¹¹⁰ and cell-free DNA⁸⁶ in pancreatic cancer. *SFRP1* hypermethylation has not previously been associated with impaired prognosis in stage IV pancreatic adenocarcinoma. However, studies of breast cancer¹¹¹ and renal cancer¹¹² have identified hypermethylation of *SFRP1* in tumour tissue as an independent risk factor for decreased overall survival. Furthermore, in our study, hypermethylation of *BMP3* and *TFPI2* were associated with impaired survival of patients with stage IV disease. The *BMP3* gene encodes methylated bone morphogenetic protein 3, which is involved in the TGF beta pathway and influences cell proliferation, differentiation and apoptosis.^{113–115} Studies have indicated a diagnostic value of *BMP3* hypermethylation in stool from patients with pancreatic cancer¹⁰⁹ and colorectal cancer.^{109,116} We are the first to describe a prognostic value of hypermethylated *BMP3*. The *TFPI2* gene encodes tissue factor pathway inhibitor 2 protein, which is associated with cell adhesion and the clotting cascade.¹¹⁷ *TFPI2* hypermethylation has also been described in several types of cancer,^{90,118,119} including pancreatic cancer tissue¹²⁰ and IPMN tissue,¹²¹ as well as in pancreatic juice from pancreatic cancer patients.¹²² We are the first to describe a prognostic value of *TFPI2* hypermethylation in cell-free DNA for pancreatic adenocarcinoma. However, hypermethylation of *TFPI2* in hepatocellular carcinoma tumour tissue is associated with advanced cancer stage and shorter survival,¹¹⁸ in accordance with our results for pancreatic cancer. Similarly, *TFPI2* hypermethylation in the serum of melanoma patients has been suggested as a marker for metastatic disease.¹¹⁹

Based on our selected gene panel, we developed prediction models for survival of patients with pancreatic adenocarcinoma. We developed a model based on the total group of cancer patients, without considering stage classification. In addition, we developed survival prediction models according to cancer stage, with the aim of developing prognostic markers, which add knowledge about tumour biology and disease aggressiveness within each cancer stage. The prediction models enabled the stratification of patients in risk groups according to survival.

Both cancer stage-specific models contained a hypermethylated gene variable with a positive impact on survival. *SFRP2* hypermethylation had a positive impact on the prognosis of patients with stage I and II disease. A similar trend was observed for *NPTX2* hypermethylation in stage IV disease. The *NPTX2* gene encodes neuronal pentraxin 2 protein.¹²³ Previous studies have described a diagnostic value of *NPTX2* hypermethylation with regard to pancreatic cancer.^{23,124} Furthermore, *NPTX2* hypermethylation has been associated with poor prognosis of patients with glioblastoma,¹²⁵ in contrast to our findings. Various causes may underlie the conflicting findings in pancreatic cancer and glioblastoma, but this discrepancy may reflect differences in tumour biology or a varying impact of *NPTX2* hypermethylation according to cancer stage. This discrepancy may also be due to the use of different analytical methods or non-identical primer sequences, which would result in analysis of different part of the gene.

Our study indicates a biological variation within pancreatic adenocarcinoma that influences patient outcome. Our findings show that hypermethylation of some genes seems to have a positive impact on prognosis, whereas hypermethylation of other genes has a negative impact. According to our study, patients lacking hypermethylated genes in cell-free DNA, stage I and II patients with *SFRP2* hypermethylation and stage IV patients with hypermethylation of *NPTX2* appear to have less aggressive tumours, resulting in improved survival compared with other patients. These findings are consistent with a study by Thomson et al. (2015) of pancreatic adenocarcinoma tumour tissue, which described a “survival-” methylation signature associated with short survival time and a “survival+” methylation signature associated with long survival time. The Wnt signalling pathway, among others, was involved in the “survival-“ signature,¹²⁶ consistent with our finding that hypermethylation of *SFRP1* results in decreased survival. Two previous studies based on a six-gene and a 13-gene expression profile in pancreatic adenocarcinoma tissue also stratified patients into a low-risk and a high-risk group.^{127,128} Similarly, our survival prediction models enabled the stratification of patients in risk groups according to survival. The prognostic tests previously described regarding pancreatic adenocarcinoma are all tissue-based, in contrast to our survival prediction tests, which have the advantage of being blood-based. Our tests have the potential to provide prognostic information in addition to the TNM classification regarding the survival of patients with pancreatic adenocarcinoma. This would clearly benefit

patients and clinicians' therapeutic decisions and facilitate the correct choice of treatment.

Blood-based biomarkers/liquid biopsies

The diagnostic and prognostic prediction models described in study II, III and IV are all blood-based tests. Blood-based markers have several advantages over tissue-based markers. The current standard of care for diagnosing pancreatic cancer involves examination of tumour tissue either by fine needle aspiration cytology or histological examination of biopsies or surgical specimens. All invasive procedures entail a risk of complications. Blood-based tests are minimally invasive, involving only limited discomfort, and have no major complications.⁴⁹ They can easily be repeated to enable close monitoring of the disease to evaluate response to treatment or early detection of recurrence.⁵⁰

Blood-based markers for pancreatic disease are urgently needed as tumours in the pancreas may occur in areas that are difficult to access. In addition, the size of the tumour may limit the ability to sample tissue adequately, and tissue biopsies may not be an accurate representation of the tumour due to intra-tumour heterogeneity.⁵⁰ There can also be molecular differences between the primary tumour and metastatic lesions, and thus a tissue biopsy from the primary lesion most likely will not represent the metastatic lesions.⁵⁰ Markers based on hypermethylated plasma-derived cell-free DNA could potentially provide information about both the primary tumour and the metastatic lesions simultaneously.⁴⁹ In cases where tumour tissue specimens are unavailable from either the primary tumour or the metastatic lesions, blood-based markers may represent an alternative or a supplement to existing tools used in the diagnostic work-up and treatment of patients with pancreatic adenocarcinoma.⁴⁹

Other biomarkers for pancreatic cancer

In addition to hypermethylation of cell-free DNA, various approaches are available for the development of cancer biomarkers. Schultz et al. (2014) published a large comprehensive study of miRNA in whole blood as a diagnostic marker for pancreatic cancer.³⁹ They developed two diagnostic panels containing four and ten miRNAs, respectively. In combination with CA-19-9, the panels reached an AUC of 0.92 when comparing pancreatic cancer patients with healthy subject and a few patients with chronic pancreatitis. Most promising, the panel of ten miRNAs combined with CA-19-9 performed with similarly high performance (AUC of 0.91) in stage I-II patients.³⁹ Future studies evaluating the ability of miRNAs to differentiate patients with pancreatic cancer and patients with benign pancreatic disease or symptoms mimicking pancreatic cancer would be of great clinical interest.

Currently, the IMMray™ PanCan-d test [Immunovia, Lund, Sweden], which is based on a wide antibody microarray, is the most studied diagnostic test for pancreatic cancer. Four studies have been published, all reporting very high performance (AUC > 0.90) for the differentiation of healthy individuals and stage III-IV pancreatic

cancer.^{129–132} Unfortunately, the performance declined substantially when the test was used to discriminate late-stage pancreatic cancer and benign pancreatic disease (AUC of 0.86¹³⁰ and AUC of 0.70¹³¹). In addition, the AUC was only 0.71 when differentiating stage I disease from healthy individuals.¹³²

Circulating autoantibodies to phosphorylated alpha-enolase¹³³ and ezrin¹³⁴ have also been suggested as potential diagnostic biomarkers for pancreatic cancer. In combination, alpha-enolase and CA-19-9 reached an AUC of 0.95 for discriminating stage I-II pancreatic cancer from a control group of healthy subjects and patients with chronic pancreatitis.¹³³ A similar result was found for ezrin.¹³⁴

Cell-free nucleosomes have been evaluated in a single small study as diagnostic biomarkers for pancreatic cancer. An ELISA-based immunoassay platform (Nucleosomics® [Volition^{RX}, Singapore] measuring epigenetic changes managed to differentiate stage II pancreatic cancer and a control group of both healthy individuals and patients with benign pancreatic disease with an AUC of 0.92.¹³⁵

The results based on alpha-enolase, ezrin and cell-free nucleosomes are promising as they are based on early-stage disease. However, the studies only contained a limited number of patients. Similar to our findings, these results need to be validated in independent patient cohorts. Additionally, it is of utmost importance to evaluate the test performance in early-stage pancreatic cancer and a control group solely containing relevant patients (e.g., patients with chronic pancreatitis) to enable differentiation of malignant and benign molecular changes in pancreatic disease. Such studies are essential for the clinical application of a diagnostic biomarker.

6. CONCLUSIONS

6.1. STUDY I/PAPER I

None of the genes previously examined had the potential to function as an individual diagnostic marker, suggesting that a panel of several genes is needed to achieve sufficient performance. Further research is warranted before a blood-based diagnostic marker for pancreatic cancer based on promoter hypermethylation can be applied clinically.

6.2. STUDY II/PAPER II

Several genes are more frequently hypermethylated in the cell-free DNA of patients with pancreatic adenocarcinoma compared with patients with benign pancreatic disease. In addition, patients with pancreatic adenocarcinoma have a higher number of hypermethylated genes than patients with benign pancreatic disease. A panel of eight genes can distinguish between patients with pancreatic adenocarcinoma and a clinically relevant control group, indicating that hypermethylated cell-free DNA is potentially usable as a blood-based diagnostic marker for pancreatic adenocarcinoma.

6.3. STUDY III/PAPER III

DNA hypermethylation of plasma-derived cell-free DNA is detectable even in early-stage pancreatic adenocarcinoma. Hypermethylations accumulate and change during neoplastic development and with aggravating cancer stage. Panels of genes can differentiate patients with pancreatic adenocarcinoma according to cancer stage. The prediction models for cancer staging may represent a supplement to existing clinical tools in stage classification of pancreatic adenocarcinoma.

6.4. STUDY IV/PAPER IV

Hypermethylation of more than ten genes in plasma-derived cell-free DNA is an independent risk factor for decreased survival in patients with pancreatic adenocarcinoma. Furthermore, the survival of pancreatic adenocarcinoma patients is associated with promoter hypermethylation of specific genes that vary depending on cancer stage. Overall, promoter hypermethylation has a negative impact on survival. However, hypermethylation of a few specific genes seems to result in improved prognosis. Prediction models based on the gene panel enabled the stratification of patients with pancreatic adenocarcinoma in risk groups according to survival time. These prediction models may work as prognostic biomarkers that supplement the TNM classification and facilitate more personalized cancer treatment.

6.5. FINAL CONCLUSION

Plasma-derived cell-free DNA promoter hypermethylation has the potential to be used as blood-based markers for the diagnosis, stage classification and prediction of survival of pancreatic adenocarcinoma. External validation of these results, however, is required before clinical application.

7. PERSPECTIVES

Unfortunately, the incidence of pancreatic cancer has slightly increased in recent decades, and patient survival has not improved.⁹ Currently, pancreatic cancer is the 4th leading cause of cancer death worldwide. In addition, the incidence of pancreatic cancer in the US has been estimated to increase by 55% by the year 2030.¹³⁶ Sadly, the number of deaths due to pancreatic cancer may also increase dramatically over the next few decades unless substantial improvements in early diagnosis and cancer therapy emerge.

Pancreatic adenocarcinoma was previously viewed to arise primarily by genetic alterations, i.e., the activation of oncogenes and the inactivation of tumour suppressor genes.¹³⁷ However, today we know that crosstalk between genetic and epigenetic alterations, including DNA methylation, is involved in carcinogenesis and the determination of cancer subtypes.

Our studies, among many others, have shown that this knowledge has the potential to provide new diagnostic and prognostic information for use in cancer management. Unfortunately, no study has yet led to changes in clinical practice with regard to the diagnostic work-up of pancreatic cancer. Further research is warranted, and extensive validation of biomarkers are required before clinical application.

The discovery of specific epigenetic events involved in the carcinogenesis of pancreatic adenocarcinoma is of great importance because epigenetic mechanisms are reversible, in contrast to genetic changes. Therefore, epigenetic events could serve as novel therapeutic targets for pancreatic cancer, which hopefully would lead to enhanced efficacy of adjuvant and palliative therapy.⁴⁴

Overall, epigenetics can provide a basis for biomarker development for pancreatic cancer, with the potential to improve early detection, ease the diagnostic work-up and facilitate tailored treatment to hopefully improve patient survival.

8. FUTURE RESEARCH

As mentioned earlier, external validation of the results in an independent cohort is needed to verify our results. We expect to perform the first external validation on sample material from the biobank BIOPAC in collaboration with a research group at Herlev Hospital, Denmark. We plan to analyse the 28 genes in approximately 250 patients with pancreatic adenocarcinoma and in 100 patients with chronic pancreatitis. This will allow external validation of study II, III and IV to be performed simultaneously.

In addition, we plan to analyse the follow-up samples from patients with pancreatic adenocarcinoma with regard to disease relapse/recurrence and in response to both surgical and palliative treatment.

Furthermore, we plan to analyse the samples from patients with acute pancreatitis with respect to aetiology and changes in the methylation profile during the course of an acute inflammatory reaction of the pancreas. We have planned a similar approach for patients with chronic pancreatitis with the additional purpose of identifying patients with a high risk of developing pancreatic cancer.

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Appendix A.

DNA sequences for probes and primers

<i>Gene</i>		<i>DNA sequence</i>	<i>Position</i>	<i>Amplicon size</i>
TAC1 M1	NC_000007.14	ATC GTA AGG TAT TGA GTA GGC	97732062 to 97732082	64
TAC1 M2		TCT CGA TAA CTA CCG CCG	97732109 to 97732126	
TAC1 M beacon		(HEX)CGA TCG ATC C+GA AC+G C+GC TCT CGA TCG(DabcyI)	97732086 to 97732100	
TAC1 Am		TAA GGA GGT TGG GAT AAA TAT C	97732043 to 97732064	83
TAC1 Bm		TCT CGA TAA CTA CCG CCG	97732109 to 97732126	
SST M1	NC_000003.12	GCG TCG AGA TGT TGT TTT GTC	187670279 to 187670299	65
SST M2		CCA AAA CCA AAA CGA TAA ACA ACG	187670234 to 187670257	
SST M beacon		(HEX)CGA TCG ACC AAC +GC+G CAC TAA CGA TCG(DabcyI)	187670260 to 187670274	
SST Am		TAG TTC GGT TTT CGC GGC GTC	187670260 to 187670274	81
SST Bm		CCA AAA CCA AAA CGA TAA ACA ACG	187670234 to 187670257	
APC M1	NC_000005.10	AGT GCG GGT CGG GAA GC	112737732 to 112737748	91
APC M2		AAT CGA CGA ACT CCC GAC G	112737805 to 112737823	
APC M beacon		(HEX)CGC GAT CGT TG+G ATG +CG+G AAT CGC G(DabcyI)	112737773 to 112737785	
APC Am		ATT GCG GAG TGC GGG TC	112737725 to 112737741	98
APC Bm		AAT CGA CGA ACT CCC GAC G	112737805 to 112737823	
MLH1 M1	NC_000003.12	TGG TTT TTT GGC GTT AAA ATG TC	36993529 to 36993552	94
MLH1 M2		AAA TAA CTT CCC CCG CCG	36993606 to 36993623	
MLH1 M beacon		(HEX)CGC GAT CTC +GTC CAA CC+G CC+G AAT ATC CGC(DabcyI)	36993569 to 36993592	
MLH1 Am		TGG TTT TTT GGC GTT AAA ATG TC	36993529 to 36993552	123
MLH1 Bm		CAT CTC TTT AAT AAC ATT AAC TAA CCG	36993626 to 36993652	
SFRP1 M1	NC_000008.11	GGA GTT GAT TGG TTG CGC	41309508 to 41309525	90
SFRP1 M2		CGC GAC ACT AAC TCC G	41309435 to 41309450	
SFRP1 M beacon		(HEX)CGC GAT G+GT+CG +GTC G+TA ATC GCG(DabcyI)	41309482 to 41309493	
SFRP1 Am		GAG GCG ATT GGT TTT CGC	41309567 to 41309584	149
SFRP1 Bm		CGC GAC ACT AAC TCC G	41309435 to 41309450	
CHFR M1	NC_000012.12	GTT TCG GTT TTA GTT TCG TAT TTC	132887175 to 132887198	102
CHFR M2		CGA CTC CTA CGT CTA AAC CCG	132887257 to 132887277	
CHFR M beacon		(HEX)CGC GAT CCG +CA+C GT+C CAT CGC G(DabcyI)	132887235 to 132887244	
CHFR Am		GTT TCG GTT TTA GTT TCG TAT TTC	132887175 to 132887198	111
CHFR Bm		CCC TAA AAA CGA CTC CTA CG	132887267 to 132887286	
RASSF1A M1	NC_000003.12	GGG AGG CGT TGA AGT C	50340882 to 50340897	76
RASSF1A M2		GTA CTT CGC TAA CTT TAA ACG	50340821 to 50340841	
RASSF1A M beacon		(HEX)CGC GAT TCG +TT+C G+GT TCG CTC GCG(DabcyI)	50340846 to 50340859	
RASSF1A Am		GGG AGG CGT TGA AGT C	50340882 to 50340897	115
RASSF1A Bm		A ATA AAC TCA AAC TCC CCC G	50340782 to 50340801	
CDKN2A M1	NC_000009.12	TTT CGA GTA TTC GTT TAT AGC	21975019 to 21975036	111
CDKN2A M2		TTT CTT CCT CCG ATA CTA ACG	21974925 to 21974945	
CDKN2A M beacon		(HEX)CGA CGT G+AA +AGA +TAT CG+C G+GT ACG TCG(DabcyI)	21974988 to 21975002	
CDKN2A Am		TGT TCG GAG TTA ATA GTA TTT TTT TC	21975033 to 21975058	133
CDKN2A Bm		TTT CTT CCT CCG ATA CTA ACG	21974925 to 21974945	
RARB M1	NC_000003.12	GGG TAT CGT CGG GGT AGA TTC	25428402 to 25428423	113
RARB M2		TCG ACC AAT CCA ACC GAA ACG	25428495 to 25428515	
RARB M beacon		(HEX)CGC GAC GAA +TA+C GTT +CCG AAT CGC G(DabcyI)	25428421 to 25428435	
RARB Am		AGT AGG GTT TGT TTG GGT ATC	25428388 to 25428408	127
RARB Bm		TCG ACC AAT CCA ACC GAA ACG	25428495 to 25428515	
ESR1 M1	NC_000006.12	GGG ATT GTA TTT GTT TTC GTC	151807705 to 151807725	104
ESR1 M2		ACG CAA CGC ATA TCC CG	151807793 to 151807809	
ESR1 M beacon		(HEX)CGC GAT GAA +CGA +CCC G+AC GAT CGC G(DabcyI)	151807722 to 151807735	
ESR1 Am		GTT TTG GGA TTG TAT TTG TTT TC	151807700 to 151807722	109
ESR1 Bm		ACG CAA CGC ATA TCC CG	151807793 to 151807809	
BRCA1 M1	NC_000017.11	TCG TGG TAA CGG AAA AGC GCG	43125409 to 43125429	83
BRCA1 M2		CCG TCC AAA AAA TCT CAA CG	43125346 to 43125365	
BRCA1 M beacon		(HEX)CGA TCG G+CG GCG +TG+A GCG ATC G(DabcyI)	43125362 to 43125371	
BRCA1 Am		GT TTT TTG GTT TTC GTG GTA AC	43125420 to 43125441	114
BRCA1 Bm		AAA CCC CAC AAC CTA TCC CCC G	43125327 to 43125348	
MESTV2 M1	NC_000007.14	CGA CGT TTT AGT TTC GAG TC	130486250 to 130486269	86
MESTV2 M2		CGC TTC CTA AAA CCA AAA ATT CTC G	130486312 to 130486336	
MESTV2 M beacon		(HEX)CGA TCG G+TG +GT+C G+GG TTC GAT CG(DabcyI)	130486278 to 130486289	
MESTV2 Am		GCG ATG GGT TTG TGC GC	130486225 to 130486242	130
MESTV2 Bm		GAA AAA CCG ATT ACG CAT ACG	130486337 to 130486355	
MGMT M1	NC_000010.11	GAT ATG TTG GGA TAG TTC GC	129467213 to 129467232	119
MGMT M2		GCA CTC TTC CGA AAA CGA AAC G	129467311 to 129467332	
MGMT M beacon		(HEX)CGC GAT CG+T ATC G+TT +TG+C GAT +TTA TCG CG(DabcyI)	129467279 to 129467294	
MGMT Am		GAT ATG TTG GGA TAG TTC GC	129467213 to 129467232	129
MGMT Bm		AAA AAA CTC CGC ACT TCC G	129467322 to 129467342	
SEPT9v2 M1	NC_000017.11	GTT TAG TAT TTA TTT TCG AAG TTC	77373542 to 77373560	

CELL-FREE DNA PROMOTER HYPERMETHYLATION AS BLOOD-BASED MARKERS FOR PANCREATIC ADENOCARCINOMA.

SEPT9v2 M2		CCTCCG CGC GAC CCG	77373467 to 77373481	91
SEPT9v2 M beacon		(FAM)CGA CGT ATT TAG TTG CGC GTT GAT CGA CGT CG(DabcyI)	77373511 to 77373530	
SEPT9v2 Am		GTT TAG TAT TTA TTT TCG AAG TTC	77373542 to 77373560	
SEPT9v2 Bm		GCC GAA AAC GCT TCC TCG	77373442 to 77373459	118
VIM M1	NC_000010.11	ATA TTT ATC GCG TTT TCG TTC	17229337 to 17229357	
VIM M2		ACG AAC CTA ATA AAC ATA ACT ACG	17229416 to 17229439	102
VIM M beacon		(FAM)CGA CGT GTT CGC GTT ATC GTC GAC GTC G(DabcyI)	17229377 to 17229395	
VIM Am		GAG GTT TTC GCG TTA GAG AC	17229296 to 17229315	
VIM Bm		ACG AAC CTA ATA AAC ATA ACT ACG	17229416 to 17229439	143
EYA2 M1	NC_000020.11	CGG AGG TAG CGG TAA C	46894866 to 46894881	
EYA2 M2		CGA TAC GAA CGA ACG AAC G	46894941 to 46894959	93
EYA2 M beacon		(FAM)CGC GAT TTC GGT TTC GTC GGA TTC GTA TCG CG(DabcyI)	46894914 to 46894933	
EYA2 Am		AGG AGG CGG AGG TAG C	46894860 to 46894875	
EYA2 Bm		CGA CGC GAT ACG AAC G	46894949 to 46894964	104
BMP3 M1	NC_000004.12	AGT GGA GAC GGC GTT C	81031024 to 81031039	
BMP3 M2		CTT ACT ACG CTA ACC CAA CG	81031101 to 81031120	96
BMP3 M beacon		(FAM)CGT CGA GCG GGT GAG GTT CGC GTA TCG ACG(DabcyI)	81031052 to 81031069	
BMP3 Am		TAG CGT TGG AGT GGA GAC	81031015 to 81031032	
BMP3 Bm		CCA ACC CCA CTT ACT ACG	81031112 to 81031129	114
ALX4 M1	NC_000011.10	TTT TTC GGA GGC GAT AAG TTC	44309934 to 44309954	
ALX4 M2		CGA ACC CGA CT TTA ACG	44309869 to 44309886	85
ALX4 M beacon		(FAM)CGC GAT TGT CGG TCG TCG TTA AAG TAT CGC G(DabcyI)	44309902 to 44309920	
ALX4 Am		GTC GGG AGG GTT CGT C	44309968 to 44309983	
ALX4 Bm		CGA ACC CGA CTC TTA ACG	44309869 to 44309886	114
SFRP2 M1	NC_000004.12	GTT TTT CGG AGT TGC GCG C	153789028 to 153789046	
SFRP2 M2		CCG AAA AAC TAA CAA CCG ACG	153788948 to 153788968	98
SFRP2 M beacon		(HEX)CGA CGT TTG TAG CGT TTC GTT CGC GTT GTT ACG TCG(DabcyI)	153789000 to 153789023	
SFRP2 Am		GTT TTT CGG AGT TGC GC GC	153789028 to 153789046	
SFRP2 Bm		CTC TTC GCT AAA TAC GAC TCG	153788922 to 153788942	124
NEUROG1 M1	NC_000005.10	GTT GAT TTT ATC GTC GGC	135535925 to 135535942	
NEUROG1 M2		CTC GCC TAC AAA AAC CAC G	135535879 to 135535897	63
NEUROG1 M beacon		(HEX)CGC GAT GCC C+GA CC+G ATC TCC TAA ATC GCG(DabcyI)	135535899 to 135535916	
NEUROG1 Am		GTT TAT ACG AGT TGA TTT GAT C	135535931 to 135535952	
NEUROG1 Bm		CTT AAC CTA ACC TCC TCG	135535860 to 135535882	92
NTPX2 M1	NC_000007.14	AGG TTA GAG TGT CGA GTA GC	98617280 to 98617299	
NTPX2 M2		TCG AAA ATC GCG TAC ACC G	98617342 to 98617360	80
NTPX2 M beacon		(HEX)CGC GAT CGG TG+C GGT TGT GAG A+CG GTG ATC GCG(DabcyI)	98617306 to 98617322	
NTPX2 Am		TTC GGT AGG TTA GAG TGT C	98617274 to 98617291	
NTPX2 Bm		CTA TCG TCT CGA AAA TCG CG	98617349 to 98617368	94
TFPI2 M1	NC_000007.14	TAT TTT TTA GGT TTC GTT TCG GC	93890809 to 93890831	
TFPI2 M2		AAA CGA CCC GAA TAC CCG	93890759 to 93890776	72
TFPI2 M beacon		(HEX)CGC GAT CGT CGG T+CG GA+C GTT CGT TGA TCG CG(DabcyI)	93890787 to 93890804	
TFPI2 Am		TAT TTT TTA GGT TTC GTT TCG GC	93890809 to 93890831	
TFPI2 Bm		CGA CTT TCT ACT CCA AAC G	93890745 to 93890763	86
BNC1 M1	NC_000015.10	GTA GGT AGT TAG TTG GTT TTC	83284403 to 83284423	
BNC1 M2		GAA ACA AAC GAC CCG AAA CG	83284467 to 83284486	83
BNC1 M beacon		(FAM)CGC GAT CGT ATT TA+C GGG AGT +CGG AGT TTG ATC GCG(DabcyI)	83284440 to 83284461	
BNC1 Am		GTA GGT AGT TAG TTG GTT TTC	83284403 to 83284423	
BNC1 Bm		GCG AAA ATT CTC TAT ACG	83284491 to 83284505	102
CDKN2B M1	NC_000009.12	TAT TGT ACG GGG TTT TAA GTC	22009107 to 22009127	
CDKN2B M2		TTC CCT TCT TTC CCA CG	22009019 to 22009035	108
CDKN2B M beacon		(HEX)CGC GAT CGA +CGA +CGG GAG GGT AAT GGA TCG CG(DabcyI)	22009082 to 22009099	
CDKN2B Am		GGT CGT TCG GTT ATT GTA C	22009120 to 22009138	
CDKN2B Bm		TTC CCT TCT TTC CCA CG	22009019 to 22009035	119
WNT5A M1	NC_000003.12	CGT GGA ATA GTT GTT TGC	55487294 to 55487311	
WNT5A M2		TTA AAA CAA AAC TAA AAT ACG	55487177 to 55487197	134
WNT5A M beacon		(HEX)CGC GAT CAA CCT AAT C+GA AAC +GCA ACT AAA GAT CGC G(DabcyI)	55487247 to 55487269	
WNT5A Am		CGT GGA ATA GTT GTT TGC	55487294 to 55487311	
WNT5A Bm		CGA ACC TAA ACT CCC G	55487159 to 55487174	152
PENK M1	NC_000008.11	AGG CGA TTT GAG TCG TTT TTA C	56446123 to 56446144	
PENK M2		GAC AAC CTC AAC AAA AAA TCG	56446032 to 56446052	112
PENK M beacon		(HEX)CGC GAT CAA AGT TGT +CGG T+CG GGA GG ATC GCG(DabcyI)	56446096 to 56446113	
PENK Am		CGG GTT ATT TCG GGA ATC	56446148 to 56446165	
PENK Bm		GAC AAC CTC AAC AAA AAA TCG	56446032 to 56446052	133
H1C1 M1	NC_000017.11	TTC GGT TTT CGC GTT TTG TTC	2056080 to 2056100	
H1C1 M2		CGA AAA CTA TCA ACC CTC G	2056153 to 2056171	91
H1C1 M beacon		(FAM)CGC GAC GGT CGT CGT TCG GGT TCG CG (DabcyI)	2056131 to 2056146	
H1C1 Am		GAT ATA ACG TTT TTT TCG CGT C	2056054 to 2056075	
H1C1 Bm		ATA CCC GCC CTA ACG CCG	2056179 to 2056196	142
GSTP1 M1	NC_000011.10	TCG GGG TGT AGC GGT C	67583673 to 67583688	
GSTP1 M2		CCC AAT ACT AAA TCA CGA CG	67583741 to 67583760	87
GSTP1 M beacon		(HEX)CGCGAT GTC G+G+C GGG AGT TCG ATC GCG (DabcyI)	67583701 to 67583715	
GSTP1 Am		AGG GCG TTT TTT TGC GGT C	67583649 to 67583667	
GSTP1 Bm		CCC AAT ACT AAA TCA CGA CG	67583741 to 67583760	111
*MESTV1 M1	NC_000007.14	CGG GGT AAT TAG TAT ATT TC	130492085 to 130492107	
*MESTV1 M2		GCT ACG ACA CTA CGC TTA CG	130492135 to 130492159	74

APPENDIX A.

*MESTv1 M beacon	(HEX)CGC GAT CGG +TA+G T+TG +CGT TAT CGC G(Dabcyl)	130492121 to 130492133	
*MESTv1 U1	TGT TGT GGT AAT TAG TAT ATT TT	130492088 to 130492107	
*MESTv1 U2	CAA CCA CTC CAA CAT ACA CTA CA	130492154 to 130492171	83
*MESTv1 U beacon	(FAM)CGC GAG +TA+G T+TG +TG+T TT+T GTT CGC G(Dabcyl)	130492123 to 130492137	
**MESTv1 A	GGT TTT AAA AGT T/CGG TGT TTA TT	130492052 to 130492074	
**MESTv1 B	CCI AAC AAC TAC AAC CAC TCC	130492162 to 130492182	130

a. * Hemimethylated reference gene MEST transcript variant 1

b. ** Un-methylated primer for the reference gene MEST transcript variant 1

M1: Methylation-specific forward primer for the array (inner primer)

M2: Methylation-specific reverse primer for the array (inner primer)

M beacon: Methylation-specific probe

Am: Methylation-specific forward primer for the nested/semi-nested PCR (outer primer/first round of PCR)

Bm: Methylation-specific reverse primer for the nested/semi-nested PCR (outer primer/first round of PCR)

Appendix B.

Characteristics of genes used in the gene panel

<i>Gene</i>	<i>Mechanism of action</i>
<i>ALX4</i>	<ul style="list-style-type: none"> - Expressed in the mesenchymal cells of developing bones, limbs, hair, teeth, and mammary tissue. - May be involved in the epithelial to mesenchymal transition in cancer.
<i>APC</i>	<ul style="list-style-type: none"> - Encodes a tumour suppressor protein that acts as an antagonist of the Wnt/β-catenin-pathway. - Involved in cell migration, adhesion, transcriptional activation, apoptosis and angiogenesis. - Defects in the gene cause familial adenomatous polyposis coli.
<i>BMP3</i>	<ul style="list-style-type: none"> - Encodes a protein belonging to the TGF-β superfamily, which can bind to TGF-β receptors, leading to recruitment and activation of SMAD family transcription factors and, regulating gene expression. - Induces bone formation.
<i>BNC1</i>	<ul style="list-style-type: none"> - Encodes a zinc finger protein present in the basal cell layer of the epidermis and in hair follicles. - Regulates keratinocyte proliferation. - May be a regulator of rRNA transcription.
<i>BRCA1</i>	<ul style="list-style-type: none"> - Encodes a nuclear phosphoprotein that plays a role in maintaining genomic stability. - Acts as a tumour suppressor. - Is part of the BRCA1-associated genome surveillance complex, which is associated with RNA polymerase II and interacts with histone deacetylase complexes. - Plays a role in transcription and DNA repair. - Mutations in BRCA1 are involved in inherited breast and ovarian cancers.
<i>CDKN2A</i>	<ul style="list-style-type: none"> - Encodes tumour suppressor proteins: P16 and P14. - P16 is an inhibitor of CDK4 and CDK6 that indirectly prevents phosphorylation of the retinoblastoma protein and consequently arrests the cell cycle. - P14 is an ARF product that functions as a stabilizer of the tumour suppressor protein p53.
<i>CDKN2B</i>	<ul style="list-style-type: none"> - The gene lies adjacent to the tumour suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumours. - Encodes a cyclin-dependent kinase inhibitor that forms a complex with CDK4 or CDK6 and prevents the activation of CDK kinases. - The protein is a cell growth regulator that controls cell cycle G1 progression.
<i>CHFR</i>	<ul style="list-style-type: none"> - Encodes an E3 ubiquitin-protein ligase. - Is involved in regulating cell cycle entry into mitosis.
<i>ESR1</i>	<ul style="list-style-type: none"> - Encodes an oestrogen receptor involved in DNA binding and activation of transcription. - Oestrogen receptors are involved in breast cancer, endometrial cancer, and osteoporosis. - May be involved in angiogenesis and lymphangiogenesis.
<i>EYA2</i>	<ul style="list-style-type: none"> - Encodes a member of the eyes absent (EYA) family of proteins. - The protein may play a role in eye development. - May act as a transcriptional activator.
<i>GSTP1</i>	<ul style="list-style-type: none"> - Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification. - GSTP1 proteins are thought to play a role in susceptibility to cancer and other diseases.
<i>HIC1</i>	<ul style="list-style-type: none"> - Encodes a transcriptional repressor. - Is involved in the TGF-β signalling regulation of angiogenesis in cancer. - Hypermethylation or deletion has been associated with different tumours.

CELL-FREE DNA PROMOTER HYPERMETHYLATION AS BLOOD-BASED MARKERS FOR PANCREATIC ADENOCARCINOMA.

<i>MGMT</i>	-	Encodes an enzyme involved in DNA repair.
	-	Cells lacking MGMT expression have induced angiogenic expression.
<i>MEST</i>	-	Encodes a member of the alpha/beta hydrolase superfamily.
	-	Is imprinted, exhibiting preferential expression from the parental allele in foetal tissue and isoform-specific imprinting in lymphocytes.
	-	The loss of imprinting of this gene has been linked to certain types of cancer and may be due to promoter switching.
<i>MLH1</i>	-	Encodes a protein involved in the DNA mismatch repair system.
	-	Is also involved in DNA damage signalling, a process that induces cell cycle arrest and can lead to apoptosis in case of major DNA damages.
<i>NEUROG1</i>	-	Encodes a transcriptional regulator involved in neuronal differentiation.
	-	Is involved in the regulation of the Wnt/ β -catenin pathway and target gene transcription.
<i>NPTX2</i>	-	Encodes a member of the neuronal pentraxins.
	-	The protein is related to C-reactive protein.
	-	Plays a role in excitatory synapse formation.
	-	The protein is upregulated in Parkinson's disease.
<i>PENK</i>	-	Encodes a preproprotein that is processed to multiple protein products, including Met- and Leu-enkephalins.
	-	Mimics the effects of opiates by binding to opioid receptors
<i>RARB</i>	-	Encodes a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators.
	-	The receptor binds retinoic acid, regulating cell growth and differentiation.
<i>RASSF1A</i>	-	Encodes a tumour suppressor protein.
	-	Involved in DNA repair, cell cycle control and apoptosis.
<i>SEPT9</i>	-	Encodes a tumour suppressor protein that is a member of the septin family.
	-	Involved in cytokinesis, cell cycle control, cell division and angiogenesis.
<i>SFRP1</i>	-	Encodes a member of the SFRP family.
	-	Modulators of the Wnt/ β -catenin pathway.
<i>SFRP2</i>	-	Encodes a member of the SFRP family.
	-	Modulators of the Wnt/ β -catenin pathway.
<i>SST</i>	-	Encodes the hormone somatostatin.
	-	Somatostatin is expressed throughout the body and inhibits the release of numerous secondary hormones by binding to somatostatin receptors.
	-	The hormone is an important regulator of the endocrine system.
<i>TAC1</i>	-	Encodes four products of the tachykinin peptide hormone family: substance P, neurokinin A, neuropeptide K, and neuropeptide γ .
	-	Acts as a neurotransmitter that interact with nerve receptors and smooth muscle cells.
<i>TFPI2</i>	-	Encodes a member of the kunitz-type serine proteinase inhibitor family.
	-	The protein can inhibit a variety of serine proteases including factor VIIa/tissue factor, factor Xa, plasmin, trypsin, chymotrypsin and plasma kallikrein. Involved in angiogenesis.
	-	Is as a tumour suppressor gene in several types of cancer.
<i>VIM</i>	-	Encodes a member of the intermediate filament family.
	-	Is involved in maintaining cell shape and integrity of the cytoplasm and stabilizing the cytoskeleton.
	-	It functions as an organizer of numerous of critical proteins involved in attachment, migration, and cell signalling.
<i>WNT5A</i>	-	Encodes a member of the Wnt/ β -catenin pathway.

Gene functions are cross-matched with the ref-seq database on www.ncbi.gov

Appendix C.

Hypermethylation of each gene by patient group

Gene	Pancreatic cancer (N = 95)			Screened negative (N = 27)			Chronic pancreatitis (N = 97)			Acute pancreatitis (N = 59)		
	n	%	95% CI	n	%	95% CI	n	%	95% CI	n	%	95% CI
<i>ALX4</i>	17	17.84	(10.78-27.10)	2	7.41	(0.91-24.29)	4	4.12	(1.13-10.22)	1	1.69	(0.04-9.09)
<i>APC</i>	78	82.11	(72.90-89.22)	12	44.44	(25.48-64.67)	53	54.64	(44.21-64.78)	40	67.80	(54.36-79.38)
<i>BMP3</i>	32	33.68	(24.31-44.11)	5	18.52	(6.30-38.08)	3	3.09	(0.64-8.77)	6	10.17	(3.82-20.8)
<i>BNC1</i>	34	35.79	(26.21-46.30)	2	7.41	(0.91-24.29)	5	5.15	(1.69-11.62)	4	6.78	(1.88-16.46)
<i>BRCA1</i>	10	10.53	(5.16-18.51)	4	14.81	(4.19-33.73)	7	7.22	(2.95-14.30)	19	32.20	(20.62-45.64)
<i>CDKN2A</i>	6	6.32	(2.35-13.24)	1	3.70	(0.09-18.97)	2	2.06	(0.25-7.25)	7	11.86	(4.91-22.93)
<i>CDKN2B</i>	12	12.63	(6.70-21.03)	2	7.41	(0.91-24.29)	5	5.15	(1.69-11.62)	7	11.86	(4.91-22.93)
<i>CHFR</i>	1	1.05	(0.03-5.73)	0	0	(0.00-12.77)	3	3.09	(0.64-8.77)	1	1.69	(0.04-9.09)
<i>ESR1</i>	74	77.89	(68.21-85.77)	17	62.96	(42.37-80.60)	59	60.82	(50.39-70.58)	45	76.27	(63.41-86.38)
<i>EYA2</i>	13	13.68	(7.49-22.26)	0	0	(0.00-12.77)	8	8.25	(3.63-15.61)	9	15.25	(7.22-26.99)
<i>GSTP1</i>	3	3.16	(0.66-8.95)	0	0	(0.00-12.77)	1	1.03	(0.03-5.61)	0	0	(0-6.06)
<i>HIC1</i>	15	15.79	(9.12-24.70)	0	0	(0.00-12.77)	6	6.19	(2.30-12.98)	4	6.78	(1.88-16.46)
<i>MESTv2</i>	75	78.95	(69.38-86.64)	12	44.44	(25.48-64.67)	57	58.76	(48.31-68.67)	39	66.10	(52.61-77.92)
<i>MGMT</i>	5	5.26	(1.73-11.86)	0	0	(0.00-12.77)	3	3.09	(0.64-8.77)	0	0	(0-6.06)
<i>MLH1</i>	14	14.74	(8.30-23.49)	6	22.22	(8.62-42.26)	7	7.22	(2.95-14.30)	17	28.81	(17.76-42.07)
<i>NPTX2</i>	71	74.74	(64.78-83.10)	17	62.96	(42.37-80.60)	41	42.27	(32.30-52.72)	29	49.15	(35.89-62.50)
<i>NEUROG1</i>	10	10.53	(5.16-18.51)	3	11.11	(2.35-29.16)	6	6.19	(2.30-12.98)	4	6.78	(1.88-16.46)
<i>RARB</i>	44	46.32	(36.02-56.85)	12	44.44	(25.48-64.67)	28	28.87	(20.11-38.95)	27	45.76	(32.72-59.24)
<i>RASSF1A</i>	40	42.11	(32.04-52.67)	4	14.81	(4.19-33.73)	11	11.34	(5.80-19.39)	10	16.95	(8.44-28.97)
<i>SFRP1</i>	42	44.21	(34.02-54.77)	7	25.93	(11.11-46.28)	17	17.53	(10.55-26.57)	11	18.64	(9.69-30.91)
<i>SFRP2</i>	37	38.95	(29.11-49.50)	5	18.52	(6.30-38.08)	25	25.77	(17.42-35.65)	4	6.78	(1.88-16.46)
<i>SEPT9v2</i>	14	14.74	(8.30-23.49)	0	0	(0.00-12.77)	3	3.09	(0.64-8.77)	1	1.69	(0.04-9.09)
<i>SST</i>	61	64.21	(53.72-73.79)	16	59.26	(38.80-77.61)	30	30.93	(21.93-41.12)	15	25.42	(14.98-38.44)
<i>TFPI2</i>	22	23.16	(15.12-32.94)	1	3.70	(0.09-18.97)	2	2.06	(0.25-7.25)	0	0	(0-6.06)
<i>TAC1</i>	56	58.95	(48.38-68.94)	4	14.81	(4.19-33.73)	34	35.05	(25.64-45.41)	15	25.42	(14.98-38.44)
<i>VIM</i>	3	3.16	(0.66-8.95)	0	0	(0.00-12.77)	0	0	(0-3.73)	0	0	(0-6.06)
<i>WNT5A</i>	8	8.42	(3.71-15.92)	0	0	(0.00-12.77)	1	1.03	(0.03-5.61)	0	0	(0-6.06)
<i>PENK</i>	2	2.11	(0.26-7.40)	0	0	(0.00-12.77)	0	0	(0-3.73)	0	0	(0-6.06)

CI: Confidence interval.

Appendix D.

Hypermethylation of each gene by cancer stage

Gene	Stage I (N = 11)			Stage II (N = 29)			Stage III (N = 13)			Stage IV (N = 42)		
	n	%	95%CI	n	%	95%CI	n	%	95%CI	n	N	95%CI
<i>ALX4</i>	1	9.09	(0.23-41.28)	2	6.90	(0.85-22.77)	0	0	(0.00-24.71)	14	33.33	(19.57-49.55)
<i>APC</i>	8	72.73	(39.03-93.98)	24	82.76	(64.23-94.15)	10	76.92	(46.19-94.96)	36	85.71	(71.46-94.57)
<i>BMP3</i>	1	9.09	(0.23-41.28)	9	31.03	(15.28-50.83)	5	38.46	(13.86-68.42)	17	40.48	(25.63-56.72)
<i>BNC1</i>	1	9.09	(0.23-41.28)	5	17.24	(5.85-35.77)	4	30.77	(9.09-61.43)	24	57.14	(40.96-72.28)
<i>BRCA1</i>	1	9.09	(0.23-41.28)	4	13.79	(3.89-31.66)	2	15.38	(1.92-45.45)	3	7.14	(1.50-19.48)
<i>CDKN2A</i>	1	9.09	(0.23-41.28)	0	0.00	(0.00-11.94)	0	0	(0.00-24.71)	5	11.90	(3.98-25.63)
<i>CDKN2B</i>	2	18.18	(2.28-51.78)	5	17.24	(5.85-35.77)	2	15.38	(1.92-45.45)	3	7.14	(1.50-19.48)
<i>CHFR</i>	0	0.00	(0.00-28.49)	1	3.45	(0.09-17.76)	0	0	(0.00-24.71)	0	0.00	(0.00-8.41)
<i>ESR1</i>	7	63.64	(30.79-89.07)	21	72.41	(52.76-87.27)	11	84.62	(54.55-98.08)	35	83.33	(68.64-93.03)
<i>EYA2</i>	2	18.18	(2.28-51.78)	4	13.79	(3.89-31.66)	1	7.69	(0.19-36.03)	6	14.29	(5.43-28.54)
<i>GSTP1</i>	0	0.00	(0.00-28.49)	0	0.00	(0.00-11.94)	1	7.69	(0.19-36.03)	2	4.76	(0.58-16.16)
<i>HIC1</i>	0	0.00	(0.00-28.49)	4	13.79	(3.89-31.66)	0	0	(0.00-24.71)	11	26.19	(13.86-42.04)
<i>MESTv2</i>	8	72.73	(39.03-93.98)	24	82.76	(64.23-94.15)	9	69.23	(38.57-90.91)	34	80.95	(65.88-91.40)
<i>MGMT</i>	1	9.09	(0.23-41.28)	0	0.00	(0.00-11.94)	1	7.69	(0.19-36.03)	3	7.14	(1.50-19.48)
<i>MLH1</i>	0	0.00	(0.00-28.49)	3	10.34	(2.19-27.35)	3	23.08	(5.04-53.81)	8	19.05	(8.60-34.40)
<i>NPTX2</i>	9	81.82	(48.22-97.72)	19	65.52	(45.67-82.06)	8	61.54	(31.58-86.14)	35	83.33	(68.64-93.03)
<i>NEUROG1</i>	1	9.09	(0.23-41.28)	1	3.45	(0.09-17.76)	1	7.69	(0.19-36.03)	7	16.67	(6.97-31.36)
<i>RARB</i>	5	45.45	(16.75-76.62)	13	44.83	(26.45-64.31)	5	38.46	(13.86-68.42)	21	50.00	(34.19-65.81)
<i>RASSF1A</i>	5	45.45	(16.75-76.62)	10	34.48	(17.94-54.33)	6	46.15	(19.22-74.87)	19	45.24	(29.85-61.33)
<i>SFRP1</i>	4	36.36	(10.93-69.21)	12	41.38	(23.52-61.06)	4	30.77	(9.09-61.43)	22	52.38	(36.42-68.00)
<i>SFRP2</i>	4	36.36	(10.93-69.21)	10	34.48	(17.94-54.33)	2	15.38	(1.92-45.45)	21	50.00	(34.19-65.81)
<i>SEPT9v2</i>	0	0.00	(0.00-28.49)	1	3.45	(0.09-17.76)	0	0	(0.00-24.71)	13	30.95	(17.62-47.09)
<i>SST</i>	6	54.55	(23.38-83.25)	15	51.72	(32.53-70.55)	5	38.46	(13.86-68.42)	35	83.33	(68.64-93.03)
<i>TFPI2</i>	4	36.36	(10.93-69.21)	2	6.90	(0.85-22.77)	1	7.69	(0.19-36.03)	15	35.71	(21.55-51.97)
<i>TAC1</i>	5	45.45	(16.75-76.62)	14	48.28	(29.45-67.47)	5	38.46	(13.86-68.42)	32	76.19	(60.55-87.95)
<i>VIM</i>	1	9.09	(0.23-41.28)	0	0.00	(0.00-11.94)	0	0	(0.00-24.71)	2	4.76	(0.58-16.16)
<i>WNT5A</i>	1	9.09	(0.23-41.28)	0	0.00	(0.00-11.94)	2	15.38	(1.92-45.45)	5	11.90	(3.98-25.63)
<i>PENK</i>	0	0	(0.00-28.49)	0	0.00	(0.00-11.94)	0	0	(0.00-24.71)	2	4.76	(0.58-16.16)

CI: Confidence interval.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

Appendix E.

Hazard ratio for each gene based on univariate Cox regression analysis

Gene	All stages (N = 95)			Stage I/II (N = 40)			Stage III (N = 13)			Stage IV (N = 42)		
	HR	P-value	95% CI	HR	P-value	95% CI	HR	P-value	95% CI	HR	P-value	95% CI
<i>ALX4</i>	1.43	0.20	(0.83-2.47)	0.82	0.78	(0.19-3.43)	1.00	-	-	0.96	0.91	(0.50-1.86)
<i>APC</i>	0.99	0.97	(0.58-1.70)	0.88	0.76	(0.38-2.01)	0.40	0.21	(0.09-1.69)	1.34	0.51	(0.56-3.19)
<i>BMP3</i>	1.41	0.13	(0.91-2.18)	0.80	0.59	(0.37-1.77)	0.71	0.58	(0.21-2.41)	3.21	0.00	(1.58-6.53)
<i>BNC1</i>	2.10	0.00	(1.36-3.25)	1.26	0.61	(0.52-3.06)	1.93	0.30	(0.55-6.75)	1.69	0.11	(0.88-3.21)
<i>BRCA1</i>	0.76	0.44	(0.38-1.52)	0.88	0.82	(0.31-2.52)	0.00	1.00	-	2.42	0.16	(0.70-8.34)
<i>CDKN2B</i>	0.80	0.49	(0.42-1.51)	0.79	0.59	(0.33-1.90)	1.18	0.84	(0.25-5.63)	1.82	0.33	(0.55-6.03)
<i>CHFR</i>	0.38	0.34	(0.05-2.76)	0.53	0.53	(0.07-3.90)	1.00	-	-	1.00	-	-
<i>ESR1</i>	1.21	0.45	(0.74-1.99)	0.89	0.75	(0.44-1.82)	0.68	0.64	(0.14-3.40)	1.27	0.57	(0.56-2.89)
<i>EYA2</i>	1.41	0.26	(0.78-2.55)	1.93	0.15	(0.79-4.71)	0.54	0.57	(0.07-4.37)	1.31	0.54	(0.55-3.16)
<i>GSTP1</i>	6.91	0.00	(2.08-22.96)	1.00	-	-	*	1.00	-	2.33	0.26	(0.54-9.99)
<i>HIC1</i>	1.37	0.27	(0.78-2.39)	1.49	0.46	(0.51-4.34)	1.00	-	-	0.92	0.82	(0.45-1.88)
<i>MEST1v2</i>	1.45	0.16	(0.86-2.45)	1.97	0.13	(0.81-4.79)	1.88	0.36	(0.49-7.22)	1.21	0.63	(0.56-2.64)
<i>MGMT</i>	2.21	0.09	(0.88-5.54)	3.02	0.29	(0.39-23.38)	0.71	0.75	(0.09-5.71)	3.45	0.06	(0.96-12.44)
<i>MLH1</i>	1.85	0.04	(1.03-3.32)	1.54	0.49	(0.46-5.18)	0.95	0.94	(0.24-3.70)	1.79	0.15	(0.81-3.96)
<i>NPTX2</i>	1.05	0.85	(0.65-1.68)	1.12	0.75	(0.55-2.29)	0.70	0.55	(0.22-2.26)	0.62	0.26	(0.27-1.42)
<i>NEUROG1</i>	1.41	0.32	(0.72-2.74)	2.51	0.22	(0.57-11.00)	0.38	0.37	(0.05-3.13)	0.85	0.70	(0.38-1.93)
<i>RARB</i>	1.07	0.73	(0.71-1.62)	1.03	0.93	(0.53-1.99)	1.64	0.42	(0.49-5.43)	0.98	0.95	(0.53-1.82)
<i>RASSF1A</i>	1.30	0.22	(0.86-1.97)	1.35	0.39	(0.68-2.68)	1.08	0.90	(0.34-3.49)	1.33	0.38	(0.70-2.51)
<i>SFRP1</i>	2.11	0.00	(1.38-3.23)	1.60	0.17	(0.82-3.13)	3.50	0.08	(0.86-14.22)	4.57	0.00	(2.02-10.34)
<i>SFRP2</i>	0.73	0.17	(0.46-1.14)	0.31	0.01	(0.14-0.71)	2.47	0.28	(0.48-12.86)	1.08	0.81	(0.58-2.02)
<i>SEPT9v2</i>	2.37	0.00	(1.32-4.27)	3.37	0.25	(0.43-26.37)	1.00	-	-	1.22	0.55	(0.63-2.38)
<i>SST</i>	1.63	0.03	(1.06-2.51)	1.15	0.67	(0.60-2.23)	2.44	0.15	(0.72-8.33)	1.67	0.23	(0.73-3.80)
<i>TFPI2</i>	2.22	0.00	(1.34-3.68)	1.39	0.50	(0.53-3.63)	5.48	0.17	(0.50-60.52)	2.59	0.01	(1.25-5.39)
<i>TAC1</i>	1.44	0.09	(0.95-2.20)	1.06	0.87	(0.55-2.04)	1.28	0.69	(0.37-4.45)	1.69	0.16	(0.81-3.52)
<i>VIM</i>	1.55	0.46	(0.49-4.94)	1.20	0.86	(0.16-8.94)	1.00	-	-	1.89	0.39	(0.45-8.00)
<i>WNT5A</i>	2.32	0.03	(1.09-4.94)	3.02	0.29	(0.39-23.38)	7.05	0.05	(0.97-51.19)	1.05	0.91	(0.41-2.72)
<i>CDKN2A</i>	1.71	0.22	(0.73-3.97)	9.24	0.05	(1.03-82.68)	1.00	-	-	0.76	0.56	(0.29-1.95)
<i>PENK</i>	2.03	0.33	(0.49-8.40)	1.00	-	-	1.00	-	-	0.96	0.95	(0.23-4.02)

Variable analyzed by simple Cox regression analysis.

Bold marks the genes with a statistically significant HR.

HR: Hazard ratio.

CI: Confidence interval.

Note: Stage is in accordance with The American Joint Committee on Cancer (AJCC) stage classification.

*One patients with stage III disease had hypermethylation of *GSTP1*. This patient died only eight days after the diagnosis, resulting in a HR of 19.32×10^{16} (p-value = 1) for *GSTP1* hypermethylation in stage III disease.

Published papers and submitted manuscripts

- I. DNA Hypermethylation as a Blood-Based Marker for Pancreatic Cancer: A Literature Review. *Pancreas*, 2015, Vol 44, p1036-1045.

- II. Cell-free DNA Promoter Hypermethylation in Plasma as a Diagnostic Marker for Pancreatic Adenocarcinoma. *Clinical Epigenetics*, 2016, Vol 8, p 117.

- III. Promoter Hypermethylation in Plasma-Derived Cell-Free DNA as a Prognostic Marker for Pancreatic Adenocarcinoma Staging. *Submitted for publication, International Journal of Cancer*, November 2016.

- IV. Cell-Free DNA Promoter Hypermethylation in Plasma as a Predictive Marker for Survival of Patients with Pancreatic Adenocarcinoma. *Submitted for publication, Oncotarget*, December 2016.

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