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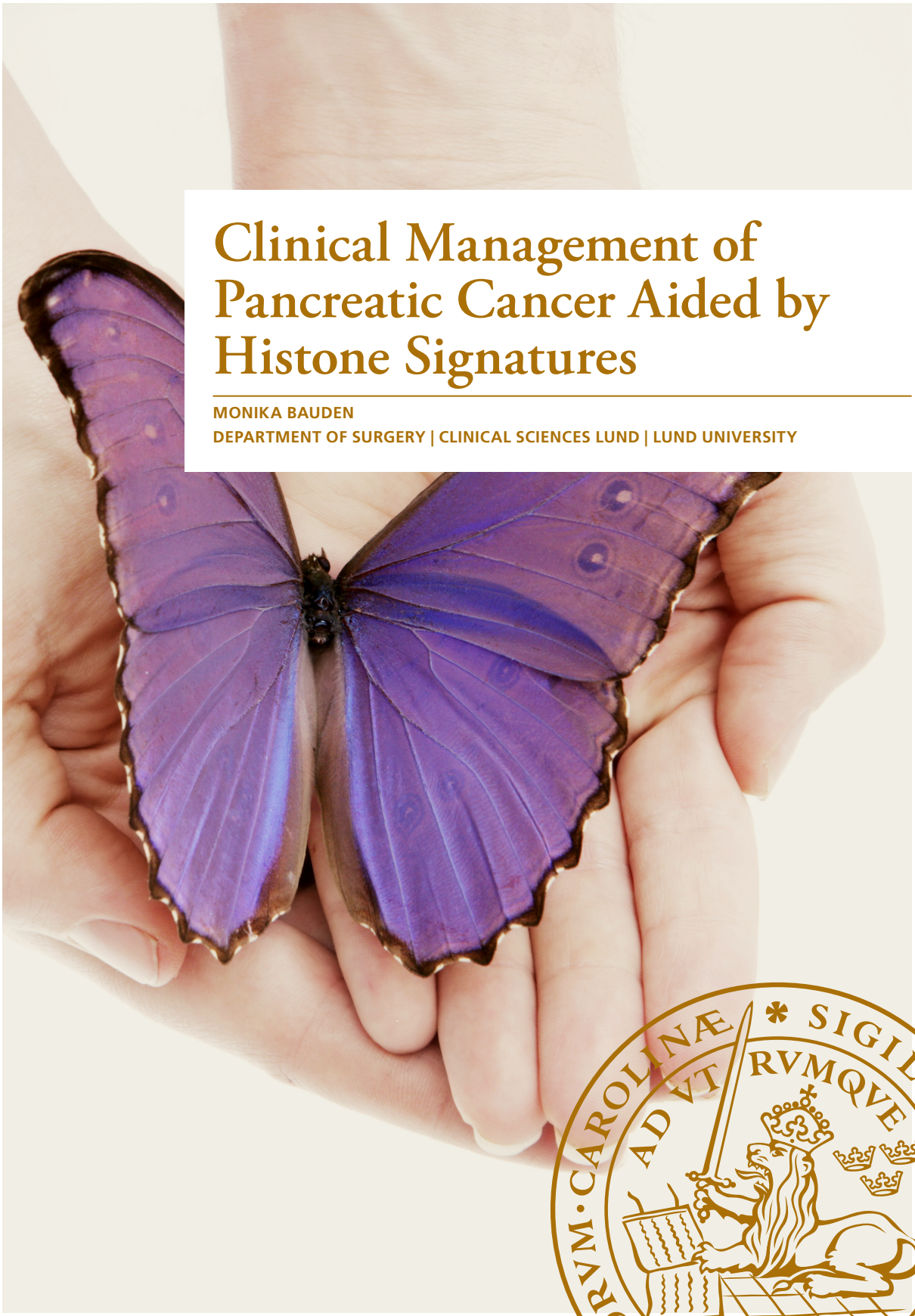

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Monika Bauden

LUND
UNIVERSITY

DOCTORAL DISSERTATION
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Faculty opponent
Professor Malin Sund, M.D. Ph.D.
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Title : **Clinical management of pancreatic cancer aided by histone signatures**

Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is considered as one of the most aggressive cancers. Despite its relatively low incidence, the prognosis is extremely poor, with a 5-year survival rate less than 7 %. In Sweden, around 1300 patients are diagnosed with PDAC each year and the incidence is projected to increase during the next decades. Compared to other cancers, the survival rate for patients with PDAC has not significantly improved over the past 50 years. Despite extensive research, there is still an unmet need to identify new PDAC specific biomarkers with a robust diagnostic, predictive or prognostic potential that could contribute to a reliable diagnosis or aid in the choice of treatment. Epigenetic modifications have long been recognized as significant contributors to the pathogenesis of PDAC and hence offer new opportunities for detecting and managing the disease.

Aims: The first part of the thesis was aimed to re-evaluate the significance of tumor size for predicting clinical outcome. The main objective of the thesis was to identify and develop nucleosome associated epigenetic biomarkers in pancreatic cancer for diagnostic or prognostic purposes.

Methods: In summary, methodologies employed in this thesis included liquid chromatography-tandem mass spectrometry (LC-MS/MS) in order to profile histone variants and histone-related modifications in patient material and normal pancreas. Immunohistochemistry (IHC) was used as a complementary method to provide further insight regarding the distribution of the PDAC specific histone marker candidate in tumor tissue. ELISA-based Nucleosomics® was used to investigate the potential of the epigenetic panel to improve the serum diagnosis of pancreatic cancer.

Results and Conclusions: The results from paper I confirmed that the probability of metastatic spread increases with larger primary tumor size. However, even small pancreatic tumors possess a high metastatic capacity. The prognostic impact of tumor size was restricted solely to patients with localized disease.

The results from paper II indicated that a panel of cell free serum nucleosomes associated with epigenetic markers may discriminate pancreatic cancer from healthy controls and patients with benign pancreatic disease. The epigenetic profile of nucleosomes in serum detected by non-invasive NuQ® immuno-assay may thus represent a new strategy for the diagnosis of pancreatic cancer.

The results from paper III suggested that formalin-fixed paraffin-embedded (FFPE) tissue processing may result in irreversible chemical modifications of histone proteins which correspond in mass shift of important post translational modifications (PTMs). A careful selection of experimental material prior to investigational mass spectral analysis, as well as manual examination of detected modifications should be made, to eliminate an incorrect interpretation of the data and false positive results.

A profile of differentially expressed histone protein variants was identified in fresh-frozen PDAC specimens and presented in paper IV. The obtained results indicated that the expression of histone variant H1.3 in PDAC tumors was associated with poor survival, and H1.3 may thus be considered as a tissue specific prognostic factor.

Key words: pancreatic ductal adenocarcinoma, epigenetic biomarkers, prognostic factors, LC-MS/MS		
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Till mina tjejer, Erika och Inez

“Started from the bottom, now we’re here”

— Drake —

Content

List of papers

Following papers are included in the thesis and will be referred to in the text by the corresponding Roman numerals.

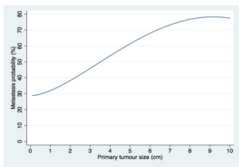
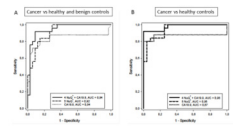
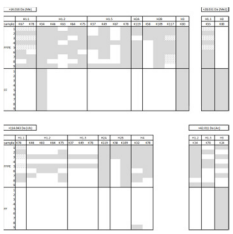
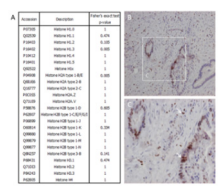
I. Ansari D, **Bauden M**, Bergström S, Rylance R, Marko-Varga G, Andersson R. Relationship between tumour size and outcome in pancreatic ductal adenocarcinoma. Br J Surg 2017;104:600-607.

II. **Bauden M**, Pamart D, Ansari D, Herzog M, Eccleston M, Micallef J, Andersson B, Andersson R. Circulating nucleosomes as epigenetic biomarkers in pancreatic cancer. Clin Epigenetics 2015;7:106.

III. **Bauden M**, Kristl T, Andersson R, Marko-Varga G, Ansari D. Characterization of histone-related chemical modifications in formalin-fixed paraffin-embedded and fresh-frozen human pancreatic cancer xenografts using LC-MS/MS. Lab Invest 2016;97:279-288.

IV. **Bauden M**, Kristl T, Sasor A, Andersson B, Marko-Varga G, Andersson R, Ansari D. Histone profiling reveals the H1.3 histone variant as a prognostic biomarker for pancreatic ductal adenocarcinoma. BMC Cancer 2017; In press.

Thesis at a glance

Study	Aim of the study	Methods	Conclusion
I 	To evaluate the relationship between PDAC tumor size and risk of metastasis and death at a population level.	An analysis of 58 728 patients with PDAC from the Surveillance, Epidemiology and End Results (SEER) database was conducted.	Pancreatic cancer possesses a high metastatic capacity even in small tumors. The prognostic impact of tumor size is restricted to patient with localized disease.
II 	To investigate the diagnostic potential of nine nucleosomal markers associated with epigenetic onco-modifications using novel NuQ®- assays platform.	A NuQ®- assay platform was used to evaluate serum samples from patients with resectable pancreatic cancer, benign pancreatic disease, and healthy individuals.	Epigenetic profiling of cf-nucleosomes in serum detected by a non-invasive NuQ® immuno-assay may provide a novel tool for the diagnosis of pancreatic cancer.
III 	To investigate the suitability of differently preserved biobank-archived tissues for analysis of post-translational modifications (PTMs) important in pancreatic cancer progression.	The mass spectrum of histone proteins extracted from nine human pancreatic tumor xenografts, preserved as FFPE and FF tissue samples, were scanned for modifications with a defined mass shift of +14.016 Da, +28.031 Da, +42.011 Da or +114.043 Da, corresponding to important PTMs.	FFPE tissue processing may result in irreversible chemical modifications of histone proteins which correspond to a mass shift of important PTMs and may thus be incorrectly interpreted as endogenously created post-translational modifications.
IV 	To profile histone variants and histone related modifications in pancreatic tumor tissue in order to elucidate their diagnostic or prognostic potential.	LC-MS/MS was used to assess the profile of histone variants and histone related PTMs from PDAC tissue and normal pancreas biopsies. The prognostic value of the selected PDAC specific histone variant H1.3 was evaluated in an external patient cohort.	The expression of the histone variant H1.3 in pancreatic tumors is associated with poor survival and may function as a prognostic factor.

Abstract

Background: Pancreatic ductal adenocarcinoma (PDAC) is considered as one of the most aggressive cancers. Despite its relatively low incidence, the prognosis is extremely poor, with a 5-year survival rate less than 7 %. In Sweden, around 1300 patients are diagnosed with PDAC each year and the incidence is projected to increase during the next decades. Compared to other cancers, the survival rate for patients with PDAC has not significantly improved over the past 50 years. Despite extensive research, there is still an unmet need to identify new PDAC specific biomarkers with a robust diagnostic, predictive or prognostic potential that could contribute to a reliable diagnosis or aid in the choice of treatment. Epigenetic modifications have long been recognized as significant contributors to the pathogenesis of PDAC and hence offer new opportunities for detecting and managing the disease.

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Populärvetenskaplig sammanfattning

I Sverige får årligen cirka 1300 patienter diagnosen pankreascancer. Det är en allvarlig tumörform där långtidsöverlevnaden är lika dyster idag som den var för 50 år sedan. En viktig anledning till detta är försenad diagnos då det saknas tidiga sjukdomstecken och metoder att upptäcka tumören innan den har hunnit sprida sig till andra organsystem. Endast 10-20 % av de patienter som får diagnosen pankreascancer är aktuella för potentiellt kurativ kirurgi.

Det finns ett stort behov av såväl noggrannare och tidigare diagnostik så som individualiserad prognostisering och behandling. I klinisk praxis används tumörmarkören CA 19-9, som dock saknas hos ca 10 % av patienter med pankreascancer på grund av genetiska betingelser. Träffsäkerheten minskar dessutom för cancer i tidigt stadium. Denna markör är därför otillförlitlig och lämpar sig inte i screeningssyfte. Nya tumörspecifika markörer som kan mätas i cirkulationen skulle kunna bidra till snabbare och säkrare diagnos. I dagsläget saknas även markörer som kan prognostisera sjukdomsförloppet hos den enskilda patienten och styra val av behandling.

Den aktuella avhandlingen belyser aspekter på pankreascancer ur ett populationsperspektiv. I första delen av avhandlingen granskas sambandet mellan tumörstorlek och sjukdomsstadium vid diagnos, metastaseringsfrekvens samt långtidsutfall. I andra delen av avhandlingen identifieras och evalueras nya epigenetiska biomarkörer i serum och vävnad, dels för icke-invasiv diagnostik (blodprov) och dels för vävnadsprognostisering.

I delarbete I analyseras uppemot 60 000 patienter med pankreascancer från det amerikanska cancerregistret SEER. I denna studie visas att en tredjedel av patienterna uppvisar fjärrmetastaser redan när primärtumören har nått 5 mm i storlek. Detta erhållna utfallet pekar på att pankreascancer inte alltid är en långsam sjukdomsprocess som utvecklas genom förstadier och gradvis ackumulering av genetiska mutationer. I vissa fall fortskrider sjukdomen snabbt där de drivande mutationerna sannolikt uppkommer samtidigt. Resultatet från delarbete I visar dock att kirurgi fortfarande utgör enda möjligheten till bot.

I delarbete II beskrivs serumanalys av cellfria nukleosomer för icke-invasiv diagnostik av kirurgiskt behandlingsbar pankreascancer med hjälp av innovativ ELISA baserad teknik. Här visas för första gången att nukleosom-associerade epigenetiska modifikationer kan användas för att särskilja pankreascancer från friska individer och från patienter med godartad pankreassjukdom.

I delarbete III används högupplöst masspektrometri för att karaktärisera kemiska modifikationer av histoner i pankreastumörer utvecklade i en djurmodell. Resultaten visar att formalinfixering av vävnaden kan resultera i kemiska modifikationer som

viktmässigt överensstämmer med kroppsegna post-translationella modifikationer och kan följaktligen misstolkas. Färskfrusen vävnad är att därför att föredra vid sökande efter nya histonmodifikationer i vävnad för att undvika falska resultat.

I delarbete IV utförs masspektrometrisk histonprofilering av pankreastumörer och normal pankreasvävnad med syfte att identifiera lämpliga biomarkörkandidater. Histonvariant H1.3 påvisas som tumörspecifik och utvärderas i en större extern kohort som prognostisk markör efter kirurgisk resektion. Resultaten visar att patienter som uttrycker H1.3 har signifikant sämre överlevnad. H1.3 bedöms därför vara utav värde att vidare undersökas som en prognostisk biomarkör.

Vědeckopopulární shrnutí

Duktální adenokarcinom pankreasu, neboli rakovina slinivky břišní, je jedním z nejzávažnějších typů zhoubných nádorů. Klinicky je karcinom pankreasu obvykle dlouho asymptomatický a současnými prostředky často diagnostikován až v pokročilém stádiu. Z tohoto důvodu je léčebná radikální resekce možná pouze pro malé množství diagnostikovaných pacientů. I přestože je rakovina slinivky břišní poměrně vzácné onemocnění, ukončí ročně mnoho lidských životů. Šance na vyléčení je malá a prognóza je stejně bezútěšná dnes jako před čtyřiceti lety. Bez brzkého vědeckého průlomu se již během následujících let tato agresivní nemoc zařadí na druhou příčku z hlediska nejčastější příčiny úmrtí na nádorová onemocnění. Mezi hlavní důvody tohoto negativního trendu jsou zařazeny nedostatečné metody pro včasnou diagnózu v kombinaci s léčebnými režimy, které jsou v současné době bohužel stále neuspokojivé.

Přesnější a včasnější diagnóza, individualizovaná prognostika, jakož i léčba šitá na míru patří k nejzásadnějším parametrům pro zlepšení této vážné situace.

Při neinvazivní diagnostice se dnes v klinické praxi všeobecně používá sérové vyšetření nádorových markerů, jako je například CA 19-9, které se nachází v krvi pacientů s karcinomem pankreasu. Tento marker je však nespolehlivý a pro diagnostiku vhodný pouze pro vysoce rizikové pacienty. Odhalení nových specifických nádorových markerů měřitelných v cirkulačním oběhu by mohlo přispět k rychlejší a spolehlivější diagnóze, individuální předpovědi průběhu onemocnění a k volbě nejvhodnější léčby.

Tato současná disertační práce vyzdvihuje aspekty rakoviny slinivky z populačního hlediska. V první části této práce je zkoumán objem nádoru v korelaci ke stadiu onemocnění při dignóze, frekvenci metastáz a dlouhodobým klinickým dopadem. Druhá část této práce se zabývá odhadem nových epigenetických biomarkerů vhodných pro neinvazivní diagnostiku a pro prognostiku.

V první studii bylo analyzováno téměř šedesát tisíc pacientů s karcinomem pankreasu registrovaných v Americké databázi SEER. Výsledky této studie ukazují, že rakovina slinivky není vždy proces pomalý, způsobený postupným hromaděním genetických mutací. Vzdálené metastázy byly pozorovány u podstané skupiny pacientů s nádorem nedosahujícím velikosti větší než pět milimetrů. To pravděpodobně znamená, že u těchto pacientů se rakovina slinivky břišní vyvíjí rychle a šíří brzy. V tomto případě nastávají odpovědné mutace pravděpodobně současně a nikoliv postupně. V každém případě zůstává chirurgie jediným způsobem potenciálního vyléčení.

Druhá studie popisuje sérový test bezbuněčných nukleozomů vhodný pro neinvazivní diagnostiku slinivky pomocí inovativní techniky zvané imunosorbentní

enzymový test, ELISA. V této práci bylo dokázáno, že epigenetické modifikace nukleozomů nalezené v krevním séru mohou být použity k odlišení rakoviny pakreasu od zdravých jedinců a pacientů s benigním onemocněním slinivky.

Ve třetí studii je využita vysoko rozlišující hmotnostní spektrometrie, tak zvaná "mass spektrometrie", a charakterizována hmotnost chemických modifikací histonů, nalezených v pankreatických rakovinových nádorech, které byly vypěstovány v živém onkologickém modelovém organizmu. Výsledky ukazují, že fixace tkáně pomocí formalinu může mít za následek chemické modifikace, jejichž hmotnost je v souladu s hmotností sledované u endogenních modifikací v nádoru. V následujících pracích určených k výzkumu a objevu nových modifikací histonů ve tkáni s pomocí mass spektrometrické analýzy by mělo být upřednostňováno užití čerstvé, nebo zmrazené tkáně.

Závěrečná studie popisuje spektrometrickou profilaci histonů v hluboce zmrazené tkáni pankreatického karcinomu s cílem objevení vhodných biomarker kandidátů. Histon variant H1.3 byl v této práci identifikován jako nádor specifický histon, ověřen pomocí imunohistochemie. Ve větší externí kohortní studii byl H1.3 prokázán jako prognostický biomarker po chirurgické resekci. Výsledky této práce ukazují, že pacienti kteří exprimují H1.3 mají výrazně vyšší úmrtnost ve srovnání s H1.3 negativními pacienty. H1.3 je z tohoto důvodu hodnocen jako potencionální prognostický biomarker vhodný pro následný výzkum.

Selected abbreviations

5MC	5-methylcytosine
Ac	Acetylation
AJCC	American Joint Committee on Cancer
AU	Arbitrary unit
AUC	Area under the curve
BSA	Bovine serum albumin
CA 19-9	Carbohydrate antigen 19-9
Cfnucleosomes	Cell free nucleosomes
CT	Computed tomography
Da	Dalton
DAB	Diaminobenzidine
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferase
ETGM	European group on tumor markers
EUS	Endoscopic ultrasound
EXODIAB	Excellence of diabetes research in Sweden
FA	Formaldehyde
FDA	Food and Drug administration
FF	Fresh-frozen
FFPE	Formalin-fixed paraffin- embedded
HPLC	High performance liquid chromatography
HRP	Horse radish peroxidase
IHC	Immunohistochemistry
IPMN	Intraductal papillary mucinous neoplasm
K	Lysine
Me	Methylation
Me2	Di-methylation
Me3	Tri-methylation
MRI	Magnetic resonance imaging
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LDA	Fisher's linear discriminant analyses
LR	Logistic regression
LUDC	Lund university diabetes center
PDAC	Pancreatic ductal adenocarcinoma
PTMs	Post-translational modifications
R	Arginine
REMARK	Reporting recommendations for tumor marker prognostic studies
RT	Room temperature

ROC	Receiver operator characteristic
SEER	Surveillance, epidemiology and end results
STROBE	Strengthening the reporting of observational studies in epidemiology
Ub	Ubiquitination
UICC	Union Internationale Contre le Cancer
US	Ultrasonography

Introduction

Background

The pancreas is a glandular organ in the digestive and endocrine system, located in the abdominal cavity behind the stomach (Figure 1). Anatomically, the pancreas is primarily divided into the head, neck, body and tail. The head is adjacent to the duodenum and two main blood vessels, the superior mesenteric artery and superior mesenteric vein/portal vein. The neck of the pancreas is located in front of the portal vein and connects the head and the body. The tail extends from the body towards the spleen.

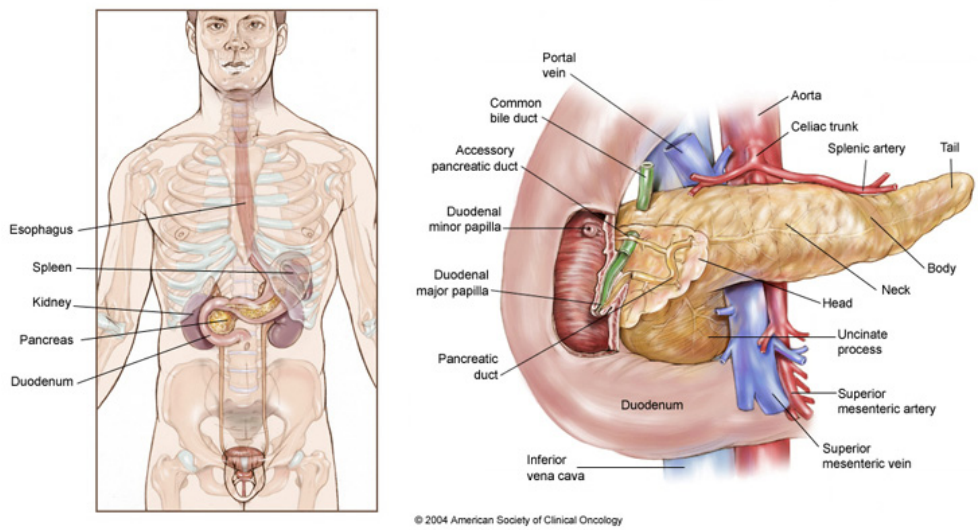

Figure 1. Anatomy of the pancreas.
Reprinted with permission from American Society of Clinical Oncology (ASCO) - Cancer.Net. Copyright © 2017 Copyright Clearance Center, Inc.

The pancreas is a secretory gland with an endocrine and an exocrine function (Figure 2). The endocrine part of the pancreas releases hormones such as insulin and glucagon involved in regulation of blood glucose levels. The exocrine part of the pancreas accounts for the majority of the pancreatic mass and plays an essential role

in the production and secretion of digestive enzymes. The exocrine pancreas consists of lobes composed of secretory acini that are connected through a ductal network. When necessary, the digestive enzymes such as amylase, lipase and peptidase are released by acinar cells into the ducts. The pancreatic ducts are restricted by a single layer of ductal epithelial cells which secrete bicarbonate rich fluid maintaining a slightly alkaline environment required for the optimal enzymatic activity. The duct system fuses into a main pancreatic duct which joins the common bile duct and enters the duodenum at the ampulla of Vater [1, 2].

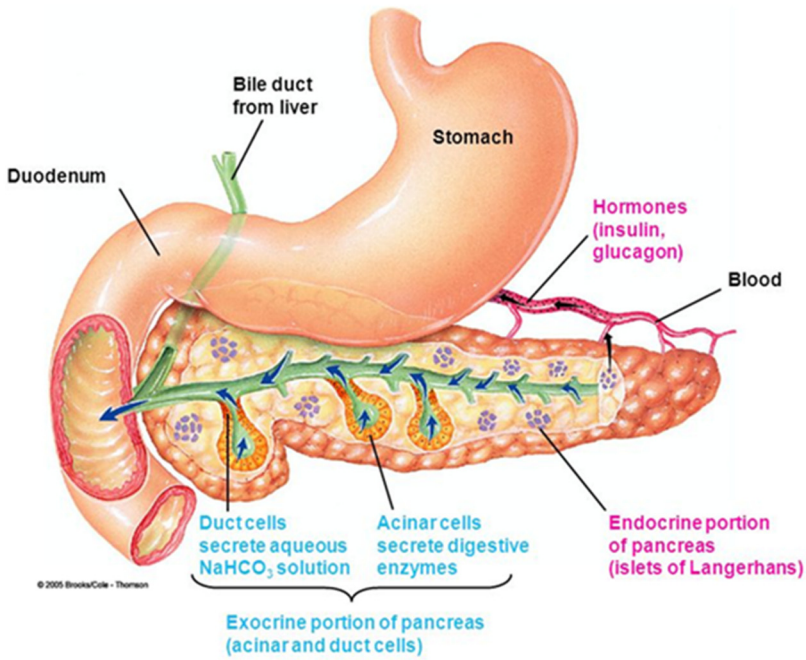

Figure 2. Exocrine pancreas.
Copyright ©2005 Brooks/Cole, Thomson Learning, Inc.

The exocrine part of the pancreas could be affected by several disorders, of which pancreatitis and pancreatic malignancies account for the most common disease conditions [2].

Pancreatic ductal adenocarcinoma

The majority of pancreatic malignancies are categorized as exocrine tumors. Within this category, 85 % of cancers are classified as adenocarcinomas arising from the pancreatic ductal cells [3].

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer, representing the third leading cause of cancer-related deaths in the Western world with a life expectancy below 10 % at 5-years [4]. In Sweden, nearly 1300 new cases of pancreatic cancer are reported each year with a similar incidence rate in both men and women [5].

The cause of pancreatic cancer is still not well defined, however, age is one of the most important risk factors. Pancreatic cancer rarely develops in patients below the age of 45 years and the average age at the time of diagnosis is 71 years [6]. Other risk factors associated with sporadic pancreatic cancer include lifestyle factors such as smoking, obesity and a high fat diet [7]. Inherited genetic susceptibility assigned to familiar cancer syndromes may predispose to pancreatic cancer. Likewise medical conditions such as chronic pancreatitis and pancreatic cysts increase the risk of pancreatic cancer [3]. PDAC is initially asymptomatic and difficult to detect. Late symptoms are usually related to locally advanced tumors or distant metastases, where the chances of cure are dramatically low.

Diagnosis

The rather vague symptoms of PDAC, such as fatigue, loss of appetite, weight loss and abdominal pain are in general not recognized as apparent indicators of pancreatic cancer, which contributes to the delayed diagnosis. PDAC is most often diagnosed in the clinical work-up of jaundice caused by the tumor obstructing the common bile duct or due to other medical issues requiring clinical attention [8]. In symptomatic patients, laboratory tests including the levels of serum biomarkers, such as carbohydrate antigen 19-9 (CA19-9), may assist to indicate a malignancy in the pancreas [9].

Imaging techniques such as computed tomography (CT), conventional ultrasonography (US), endoscopic ultrasound (EUS) or magnetic resonance imaging (MRI), remain the best validated modalities for establishment of the primary diagnosis. However, CT remains the gold standard for determining resectability. Cytological examination of a tumor biopsy is primarily restricted to inoperable cases before initiating chemotherapy [8].

Staging

Staging of PDAC is usually performed after the confirmation of the diagnosis and is primarily based on the findings from the radiological examinations. In summary, a staging system includes determination of tumor size, location and spread, which are important parameters for the assessment of an appropriate initial treatment [10]. The American Joint Committee on Cancer (AJCC) follows the standard TNM system (Tumor size, Lymph node involvement and Metastasis), which divides PDAC into stages 0, I, II, III, or IV [11].

The historic staging used by Surveillance, Epidemiology and End Results (SEER) is based on the Union Internationale Contre le Cancer (UICC) TNM classification [12].

Pancreatic tumors may be further categorized as resectable, borderline resectable, locally advanced or distant metastatic disease. Tumors classified as T1, T2 and T3 (stage I and II) are in general considered as potentially resectable and associated with a better prognosis [13, 14, 15]. (Table 1 and Table 2).

Table 1.

AJCC TNM classification system for pancreatic cancer.

Primary tumor (T)	
TX	Primary tumor cannot be assessed
T0	No evidence of primary tumor
Tis	Carcinoma in situ
T1	Tumor limited to the pancreas, ≤ 2cm in greatest dimension
T2	Tumor limited to the pancreas, > 2cm in greatest dimension
T3	Tumor extends beyond the pancreas, no involvement of
	the celiac axis or the superior mesentric artery
T4	Tumor involves the the celiac axis or the superior mesentric artery
Regional lymph nodes (N)	
NX	Regional lymph nodes cannot be assessed
N0	No regional lymph node metastasis
N1	Regional lyph node metastasis
Distant metastasis (M)	
M0	No distant metastasis
M1	Distant metastasis

Table 2.
AJCC staging groups and SEER historic staging and technical resectability.

AJCC stage grouping				SEER historic staging	Resectability
Stage	**T**	**N**	**M**		
0	Tis	N0	M0	**Localized**	**Resectable**
IA	T1	N0	M0		
IB	T2	N0	M0		
IIA	T3	N0	M0	**Regional**	
IIB	T1	N1	M0		
	T2	N1	M0		
	T3	N1	M0		**Borderline resectable**
III	T4	Any N	M0		**Borderline resectable/ unresectable**
IV	Any T	Any N	M1	**Distant**	**Unresectable**

Prognosis

According to the latest annual report provided by the American Cancer Society, the prognosis for patients diagnosed with PDAC remains very dismal. The 5-year relative survival rate for the subset of patients with metastatic disease which accounts for the majority of cases, is estimated to 3 %. For patients with the earliest stage of disease, defined as localized disease at stage 0-I, the 5-year relative survival rates increases to 29 % [4]. Median survival for patients with resectable PDAC receiving adjuvant therapy is approximately 17-23 month. For patients with metastatic disease, the survival rate decreases dramatically to 4-6 months [16].

Even though the TNM staging serves as a fundament for the estimations of prognosis, other surgical and histopathological parameters may impact the survival of patients with PDAC. Margin resection, histologic tumor grade, lymphovascular invasion, perineural and inflammatory cell infiltration are all reported to correlate with survival [17-21]. Yet, existing prognostic criteria are inadequate for the estimation of the metastatic risk for an individual patient.

PDAC is a heterogeneous disease at several levels and the outcome of the individual patient is likely dependent on the specific tumor biology that determines the path of the cancer progression and the response of the patient to the treatment [22].

Treatment

Surgical resection, followed by adjuvant chemotherapy, is the only potentially curative treatment option in pancreatic cancer [23]. However, surgery is predominantly reserved for patients with early stage of disease mainly at stage I, II and occasionally at stage III. Due to the lack of early disease specific symptoms and the absence of effective detection methods, more than 80 % of PDAC patients are diagnosed with locally advanced or metastatic tumors at a primarily unresectable stage [24]. If the tumor is not possible to resect, the main focus of the treatment is palliative care, including chemoradiotherapy and management of progressive symptoms, such as pain, jaundice and intestinal obstruction.

Standard combination chemotherapy in the form of FOLFIRINOX is currently the best alternative for patients diagnosed with metastatic disease [25]. For unresectable patients in advanced stage, a detailed molecular profile of the tumor could provide information aiding the personalized treatment and thus dramatically improve the clinical outcomes.

Pathophysiology of PDAC

The progression of pancreatic cancer is characteristically associated with activating driver mutations in the oncogene *KRAS* occurring early in the disease development. A subsequent loss of the tumor suppressors *CDKN2A/p16*^{INK4a}, *TP53* and *SMAD4* is related to the invasive carcinomas [26] (Figure 3). Nearly all patients with an invasive adenocarcinoma are, to some extent, carriers of these fundamental mutations [27]. Alongside these essential genetic alterations, occurring during the malignant transformation process, a high number of additional mutations were identified in individual tumors, making the genetic basis of pancreatic cancer extremely complex and heterogeneous [28].

The tumorigenesis of sporadic PDAC is classically presented as a gradual process involving a sequential accumulation of genetic alterations in precursor lesions that over time transform to invasive PDAC. The disease progression, from the initial mutation to the acquisition of the invasive phenotype, was estimated to extend over nearly two decades. In this disease model, distant metastases occur principally at the advanced stage of cancer, leaving a time margin for early detection [29].

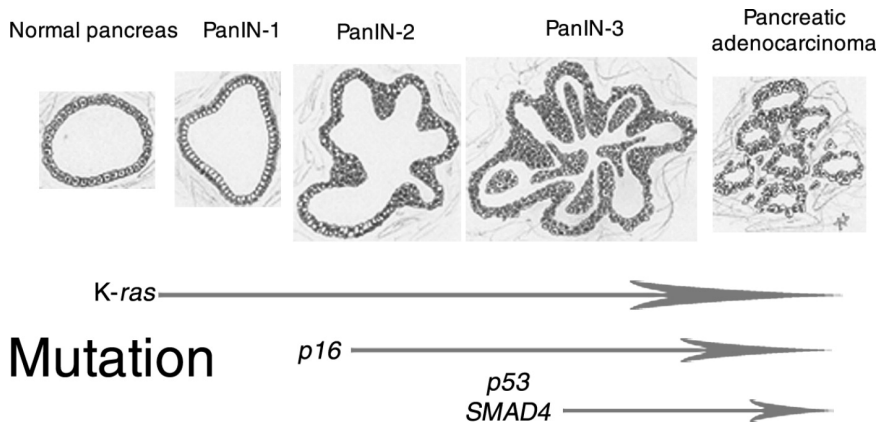

Figure 3. Progression model for pancreatic cancer.
The progression model is divided into subsequential stages associtaed with the specific gene mutation. Reprinted with permission from Taylor & Francis [6].

Another model of pancreatic cancer evolution, challenging the current model of tumorigenesis, was recently proposed. This renewed model of pancreatic cancer advocates a simultaneous acquirement of independent mutations leading to an abrupt and rapid disease progression, despite early detection [30].

Epigenetic alterations in PDAC

Aside from the well-characterized genetic mutations, epigenetic alterations, including aberrant DNA methylation and post-translational modification of histone proteins, contribute to the development of PDAC. Epigenetic modifications, as illustrated in Figure 4, may influence the accessibility of the genome for transcription and thus lead to an abnormal gene expression pattern that promotes tumor progression.

The possibility to detect the individual epigenetic changes already in the earliest precursor lesions [31] makes the epigenetic marks suitable biomarker candidates for the early detection of PDAC.

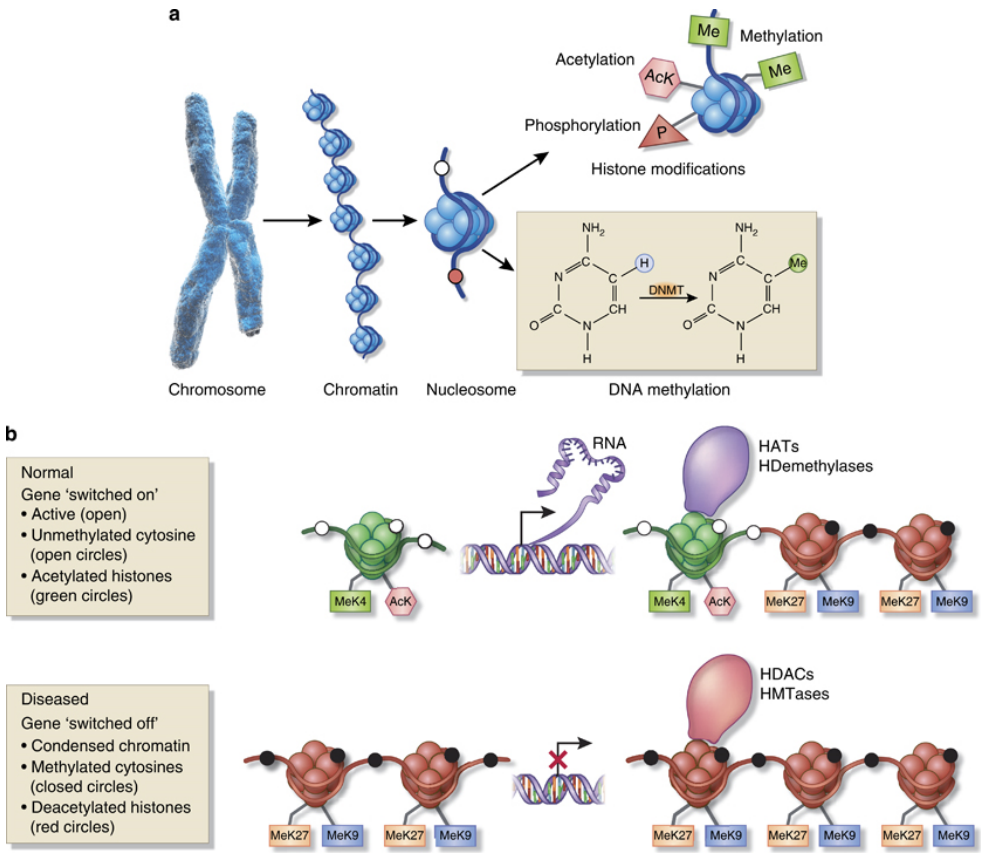

Figure 4. Mechanism of epigenetic modifications.
Epigenetic modifications of chromatin configuration (a). Epigenetic regulation of transcriptional activity (b). Reprinted with permission from Elsevier Inc. Copyright © 2011 International Society of Nephrology. Published by Elsevier Inc [32].

DNA methylation

DNA methylation at the 5-carbon position of cytosine residues (CpG sites) in the gene promotor sequence is recognized as one of the most important mechanisms of epigenetic regulation in pancreatic carcinogenesis [33]. Specifically, an excessive DNA methylation with a subsequent epigenetic gene silencing was detected at the gene promotor regions of tumor suppressor genes such as *APC, BRCA1* and $p16^{INK4a}$ [34]. Abnormal DNA methylation profiles were also found in genes associated with molecular pathways involved in pancreatic cancer progression [35]. The expression of DNA methyltransferases (DNMTs), catalyzing the transfer of a methyl group to the cytosine base in DNA, was reported to increase from normal ducts to precursor lesions to PDAC. Elevated levels of DNMTs were also found to correlate with clinical staging [33].

Histone modifications

Histones are basic units of nucleosomes, which are the elementary components of chromatin. Nucleosomes are composed of 147bp of DNA wrapped around a histone octamer consisting of two copies of each of the four main core histone variants. The higher order chromatin structure is further stabilized by linker histones. Histone proteins may be subjected to various post-translational modifications including methylation, acetylation and ubiquitination of lysine, arginine or histidine residues at the side chain of the amino acid. A divergent pattern of histone modifications interfering with the normal chromatin function, is frequently associated with disordered regulation of the specific histone modifying enzymes [36].

Acetylation

Acetylation of histone proteins is catalyzed by histone acetyltransferases (HATs) and generally results in more relaxed chromatin and transcriptional activity, whereas loss of acetylation leads to transcriptional repression and gene silencing. In pancreatic cancer, loss of histone acetylation, in response to an abnormal expression of specific histone deacetylases (HDACs), was associated with an unrestrained proliferation of pancreatic epithelia, poorly differentiated tumors as well as therapeutic resistance [37, 38].

Methylation

Histone methylation is mediated by specific histone methyltransferases (HMTs) and histone demethylases (HDMs). Methylation regulated transcriptional activation or silencing is dependent on the position of the methylated amino acid residue.
In general, di- and tri-methylation of histone 3 at lysine 4 (H3K4Me2) and (H3K4Me3) and single methylation of histone 3 at lysine 9 (H3K9Me) are associated with open chromatin and transcriptional activation. Di- and tri-methylation of histone 3 at lysine 27 (H3K27Me2 and H3K27Me3) and di- and tri-methylation of histone 3 at lysine 9 (H3K9Me2 and H3K9Me3) are, in contrast, associated with more condensed chromatin and transcriptional repression [39]. In pancreatic cancer, H3K27Me3 was implemented in epigenetic silencing of the suppressor gene $p16^{INK4A}$. H3K27 methyltransferase, enzyme enhancer of zeste homolog 2 (EZH2) was reported overexpressed in a large fraction of PDAC and associated with poor tumor differentiation and decreased survival [33, 38].

Ubiquitination

Histone ubiquitination occurs mainly on histone H2A and H2B in response to DNA damage, to aid the recruitment of the DNA repair machinery. Increased levels of ubiquitination at lysine 119 (H2AK119Ub) were recurrently found in PDAC specimen and associated with the aggressiveness of the disease [33].

Tumor biomarkers

Cancer biomarkers are collectively defined as single molecular markers, or a profile of molecular markers assessable in body fluids or tissues, that indicate a state of malignant disorder [40]. The tumor biomarkers are in general implicated for the establishment of diagnosis, prediction of survival, risk assessment or facilitating the choice of treatment [41]. (Figure 5).

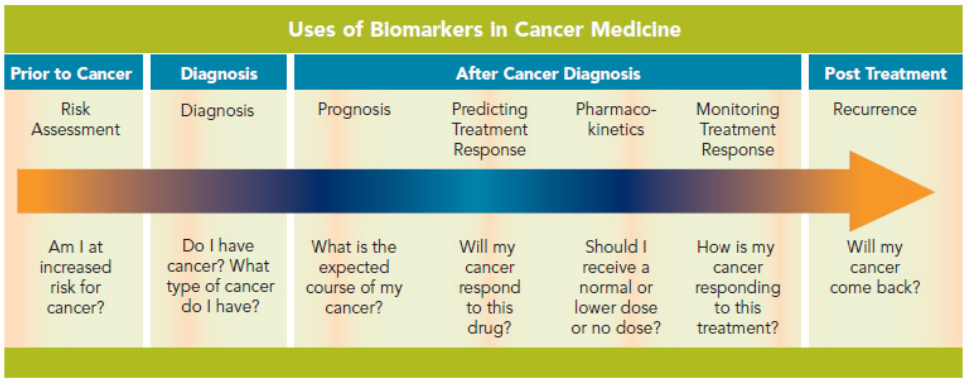

Figure 5. The use of biomarkers in cancer management.
This figure is retrieved from https://www.fournier-majoie.org/en and reprinted with the permission from Foundation Fournier-Majoie. Biomarkers in Cancer- An Introductory Guide for Advocates, Research Advocacy Network, 2010.

Tumor associated antigens, including carcinogenic embryonic antigen (CEA) and especially CA-19-9, are the only validated biomarkers for the monitoring of pancreatic cancer following tumor resection and the response to therapy. Elevated serum levels of these markers are, however, associated with other malignant and benign conditions, making them unsuitable for diagnostic purposes [42]. Although there are only two pancreatic cancer biomarkers in current clinical use, the number of potential candidates is continuously increasing. A countless amount of genetic, epigenetic or proteomic biomarkers related to the management of PDAC were reported in the literature during the last two decades [41]. Yet, despite the advances in biomarker discovery research, no pancreatic cancer specific tissue or serum biomarkers have reached clinical praxis. Nevertheless, the window of opportunities for the discovery of new biomarkers, applicable in the clinical setting, is still open.

In other respects, the large diversity of the identified signature molecules related to tumor specific alterations may provide novel insights regarding the complex tumor biology of PDAC. In the long run, the accumulated knowledge may be translated into clinical settings and reverse the poor prognosis of patients with PDAC.

Objectives

The first part of the thesis aimed to re-evaluate the significance of primary tumor size for predicting clinical outcome at the population level.

The general objective of the thesis was to identify and investigate nucleosome associated epigenetic variations as pancreatic ductal adenocarcinoma (PDAC) specific biomarkers for diagnostic or prognostic purposes.

The specific aims of the individual studies are listed below.

Study I

This study was conducted to evaluate the correlation between the size of the primary tumor and metastatic and survival rates in a large cohort of patients diagnosed with PDAC.

Study II

The study was performed to investigate the diagnostic potential of nine nucleosomal markers associated with epigenetic onco-modifications using a novel NuQ®- assay platform.

Study III

This experimental study was conducted to investigate the suitability of differently preserved biobank-archived tissue for analysis of post-translational modifications (PTMs) important in pancreatic cancer progression.

Study IV

This study was conducted to profile histone variants and histone related modifications in PDAC as possible diagnostic or prognostic biomarker candidates.

Material and methods

The design for individual studies, the study material and the methodology used in this thesis are presented and summarized in this chapter.

Study design

Study design used in papers included in this thesis is outlined in Table 3.

Table 3.
Study design overview

Paper	I	II	III	IV
Study design	Retrospective CS	Prospective CS	Experimental	Retrospective CS
Study material	Human Cancer Registry	Human serum	Human tumor xenografts	Human tissue
Cohort origin	USA	Lund, Sweden	Lund, Sweden	Lund, Sweden
Applied methods	Statistical computations	Nucleosomics®	LC-MS/MS	LC-MS/MS and IHC
No. of individuals	58 728	59 (25+ 24+10)	18 (9+9)	20 (10+10) and 72 (62+10)
Collection period	1988-2013	2012-2014	2016	2000- 2015

Abreviations: CS, cohort study; LC-MS/MS, Liquid chromatography tandem mass spectrometry; IHC, immunohistochemistry

Study populations

The USA patient cohort (Paper I)

The study cohort comprised patients diagnosed with pancreatic cancer registered between 1988 and 2013 in SEER database.

The SEER Program registry is a national program under the administration of the National Cancer Institute (NCI). SEER registry consists of 18 regional or statewide cancer registries, providing population-based information on cancer

incidence and survival in the United States, available for comprehensive epidemiology analysis.

The patient data in SEER are collected from individual cancer archives, recording patient demographics, primary tumor site, tumor morphology and stage at diagnosis, first course of treatment, and follow-up [43].

The patients included in paper I were identified and selected retrospectively, using International Classification of Diseases for oncology, 3rd edition (ICD-O-3) with a coding system for both topography and morphology. The study followed the Strengthening the reporting of observational studies in epidemiology (STROBE) guidelines [44].

The Lund patient cohort (Paper II, IV)

All patients underwent pancreatic resection with curative intent at the Department of Surgery, Skåne University Hospital in Lund, Sweden between 2000 and 2015. In paper II, blood samples were collected at the time of diagnosis, prior the therapeutic intervention. All serum samples included in the study were obtained from the local biobank.

In *paper IV*, the clinical specimens (n=10) analyzed in the first part of the study were accessed from the local biobank and selected to obtain a homogenous study population. Formalin-fixed paraffin-embedded (FFPE) tumors (n=72) used for the evaluation of prognostic biomarkers were acquired from the Department of Pathology, Skåne University Hospital in Lund, Sweden and comprised histologically proven primary PDAC cases (n=62) and benign pancreatic disease (n=10).

Healthy controls (Paper II, IV)

Age-matched serological control samples (n= 24) were collected from healthy blood donors at the local blood donation center in Lund, Sweden *(Paper II)*.

In *paper IV*, frozen tissue biopsies from pancreatic head (n=10) were obtained from healthy organ donors and acquired through the Lund University Diabetes Center (LUDC), a part of the national consortium Excellence of Diabetes research in Sweden (EXODIAB).

Biobank samples (Paper II, IV)

Serum

The blood samples from patients or heathy blood donors were collected in serum separator tubes (BD SSTII advanced, Becton Dickinson) by medically trained personnel at the Department of Surgery, Skåne University Hospital in Lund, or at the local blood donation center, respectively. After arrival to the laboratory, the samples were centrifuged at 2500x g for 10 minutes in room temperature. The obtained serum was then aliquoted and stored at -80 °C in the freezer, dedicated for the local pancreatic biobank, until further use.

Tumor tissue

Tumor biopsies were acquired from patients with suspected PDAC, undergoing pancreaticoduodenectomy. After the arrival to the laboratory, the biopsy was rinsed with distilled water and divided in half. One part of the tissue was snap-frozen and stored at -80 °C in the freezer, dedicated for the local pancreatic biobank. The remaining part was fixed in 4 % PFA for 24 hours at 4 °C, dehydrated, embedded in paraffin and stored in a cold room until analysis.

Pancreatic cancer cell derived human xenografts (Paper III)

Human xenografts (Figure 6) were generated in genetically identical NMRI-nu mice (Janvier Labs) by inoculation of human pancreatic cancer cell line Capan-1 (ATCC, USA). The cancer cells were derived from liver metastasis originating from pancreas adenocarcinoma developed in the pancreatic head.

Briefly, the appropriate amount of cultivated cells was collected from a culturing flask. After the determination of the cell concentration, 25 x 10^6 viable cells were re-suspended in 1.25 ml serum free Iscove's modified Dulbecco's culturing medium. Within one hour after harvesting, 50 µl cell suspension containing 1x10^6 tumor cells was subcutaneously injected through a fine needle into the right flank of the respective animal. The tumors were resected two weeks after the inoculation.

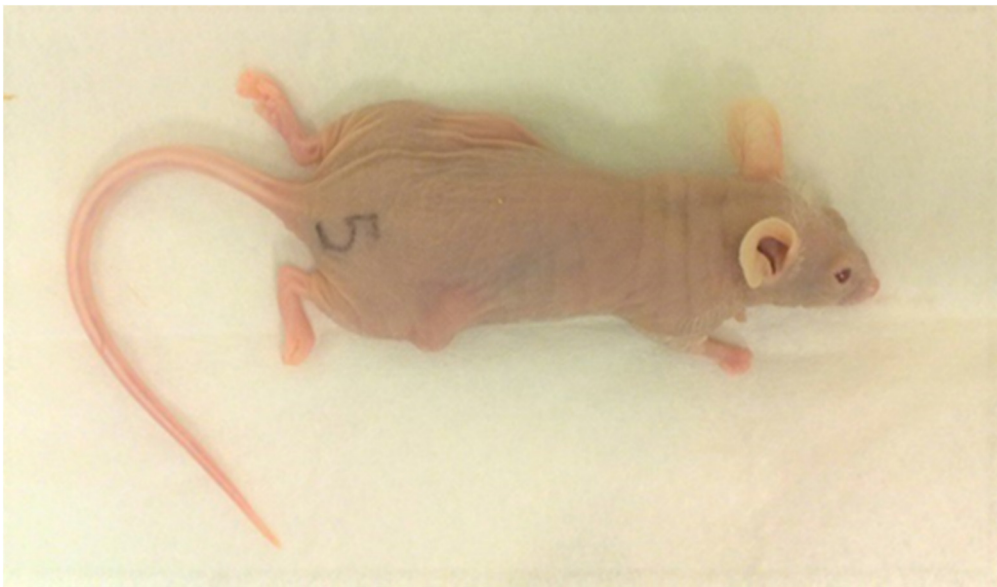

Figure 6.
Pancreatic cancer cell derived xenograft model.

Ethical approval

Studies (*Paper I, II and IV*) including human subjects were approved by the local Ethical committee for clinical research at Lund University, Sweden. Written informed consents were obtained from all subjects included in study II and IV.

The study comprising animal experiments (*Paper III*) were conducted and performed in agreement with the guidelines of the Swedish Government and the Lund University, Sweden and approved by the local ethical committee. The animals were housed in standardized pathogen free conditions in individually ventilated cages and provided unlimited access to food, drinking water, standard rodent chew and nesting material and received proper animal care.

Methodology

Nucleosomics® (Paper II)

Enzyme-Linked Immuno Sorbent Assay (ELISA) based NuQ®-assays (Belgian Volition) were used for the quantification of circulating cell free nucleosome structures, as well as defined epigenetic features, such as histone modifications, histone variants or DNA modification of interest (Figure 7).

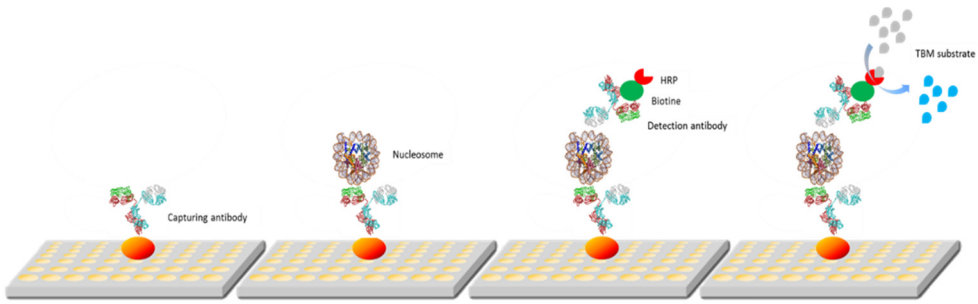

Figure 7. The general principle of the NuQ®- assay.
The assays consist of antibodies immobilized to the solid phase of the microtiter plate for the capturing of nucleosomes. Separate detection antibodies directed to bind to the specific epitope are linked to a suitable enzyme that converts an appropriate substrate to a detectable signal.

Briefly, 10 µl of 6-fold diluted serum samples were incubated overnight at 4-8 °C in 96-well nucleosome capture microtiter plates pre-coated with nucleosome capturing antibody. The next day, after the washing step, the respective plate was incubated for 90 min at room temperature (RT) with 50 µl of an appropriate biotinylated detection antibody, specific to the modification of interest or histone variant. After the incubation, the plates were washed and incubated with 50 µl of streptavidin–horseradish peroxidase (HRP = 0.25 µg/ml) for 30 min at RT. The plates were washed and a peroxidase substrate was added. Finally, after 20 min of incubation, the plates were measured using an X-Mark Microplate spectrophotometer (BioRad) to obtain optical density values. Mean imprecision of sample duplicates ranged from 2-4 %. Intra- and Inter-assay imprecision was below 11 %.

Tissue processing prior mass spectrometric analysis (Paper III, IV)

Formalin-Fixed Paraffin-Embedded tissue

Eight 10 µm tissue sections with an area up to 80 mm^2 were cut from FFPE tissue blocks, collected in 2ml maximum recovery microtubes (Axygen), deparaffinized and extracted. Briefly, the sections were incubated twice for 10 min at 97 °C in 1 ml EnVision™ FLEX retrieval solution, pH 8 (Dako, Agilent Technologies) diluted 1:50 to remove the excess of paraffin from the tissue. After the careful removal of the retrieval solution, the deparaffinized tissues were sonicated with a probe in Tris-guanidine based extraction buffer for 20 min on ice. The samples were then centrifuged to pellet the debris. The soluble proteins in the supernatant were reduced, alkylated and precipitated using standard protocols. The pelleted protein precipitates were then dissolved in ammonium bicarbonate and quantified using the BCA assay. 100 µg of protein was digested using Sequencing Grade Modified Trypsin (Promega) with an enzyme – protein ratio of 1:100. The resulting peptides were dissolved in 50 µl mobile phase A (0.1 % formic acid) and quantified using the Pierce quantitative colorimetric peptide assay.

Fresh-Frozen tissue

The tissue samples were pulverized in liquid N_2 using mortar and pestle and homogenized in ice cold extraction buffer supplemented with protease and phosphatase inhibitors. The crude homogenates were then subjected to four thaws and freeze cycles, followed by ultrasonic bath treatment for 20 min on ice and a short centrifugation to remove debris. The soluble proteins in the supernatant were processed as reported above. 130 µg of protein digested using Sequencing Grade Modified Trypsin, Mass Spec Grade Trypsin/Lys-C Mix or Sequencing grade Glu- C (Promega), at a final protein enzyme ratio of 1:100. The digests were further processed as described above. In *paper IV*, the Thermo Scientific Pierce Peptide Retention Time Calibration Mixture, consisting of 15 peptides, was added to each sample for the normalization and control of the chromatographic performance.

LC-MS/MS analysis (Paper III, IV)

The peptide analysis was performed using a high performance liquid chromatography (HPLC) system EASY-nLC 1000 connected to Q Exactive quadrupole Orbitrap mass spectrometer, equipped with a Thermo nanospray Flex ion source.

Digested peptides dissolved in mobile phase A were injected at a flow rate of 300 nl/min and separated either with a 150 min gradient of 4-40 % acetonitrile (ACN) in mobile phase A (*Paper III*) or with 132 min gradient of 5-22 % ACN in mobile phase A (*Paper IV*) using a two-column setup including an

analytical column (25 cm x 75 µm ID, particle size 2µm, pore size 100 Å, PepMap C18) and a pre-column (2 cm x 75 µm ID, particle size 2µm, pore size 100 Å, PepMap C18). Each sample was measured in duplicate in a random order.

The Q Exactive system was operated in the positive data-dependent acquisition (DDA) mode to automatically switch between the full scan MS and MS/MS acquisition. For peptide identification a full MS survey scan was performed in the Orbitrap detector. Fifteen data-dependent higher energy collision dissociation MS/MS scans were performed on the most intense precursors. The spray voltage was set to 1.75 kV with the capillary temperature of 300 °C. The S-lens radio frequency (RF) level was fixed at 50 %. The MS1 survey scans of the eluting peptides were executed with a resolution of 70 000, recording a window between m/z 400 and m/z 1 600. The automatic gain control (AGC) target was set to $1x\ 10^6$ with an injecting time of 100 ms. Normalized collision energy (NCE) for MS/MS was set at 25 % (*Paper III*) or 27 % (*Paper IV*) for all scans. The resolution of the data dependent MS2 scans was fixed at 17 500 and the values for the AGC target and inject time were $1x10^6$ and 120 ms (*Paper III*) or $5x10^5$ and 80 ms (*Paper IV*), respectively.

MS data analysis

The acquired MS/MS raw data files obtained from the combined randomized measurements were processed with Proteome Discoverer software, Version 1.4 (Thermo Fisher) to identify the histone proteins including information about number of detected peptides, sequence coverages and modifications (Figure 8).

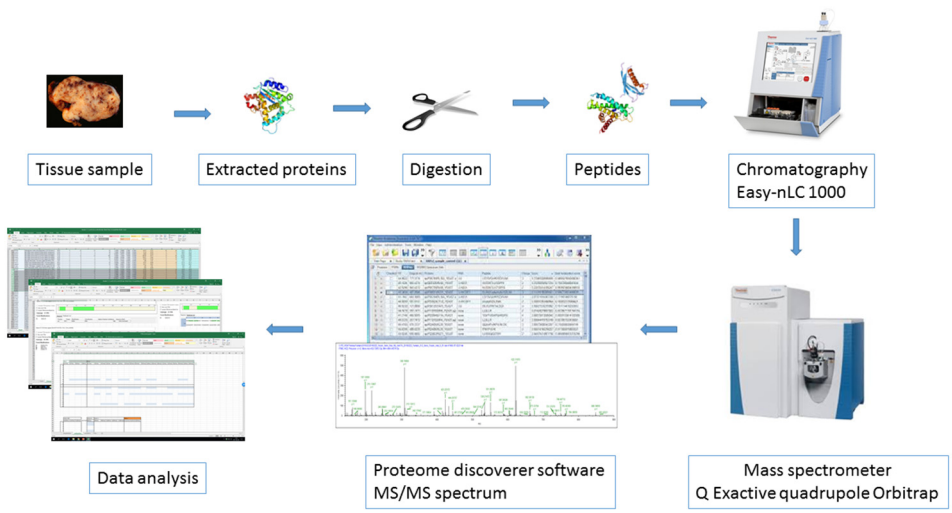

Figure 8.
MS/MS workflow for identification of proteins.

The selection of spectra was based on the following settings: min precursor mass 350 Da; max precursor mass 5 000 Da; s/n threshold 1.5. Following parameters were used for Sequest HT searches: precursor mass tolerance 10 ppm; fragment mass tolerance 0.002 Da; trypsin or Glu-C; 1 missed cleavage site; Uniprot human database; dynamic modifications: acetyl (+42.011 Da; K), methyl (+14.016 Da; K,R), dimethyl (+28.031 Da; K,R), trimethyl (+42.047 Da; K,R), glygly (+114.043 Da; K) and oxidation (+15.995 Da; M,P) and fixed modification: carbaminomethylation (+57.021 Da; C). The percolator was used for the processing node and the cutoff limit false discovery rate (FRD) value was set to 0.01. The selected spectra were then used for the identification of histone proteins and histone-related modifications.

Immunohistochemistry (Paper IV)

Immunohistochemistry (IHC) is a broadly used technique for detection of antigens in tissue sections, based on the interaction between the antigens of interest and antibodies (Figure 9). IHC is useful for the diagnosis of disease, drug development and biological research [45].

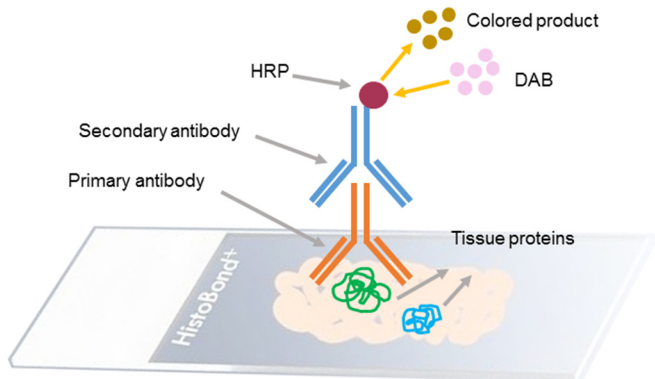

Figure 9. General principle of immunohistochemistry. Primary antibody binds to a specific antigen in tissue section. The antigen- antibody complex is completed by incubation secondary antibody conjugated with HRP enzyme which converts the chromogenic substrate, DAB, to an insoluble colored precipitate.

The formalin-fixed paraffin-embedded PDAC specimens, corresponding to fresh-frozen preserved tissue analyzed with LC-MS/MS and normal pancreatic tissue, were sectioned and stained for the presence of Histone H1.3 antigen. Tissue sections without the primary antibody were used as negative control.

Briefly, 4 µm tissue sections adhered on a microscope slide were deparaffinized using PT Link -PT 11730 for 20 min at 97 °C in 1x EnVision^{TM} FLEX retrieval

solution, pH 6 (Dako, Agilent Technologies). The slides were then rinsed with Tris-buffered saline (TBS) based washing buffer and pretreated with 5 % normal goat serum in dilution buffer (TBS with 1 % BSA) for one hour at RT, to block the unspecific binding sites. The sections were then incubated overnight at 4 °C with 5 µg/ml anti- histone H1.3 polyclonal IgG (Abcam), recognizing N–terminal amino acids 7-33 of human histone H1.3. The endogenous peroxidase was inactivated using 0.3 % hydrogen peroxide and 1 % methanol dissolved in TBS. Horseradish peroxidase (HRP) conjugated secondary antibody (Sigma-Aldrich), diluted 1:200, was then applied to tag the primary antibody. Diaminobenzidine (DAB) kit (Vector Laboratories Inc.) was used as the substrate for visualization of the antigen. The nuclear contrast was achieved with hematoxylin counterstaining. The sections were then dehydrated, cleared with xylene and mounted. The distribution of H1.3 in the tissue, the immunoreactivity, the overall staining intensity as well as the subcellular location was evaluated and representative images were taken using Olympus BX53 microscope.

Statistical analysis

Paper I

The survival was estimated and plotted using the Kaplan-Meier method. Adjustment for the confounding variables was made using the Cox proportional hazard method. Because of the large sample size, proportional hazards assumptions were tested to give more weight to the graphical tests. The obtained results were verified using a Poisson regression model. Clinically relevant confounding variables were selected and included age, gender, year of diagnosis, tumor location, histological grade, staging, surgical resection, and radiation therapy. The collinearity of variables was tested and variables with no interaction terms in the main effect model were removed.

Missing values were imputed using the chained equation multiple imputation strategy. Ten iterations for each chain as well as the imputed data were accepted. Data was analysed using Stata® MP 14.1 software (StataCorp LP, College Station, TX).

Paper II

For each assay, the data was pre-processed, by dividing the values converted to log2 scale with the standard deviation. Linear models were calculated using Logistic Regression (LR), and Fisher's Linear Discriminant analysis (LDA).

The data obtained from above stated calculations were used to determine the weighted sum of the NuQ® variables and reported as arbitrary units (AU) for the

multi-criteria evaluation and the optimal discrimination of the experimental groups. A combination of nine individual NuQ® assays was included for selection (including or excluding CA19-9 as a potential variable). Five variables were accepted as an upper limit, to avoid overtraining. Models with one to five variables were ranked by area under the Receiver Operator Characteristic curve. Equivalent Receiver Operator Characteristic (ROC) curves were obtained from LR and LDA. The analysis was calculated using the statistical programming language R.

Paper III

The results originating from LC-MS/MS analysis of paraffin embedded or fresh-frozen samples were evaluated as presence or absence of the respective histone modification and analyzed as categorical data. P-values at a significance level of 0.05 were calculated using Fisher's exact test. The results were reported as statistically significant at p-value < 0.05. The analysis was conducted using GraphPad Prism v6.0.

Paper IV

The results obtained from LC-MS/MS analysis of fresh-frozen tissue were assessed as presence or absence of the respective histone protein variant and analyzed as categorical data using Fisher's exact test.

In the analysis of H1.3 as a prognostic biomarker, the correlation between H1.3 expression and clinicopathological parameters was determined using the Mann-Whitney U test for continuous variables and Fisher's exact test or x^2 for categorical variables. The Kaplan-Meier method was used to estimate the survival for patients with positive or negative H1.3 tumor expression. P-values estimating the differences between groups were calculated using the log-rank test. Clinically relevant confounding variables were selected and included age, gender, tumor size, grading, lymph node metastasis, margin status and adjuvant chemotherapy. The adjustment for possible confounding variables was calculated using the Cox proportional hazards model. Hazard ratios were presented with 95 % confidence intervals. A value of $p < 0.05$ was considered as statistically significant. The statistical analyses were computed using Stata® MP statistical software, version 14.1.

Main results

Relationship between tumor size and outcome in pancreatic ductal adenocarcinoma

In paper I, the correlation between tumor size and clinical outcome in pancreatic ductal adenocarcinoma was investigated in a population including 58 728 patients, selected from 177 115 patients diagnosed with pancreatic cancer registered in the SEER database. The flow diagram for the selection of the study group and the characteristics of the selected patient cohort are illustrated in Figure 10.

	No of patients* (n= 58 728)
Age (years)ǂ	67.0 (11.5)
Gender ratio (F:M)	29,003 . 29 725
Tumour size	
≤0.5 cm	187 (0.3)
0.6-1 cm	430 (0.7)
1.1-1.5 cm	1,349 (2.3)
1.6-2 cm	3,791 (6.5)
>2 cm	52,971 (90.2)
Histological grade	
Well differentiated	3,142 (5.3)
Moderately differentiated	12,409 (21.1)
Poorly differentiated	11,484 (19.6)
Anaplastic	470 (0.8)
Unknown	31,223 (53.2)
Tumour location	
Head	34,792 (59.2)
Body	7,564 (12.9)
Tail	7,470 (12.7)
Pancreatic duct	356 (0.6)
Other specified parts of pancreas	758 (1.3)
Overlapping	4,752 (8.1)
Pancreas, NOS	3,036 (5.2)
Stage	
Localized	4,777 (8.1)
Regional	23,579 (40.2)
Distant	29,128 (49.6)
Unknown	1,244 (2.1)
Surgical resection	
No	43,182 (73.6)
Yes	15,398 (26.2)
Unknown	148 (0.3)
Radiation therapy	
No	43,011 (73.2)
Yes	15,717 (26.8)
Chemotherapy	
Noǂ	26,592 (45.3)
Yes	32,136 (54.7)
Time period	
1988-2006	27,601 (47.0)
2007-2013	31,127 (53.0)

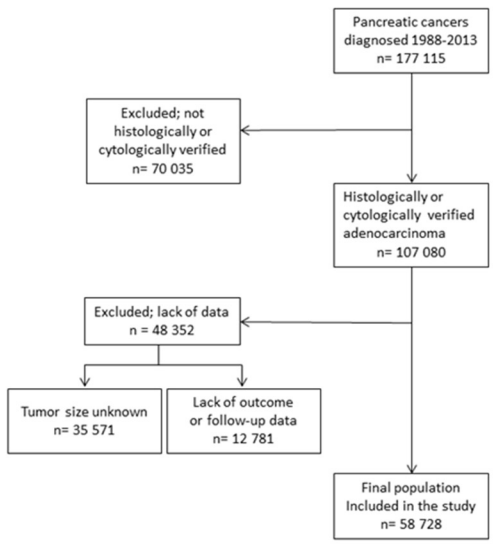

Figure 10. Flow diagram for the selection of the study group and characteristics of the selected cohort.
* indicates percentage in parentheses unless stated otherwise; ǂ values are mean (s.d.) ǂ No evidence of therapy was found in the medical records. Abbreviation: NOS, not otherwise specified.

Tumor size correlation to distant metastasis

The expanding tumor size was associated with a higher frequency of distant metastasis. As illustrated in Figure 11, 30.6 % of the primary tumors with a size equal to or below 0.5 cm presented with distant metastasis at the time of diagnosis. The frequency of metastasis increased to 73.9 % for tumors with a size of 10 cm.

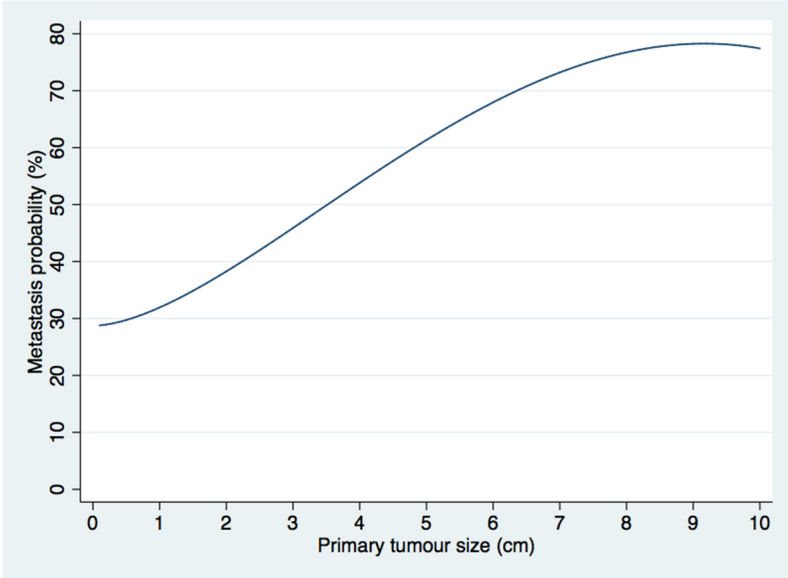

Figure 11.
Diagram illustrating the frequency of distant metastasis in relation to tumor size.

Stage dependent correlation of tumor size and survival

As presented in Figure 12, the increasing tumor size was related to decreased survival rate only in localized disease. However, patients with a regional or distant disease showed a similar pattern of survival across all tumor size categories.

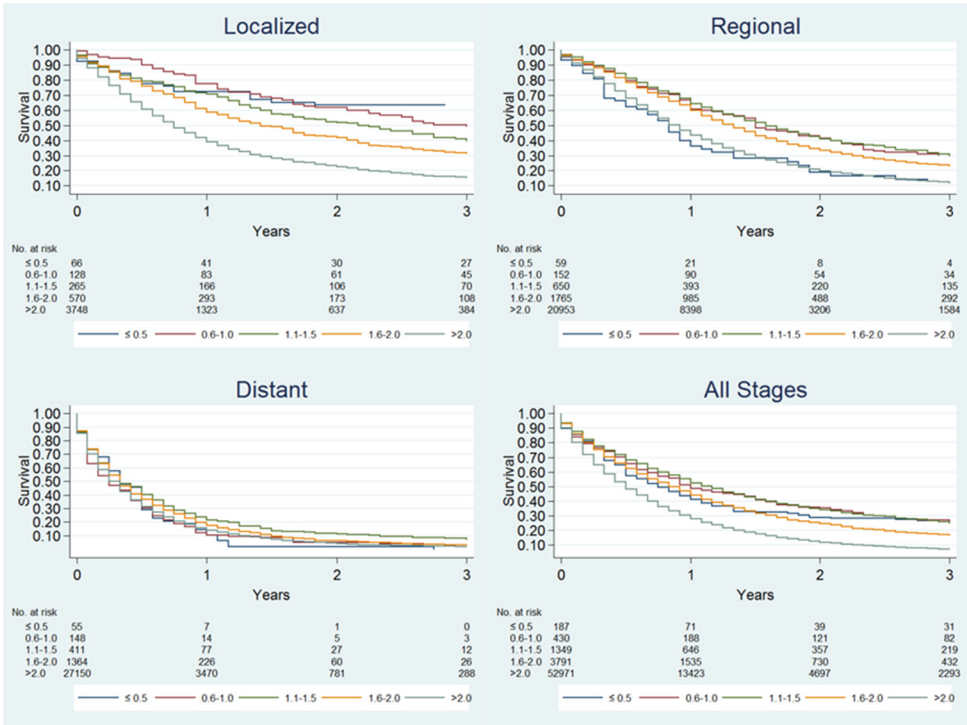

Figure 12.
Kaplan-Meier curves illustrating survival related to tumor size (cm) and stage of the disease.

Correlation of tumor size and survival, with or without surgical resection of the tumor

Overall, 5-year survival rate of patients who underwent surgical resection of the primary tumor was estimated to 16.1 % compared to 1.2 % for patients without surgery. Moreover, following surgery, the prognosis was improved for patients with smaller tumor size compared to patients with larger tumors. This trend was not observed in non-operated patients, as summarized in Figure 13.

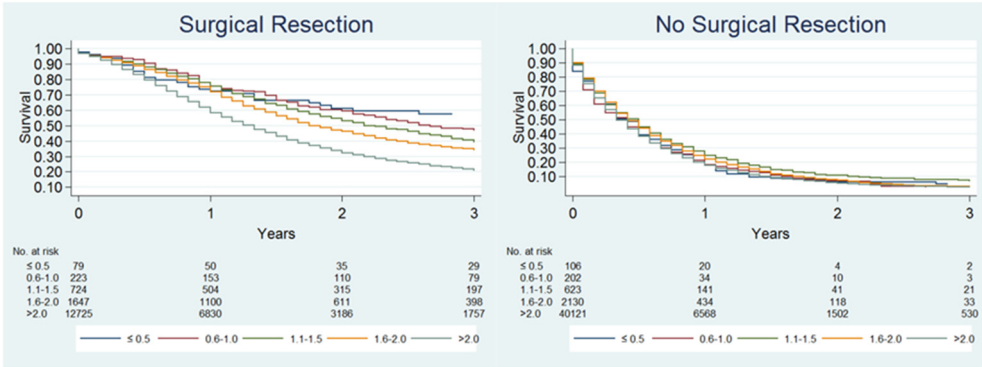

Figure 13.
Kaplan-Meier curves illustrating survival related to tumor size and surgical resection.

The prognostic impact of tumor size

Using the main Cox regression model, increasing tumor size was identified as a significant and an independent predictor of poor survival with a hazard ratio 1.04, corresponding to a mortality increase of 4 % for each cm-increase in tumor size (Table 4). This association was, however, recognized only in patients with resected tumors diagnosed with localized disease, presented in Table 5.

Table 4.
Predictors of survival identified by the main Cox regression model.

	Hazard Ratio	*P*
Age	1.01(1.01 - 1.011)	<0.001
Male gender	1.03 (1.01 - 1.05)	0.001
Tumor size (cm)	1.04 (1.04 - 1.05)	<0.001
Grade		
Well-differentiated	1.00 (reference)	
Moderately differentiated	1.21 (1.16 - 1.26)	<0.001
Poorly differentiated	1.52 (1.45 - 1.59)	<0.001
Anaplastic	1.49 (1.32 - 1.68)	<0.001
Stage		
Localized disease	1.00 (reference)	
Regional disease	1.32(1.27- 1.37)	<0.001
Distant metastases	2.07 (1.99 - 2.15)	<0.001
Location (head of pancreas vs other sites)	0.96 (0.94 - 0.98)	<0.001
Surgery	0.43 (0.42 - 0.44)	<0.001
Radiation therapy	0.90 (0.88 - 0.93)	<0.001
Chemotherapy	0.58 (0.57 - 0.59)	<0.001
Time interval (2007-2013 vs 1988-2006)	0.87 (0.85 - 0.88)	<0.001

Values of 95% confidence intervals are presented in parenthesis.

Table 5.
Prediction of survival in subgroups according to size and surgical resection of the tumor (left panel) or according to size and stage of the disease (right panel).

Tumor size (cm)	Hazard Ratio*	*P*
Surgical resection		
≤0.5	1.00 (reference)	
0.6-1.0	1.25 (0.83-1.87)	0.283
1.1-1.5	1.47 (1.01-2.14)	0.044
1.6-2.0	1.73 (1.20-2.50)	0.003
>2	2.33 (1.62-3.36)	<0.001
No surgical resection		
≤0.5	1.00 (reference)	
0.6-1.0	1.04 (0.79-1.37)	0.762
1.1-1.5	0.82 (0.65-1.05)	0.116
1.6-2.0	0.93 (0.74-1.16)	0.504
>2	1.05 (0.84-1.31)	0.679

Tumor size (cm)	Hazard Ratio*	*P*
Localized disease		
≤0.5	1.00 (reference)	
0.6-1.0	1.57 (0.94 - 2.62)	0.085
1.1-1.5	2.10 (1.31 - 3.37)	0.002
1.6-2.0	2.31 (1.46 - 3.65)	<0.001
>2	3.38 (2.16 - 5.30)	<0.001
Regional disease		
≤0.5	1.00 (reference)	
0.6-1.0	0.74 (0.52 - 1.05)	0.088
1.1-1.5	0.73 (0.54 - 1.00)	0.047
1.6-2.0	0.88 (0.65 - 1.18)	0.398
>2	1.09 (0.82 - 1.46)	0.550
Distant metastases		
≤0.5	1.00 (reference)	
0.6-1.0	1.01 (0.71 - 1.44)	0.964
1.1-1.5	0.77 (0.56 - 1.06)	0.113
1.6-2.0	0.87 (0.64 - 1.18)	0.364
>2	0.97 (0.72 - 1.31)	0.840

Values of 95% confidence intervals are presented in parenthesis.* Multivariable Cox regression analysis adjusted for age, male gender, grade, tumor location, stage, radiation therapy, chemotherapy and time interval.

The results confirm the impact of tumor size on the probability of metastasis and long term survival. The association between small tumor size and prolonged survival was, however, supported exclusively in the group of patients diagnosed with localized disease. The survival rate was not dependent on the size of the primary tumor to any further extent in patients with regional or distant metastasis.

Circulating nucleosomes as epigenetic biomarkers in pancreatic cancer

In paper II, the diagnostic potential of cancer specific epigenetic markers was investigated in serum samples from 59 individuals, including 25 patients with resectable pancreatic cancer, 10 patients with benign pancreatic disease and 24 healthy individuals. The selection of objects included in the study was based on the information obtained from the hospital records (Table 6).

Table 6.
Patient characteristics.

Diagnosis	No. of patients	Median CA19-9 level (IQR)	Median Age (IQR)	M: F
Pancreatic Cancer	25	150 kU/L (1.7 – 1494 kU/L)	69 (46-78)	15:10
Lymph node involvement	19			
Stage IIA	3			
Stage IIB	22			
Benign disease	10	31 kU/L (0.6 – 300 kU/L)	72 (58-77)	5:5
Chronic pancreatitis	4			
IPMN	2			
Serous cystadenoma	2			
Tubular adenoma in the ampulla of Vater	1			
Benign biliary stricture	1			
Healthy	24	7.3 kU/L (4-20 kU/L)	58 (48-70)	15:9

Abbreviations: IPMN, Intraductal Papillary Mucinous Neoplasms; M, Male; F, Female

Epigenetic profiling of circulating nucleosomes in serum using nucleosome assays

Profile of epigenetic markers associated with cfnucleosomes present in serum samples was analyzed using Nucleosomics®. Nine epigenetic structures were measured; 5-Methylcytosine (5MC), histone modifications H3K4Me2, H3K9Ac, H3K9Me3, H3K27Me3, H4PanAc, H2AK119Ub, as well as histone variants mH2A1.1 and H2AZ. The diagnostic potential of the specific epigenetic features was evaluated individually or as optimal panels, defined by multivariate analysis. The panels consisted of five NuQ® assays (5MC, H3K4Me2 H2AK119Ub, H2AZ and H2A1.1) or four NuQ® assays (5MC, H3K4Me2, H2AZ and H2A1.1) in combination or without CA19-9. The panels were tested in two clinical settings; cancer vs healthy controls or cancer vs control group including both benign and healthy controls.

Diagnostic performance of NuQ® assays

As shown in Table 7, no individual NuQ® assay outperformed CA 19-9. Panel combining five NuQ® assays distinguished cancer group from control groups with a higher precision than CA19-9 with an equal sensitivity at 90 % specificity. A panel combining four NuQ® assays and incorporating CA 19-9 was able to discriminate pancreatic cancer from control groups with a superior diagnostic performance.

Table 7.
Performance of individual NuQ® assays, panels of NuQ® assays and CA19-9.

	Cancer vs Controls		Cancer vs Healthy	
NuQ® Assay	**AUC**	**Sensitivity**	**AUC**	**Sensitivity**
H3K4Me2	0.52	0 %	0.53	0 %
mH2A1.1	0.58	16 %	0.64	40 %
H3K9(Ac)	0.61	12 %	0.69	44 %
H3K27Me3	0.64	40 %	0.68	40 %
H4Pan(Ac)	0.67	24 %	0.71	36 %
H2AZ	0.68	28 %	0.72	36 %
5 methyl Cytosine (5MC)	0.70	40 %	0.72	40 %
H2AK119Ub	0.70	36 %	0.78	60 %
H3K9Me3	0.77	28 %	0.81	28 %
CA19-9	0.84	72 %	0.87	80 %
4 NuQ®	0.90	64 %	0.91	68 %
5 NuQ®	0.92	72 %	0.95	84 %
4 NuQ® and CA 19-9	0.94	92 %	0.98	92 %

The receiver operator characteristic (ROC) curves for the optimal NuQ® panels with or without CA19-9 and CA19-9 alone are provided in Figure 14. In the clinical setting for the discrimination of cancer group from controls, the panel of four NuQ® assays combined with CA19-9 gave AUC of 0.94 which was significantly higher than that of CA 19-9 with an AUC of 0.84. The accuracy of the diagnostic test of four NuQ® assays combined with CA19-9, distinguishing pancreatic cancer cases from healthy individuals, increased to an AUC of 0.98 with an overall sensitivity of 92 % at 90 % specificity.

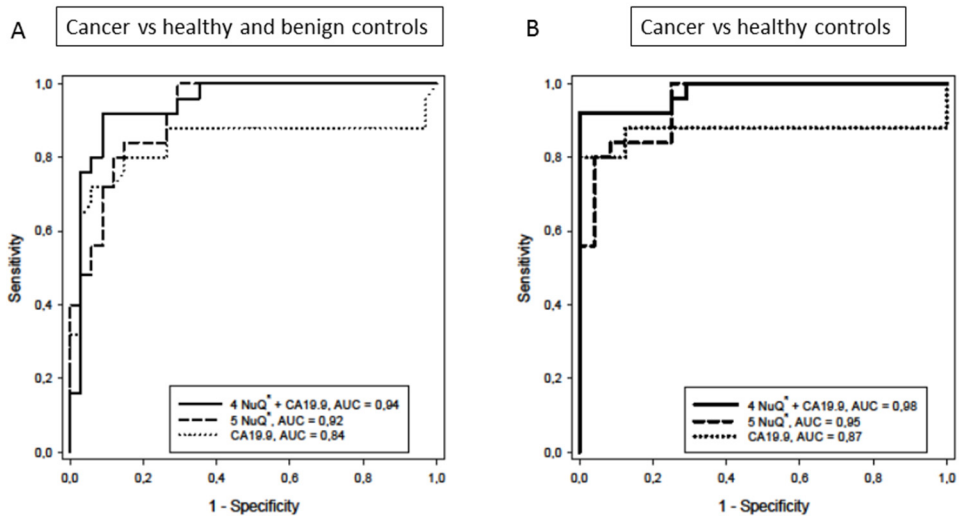

Figure 14.
ROC curves for discrimination of cancer versus healthy and benign control groups (A) and cancer versus healthy group (B).

The results in this study indicate that epigenetic profiling of cfnucleosomes in serum, using a NuQ® immunoassay-assay, may improve the diagnostic evaluation of pancreatic cancer.

Characterization of histone-related chemical modifications in formalin-fixed paraffin-embedded and fresh-frozen human pancreatic cancer xenografts using LC-MS/MS

In paper III, the MS spectrum of histone proteins extracted from nine human pancreatic tumor xenografts, preserved as formalin-fixed paraffin-embedded and fresh-frozen tissue samples, were investigated regarding modifications with a defined mass shift of +14.016 Da, +28.031Da, +42.011 Da or +114.043 Da, corresponding to methylation (Me), di-methylation (Me2), tri-methylation (Me3) acetylation (Ac) and ubiquitination (Ub), respectively. An example of apparent methylation, a modification associated with a mass shift of +14.016 Da is illustrated in Figure 15.

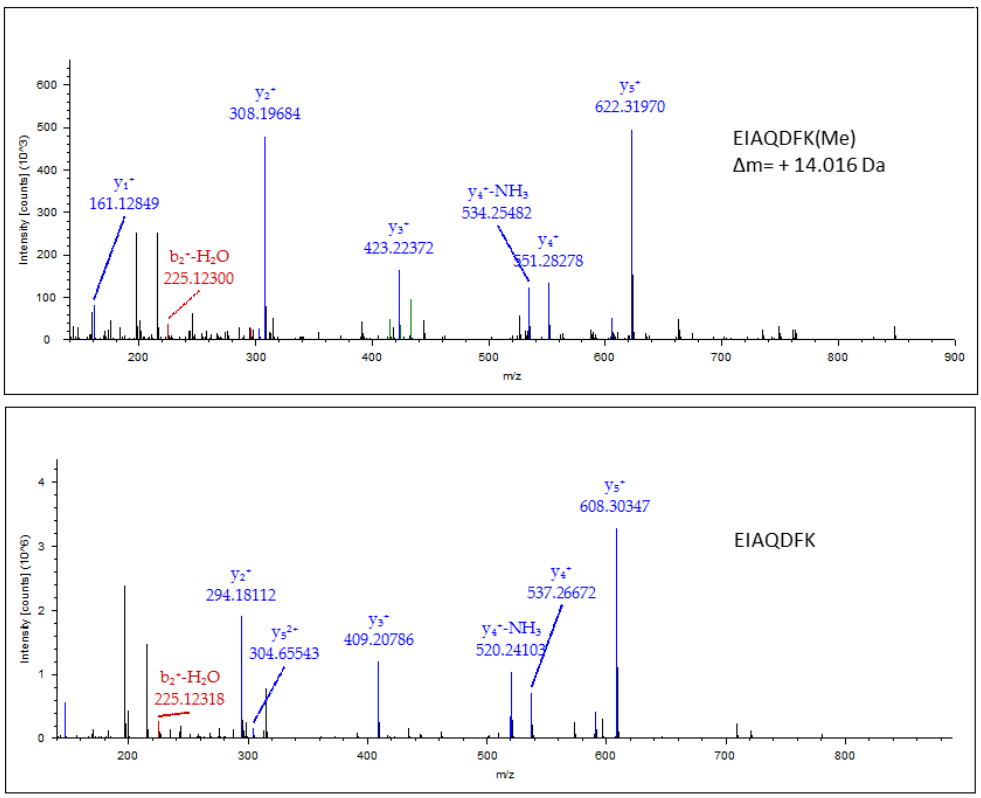

Figure 15.
MS/MS spectrum alignment illustrating a mass shift of + 14.016 Da interpreted as methylation of a lysine residue.

Identification of histone modifications differentially distributed between the groups

By comparison of peptides detected in both experimental groups, the majority of modification sites and the individual modifications were identified in FFPE samples.

The most prominent modification detected in FFPE samples was apparent methylation of lysine residue followed by ubiquitination and acetylation, as shown in Figure 16.

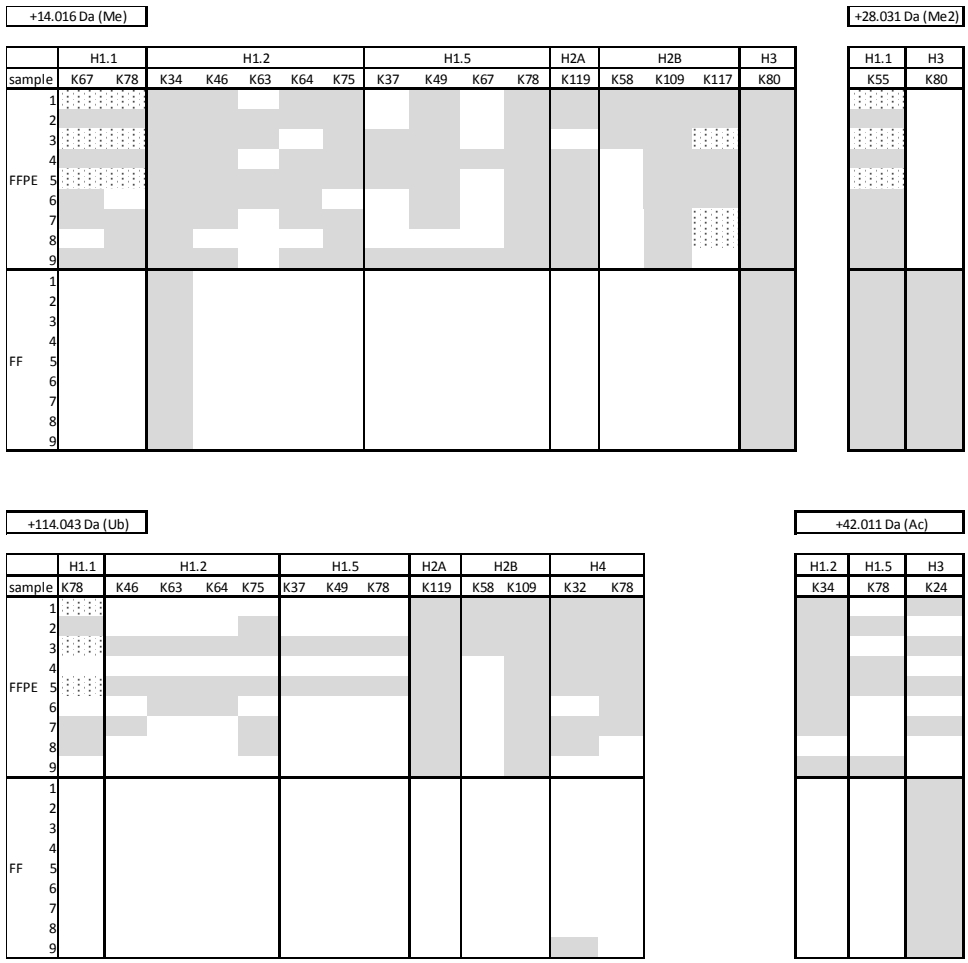

Figure 16. Graphical summary of individual modification sites with defined mass shifts, expressed on respective histone proteins in FFPE (upper box) compared to FF samples (lower box).
Each row represents one sample and each column represents individual modification site. Presence of modifications in individual samples is presented in the gray cells. Absence of modifications is indicated in the unmarked cells. Histone variants, not detected in the sample ale visualized as dashed cells ⁞.

56 % of the detected modifications, including modifications with a mass shift of +14.016 Da, + 42.011 Da and +114.043 Da, were found significantly more frequent in FFPE group. Five individual modifications displayed a uniform pattern throughout the FFPE samples. The remaining 27 modifications were irregularly distributed within the group.

Table 8.
Histone modifications showing differences in the frequency between FF and FFPE.

Histone	Site	FFPE		FF		
		Yes	No	Yes	No	p-value
Presence of mass shift +14.016 Da (Me)						
H1.1	K67	5(6)	1(6)	0	9	**0.002**
	K78	5(6)	1(6)	0	9	**0.002**
H1.2	K34	9	0	9	0	ND
	K46	8	1	0	9	**0.0004**
	K63	4	5	0	9	0.0824
	K64	7	2	0	9	**0.0023**
	K75	8	1	0	9	**0.0004**
H1.5	K37	4	5	0	9	0.0823
	K49	8	1	0	9	**0.0004**
	K67	2	7	0	9	0.4706
	K78	9	0	0	9	**< 0.0001**
H2A	K119	8	1	0	9	**0.0004**
H2B	K58	3	6	0	9	0.2059
	K109	9	0	0	9	**< 0.0001**
	k117	5(6)	1(6)	0	9	**0.002**
H3	K80	9	0	9	0	ND
Presence of mass shift + 28.031 Da (Me2)						
H1.1	K55	6(6)	0(6)	9	0	ND
H3	K80	0	9	9	0	**< 0.0001**
Presence of mass shift + 114.043 (Ub)						
H1.1	K78	3(6)	3(6)	0	9	**0.044**
H1.2	K46	3	6	0	9	0.2059
	K63	3	6	0	9	0.2059
	K64	3	6	0	9	0.2059
	K75	5	4	0	9	0.0294
H1.5	K37	2	7	0	9	0.4706
	K49	2	7	0	9	0.4706
	K78	2	7	0	9	0.4706
H2A	K119	9	0	0	9	**< 0.0001**
H2B	K58	3	6	0	9	0.2059
	K109	9	0	0	9	**< 0.0001**
H4	K32	7	2	1	8	**0.0023**
	K78	7	2	0	9	**0.0023**
Presence of mass shift + 42.011 Da (Ac)						
H1.2	K34	8	1	0	9	**0.0004**
H1.5	K78	3	6	0	9	0.2059
H3	K24	4	5	9	0	**0.0294**

Abbreviations: ND = no difference between groups. The findings in nine FF versus nine FFPE samples are presented as presence or absence of the respective histone modification. P-values are reported at significance level of 0.05. The number of samples included in the analysis is presented in parenthesis in occasions of absent histone variants. Bold values indicate modifications with significantly higher occurrence in FFPE samples.

In the FF specimens, five modifications were homogenously present in all samples. A modification with a mass shift of +114.043 Da was observed in one sample. A modification with a mass increase of 42.011 Da located on H3K24, was significantly more pronounced in FF samples. A modification with a mass shift of +28.031 Da sited on H3K80, appeared only in the FF group.

In total, five individual modifications were detected in both FFPE and FF groups. All identified modifications and their distribution, within the respective group, are summarized in Table 8.

The results presented in this study revealed, that the occurrence of the detected lysine modifications related to histone proteins was significantly higher in FFPE samples as compared to the equivalent FF samples. Findings in this study suggest that such modifications are likely condensation products originating from FFPE tissue processing and thus false positive PTMs artefacts.

Histone Profiling Reveals the H1.3 Histone Variant as a Prognostic Biomarker for Pancreatic Ductal Adenocarcinoma

In paper IV, LC-MS/MS was used to assess the profile of histone variants and histone related PTMs, including methylation (Me), di- and trimethylation (Me2) and (Me3), acetylation (Ac) and ubiquitination (Ub) from PDAC tissue and normal pancreas biopsies. The prognostic value of the selected PDAC specific histone variant H1.3 was evaluated in an external patient cohort comprising 72 samples.

Characterization of histone variants and histone related PTMs using LC-MS/MS.

In summary, the MS based characterization of the extracted proteins resulted in the identification of 24 histone protein variants associated with linker histone H1 family and core histone families, including H2A, H2B, H3 and H4. The comparative investigation of the proteomic profile, as well as the immunohistological evaluation, indicated that histone variant H1.3 was predominantly occurring in the pancreatic cancer tissue and was thus selected for further analysis as a PDAC specific epigenetic marker. The results of histone protein identifications and the distribution of H1.3 within the pancreatic cancer tissue are summarized in Figure 17.

A

Accession	Description	Fisher's exact test p-value
P07305	Histone H1.0	1
Q02539	Histone H1.1	0.474
P16403	Histone H1.2	0.105
P16402	Histone H1.3	0.005
P10412	Histone H1.4	1
P16401	Histone H1.5	1
Q92522	Histone H1x	1
P04908	Histone H2A type 1-B/E	0.005
Q8IUE6	Histone H2A type 2-B	1
Q16777	Histone H2A type 2-C	1
P0C0S5	Histone H2A.Z	1
Q71UI9	Histone H2A.V	1
P58876	Histone H2B type 1-D	0.605
P62807	Histone H2B type 1-C/E/F/G/I	1
P06899	Histone H2B type 1-J	1
O60814	Histone H2B type 1-K	0.334
Q99880	Histone H2B type 1-L	1
Q99879	Histone H2B type 1-M	1
Q99877	Histone H2B type 1-N	1
Q8N257	Histone H2B type 3-B	0.141
P68431	Histone H3.1	0.474
Q71DI3	Histone H3.2	1
P84243	Histone H3.3	1
P62805	Histone H4	1

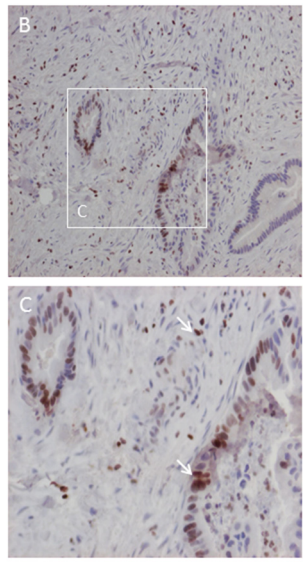

Figure 17. The comprehensive histone profile and H1.3 distribution in PDAC tissue. The profile of histone variants is summarized in (A). Positive H1.3 staining of tumor cells and TILs is indicated by the arrows and illustrated in (B) and (C).The images were magnified 10x in (B) and 20x in (C).

PTMs detected on peptides generated from histone proteins showed a consistently overlapping distribution pattern in both experimental groups, resulting in a non-significant outcome. The complex array of the PTMs is summarized in Figure 18.

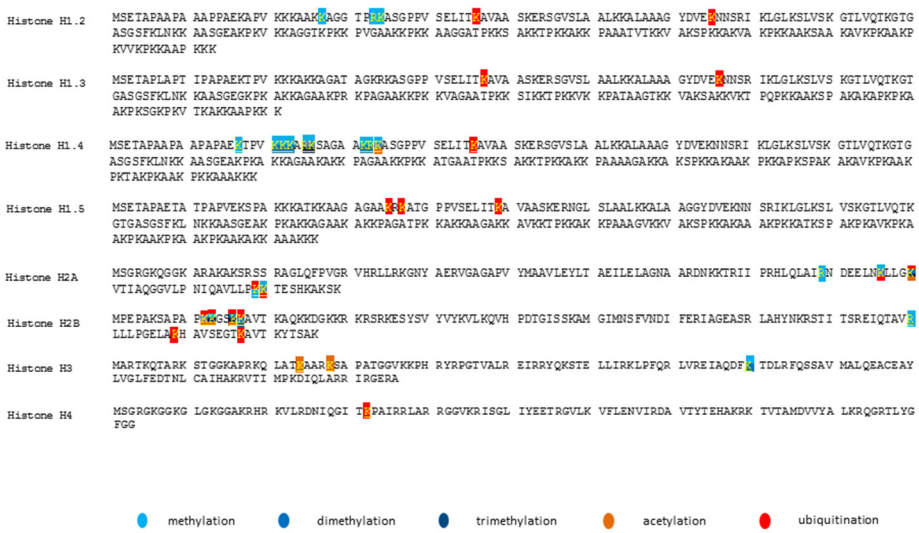

Figure 18. Distribution of histone-related PTMs.
Sequence alignments, representing the linker histone H1 variants and the main core histone families. The modified lysine and arginine residues are indicated by the color of the individual annotated modifications.

Evaluation of H1.3 as a prognostic biomarker in external PDAC cohort

In accordance with results obtained from the MS analysis, the majority of the PDAC samples exhibited intra-tumor H1.3. The subcellular expression of H1.3 in pancreatic cancer biopsies is presented in Figure 19.

H1.3 expression	
Positive n = 50 (80.6 %)	Negative n = 12 (19.4 %)
Sub-cellular compartment	No. of H1.3 positive cases (%)
Nucleus	27 (54)
Cytoplasm/Membrane/Nucleus	17 (34)
Cytoplasm/Membrane	6 (12)

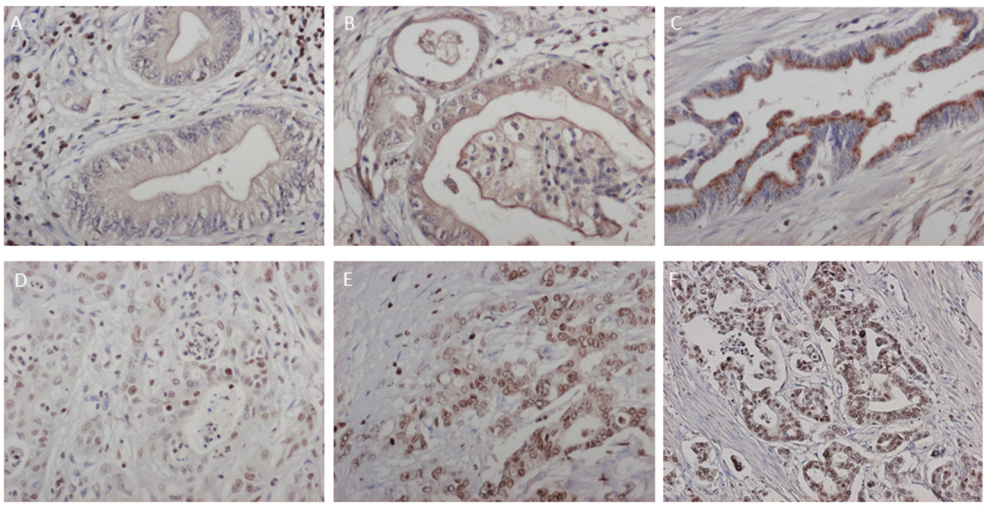

Figure 19. Subcellular expression of H1.3 in PDAC specimens.
Cytoplasm/membrane staining of H1.3 in PDAC tumor cells with mild, intermediate and intense intensity is illustrated in (A, B and C), respectively. The nuclear staining of H1.3 in PDAC tumor cells with mild, intermediate and intense intensity is illustrated in (D, E and F), respectively. The images were magnified 20x.

With the exception for the age of the patient, the expression of H1.3 was not associated with other factors, possibly influencing the clinical outcome, such as the gender, tumor size, T-stage, grade of differentiation, lymph node metastasis, vascular, perineural, adipose tissue invasion, peritumoral inflammation, resection margin status or adjuvant chemotherapy (Table 9).

Table 9.
The correlation between the H1.3 expression and clinicopathological data in resected PDAC.

		H1.3 expression		
	No. of patients (%)	**Positive n = 50 (80.6 %)**	**Negative n = 12 (19.4 %)**	**P-value**
Age (years), median [IQR]	67 [43-76]	66 [43-78]	73 [58-76]	0.012
Male gender	29 (46.8)	25 (50)	4 (33.3)	0.173
Tumor size (cm), median [IQR]	3 [0.3-8.5]	3 [1-8.5]	3 [0.3-4]	0.285
T-stage				
T2	11 (17.7)	9 (18)	2 (16.7)	nd
T3	47 (75.8)	38 (76)	9 (75)	0.942
Poor differentiation	36 (58.1)	31 (62)	5 (41.7)	0.173
Lymph node metastasis	38 (61.3)	31 (62.0)	7 (58.3)	0.815
Vascular invasion	17 (27.4)	13 (26)	4 (33.3)	0.721
Perineural invasion	37 (59.7)	27 (43.5)	10 (83.3)	0.063
Adipose tissue invasion	31 (50)	24 (48)	7 (58.3)	0.520
Inflammation	13 (21)	9 (18)	4 (33.3)	0.256

Abbreviation: IQR, interquartile range.

Patients with a positive H1.3 expression showed a median survival of 27.6 month with a 5-year survival of 11.1 % as compared to 45.6 months and a 5-year survival of 42 %, estimated in patients with a negative H1.3 expression, as illustrated in Figure 20.

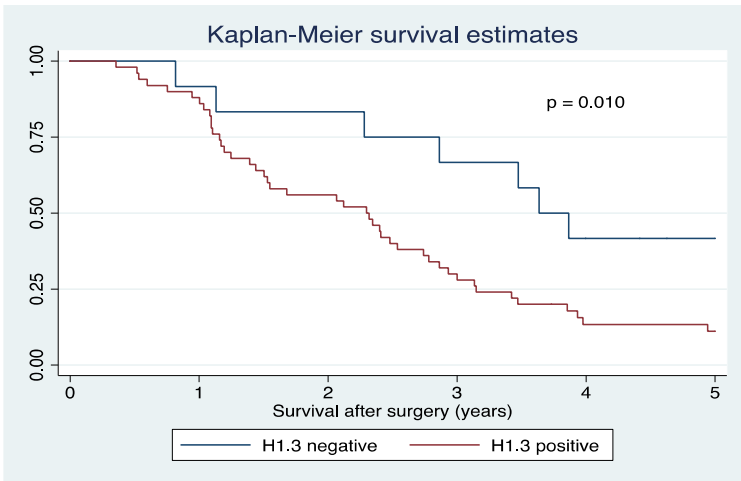

Figure 20.
Kaplan-Meier survival curves for patients with positive or negative H1.3 expression

As presented in Table 10, the positive H1.3 expression was identified as a predictor of poor survival with a hazard ratio estimated to 2.37-2.65.

Table 10.
Multivariate Cox regression analysis of H1.3 expression for the survival of patients with resected PDAC (n=62).

H1.3 expression (positive vs negative)	Hazard ratio	*P*
Unadjusted	2.37 (1.17-5.31)	0.018
Adjusted	2.65 (1.12- 6.27)	0.026

Values of 95 % confidence intervals are presented in parenthesis.

Conclusions

Major conclusions based on the results obtained from the studies of the present thesis are recapitulated below:

Paper I

Pancreatic cancer possesses a high metastatic capacity even in small tumors. The prognostic impact of tumor size is restricted to patients with localized disease.

Paper II

Epigenetic profile of cfnucleosomes in serum detected by a non-invasive NuQ® immuno-assay may represent a new strategy for the diagnosis of pancreatic cancer.

Paper III

FFPE tissue processing may result in irreversible chemical modifications of histone proteins which correspond in mass shift of important PTMs and may thus be incorrectly interpreted as endogenously created post translational modifications. A careful selection of experimental material should be made prior the investigational mass spectral analysis, as well as manual examination of detected modifications, to eliminate an incorrect interpretation of the data that may result in false positive results.

Paper IV

The expression of histone variant H1.3 in PDAC tumors is associated with poor survival and may thus function as a prognostic factor.

Discussion

Relationship between tumor size and clinical outcome in PDAC

Traditionally, tumor size has been viewed as an important prognostic factor. According to the AJCC/tumor–node–metastasis (TNM) classification system, the earliest cancer stage is defined as tumors with a maximum size of 2 cm, limited to the pancreas and free from metastases (T1N0M0) [46, 47]. Such small tumors are generally considered as technically resectable and presumably associated with a more favorable prognosis [48]. Larger tumors, on the other hand, are generally recognized as advanced tumors often accompanied by higher frequency of metastasis and associated with a poor prognosis [29, 49-51].

The results of paper I confirmed that the probability of metastatic spread increased with tumor size. On the other hand, nearly one third of primary tumors with a size below 0.5 cm presented with regional or distant spread. Evidently, certain subtypes of pancreatic cancer cells harbor an aggressive metastatic potential and disseminate from undetectable tumors. It means that shedding of tumor cells is not profoundly a late event in the progression of PDAC.

As presented in the introduction part of the thesis, the systemic advancement of pancreatic cancer was proposed to follow two basic scenarios. The classical model of linear cancer progression with a subsequent late dissemination [29] and the renewed parallel model based on genomic rearrangement patterns, resulting in a rapid systemic involvement [30].

In line with the parallel progression model, the metastatic initiator cells generally detach from the primary tumors early and infiltrate regional or distant sites before the acquisition of fully malignant phenotype [52]. Simultaneously, such disseminated tumor cells may enter a dormant state in the infiltrated organ and result in a systemic recurrence months after the surgical resection of primary tumor [53].

In summary, the findings of paper I highlighted the apparent discrepancy between the existing staging and tumor biology, which may influence the future assessment of pancreatic cancer staging and the treatment recommendations.

Concise diagnosis, including the accurate stage determination and prediction of the disease progression, are crucial for the choice of the most appropriate initial treatment and further clinical management. Specific biomarkers, taking tumor biology into account, may substantially benefit the diagnostic process and aid the classification of patients with specific tumor phenotypes into prognostic subgroups. The addition of such PDAC specific markers in the daily clinical practice may bring the implementation of the tailored way of disease management one step further.

Meanwhile, PDAC should be considered as a systemic disease at any time of diagnosis and an appropriate systemic treatment should be administered as soon as possible to target undetectable micrometastases [54].

Diagnostic value of NuQ® immuno-assay in PDAC

Nucleosomal markers, included in the panel of NuQ® assays performed in study II, are all involved in cellular events modifying gene transcription or stability of the genome. Alternatively spliced histone variants, macroH2A.1and H2A.Z mediate chromatin rearrangements to regulate DNA damage responses [55]. Histone related PTM, H2AK119Ub, is one of the most abundant histone modifications in mammalian cells associated with inhibition of transcription initiation by preventing H3K4 methylation [56].

These markers could be easily combined to produce highly accurate diagnostic tests with the possibility to incorporate CA19-9, which is currently in clinical use. Like CA19-9, the levels of cfnucleosome biomarkers could be determined using a simple convenient ELISA based test, requiring only a small volume of serum.

The results indicated that the panel of serum nucleosomes associated with epigenetic markers may differentiate patients with pancreatic cancer from healthy individuals with a higher precision than CA19-9. The panel of NuQ® assays was also able to discriminate patients with cancer from benign pancreatic diseases and by that overcome the limitation of CA19-9 which is frequently increased in patients with benign extrahepatic cholestatic jaundice [57].

To this point, despite the excellent accuracy of the NuQ® assay panel to discriminate patients with PDAC, the potential screening sensitivity is insufficient beyond high risk patients.

To improve the performance of such a diagnostic test panel, alternative assays including nucleosome associated epigenetic modifications specific for PDAC would be essential. Customized assays, enabling the detection of all PDAC specific biomarkers in a single analysis, would however be desirable to facilitate the clinical use of a particular diagnostic panel.

LC-MS/MS analysis of nucleosome associated epigenetic modifications

Archival formalin-fixed paraffin-embedded tissue material is commonly utilized for mass spectrometry based profiling of post-translational modifications on histone proteins [58]. Formalin used for fixation of the tissue specimen may, however, induce irreversible chemical alterations of histone proteins, introducing various shifts in masses [59] that could be incorrectly interpreted as endogenously created post-translational modifications.

The results in paper III suggested that histone modifications, exclusively present in FFPE processed tissue, were most likely condensation products resulting from formaldehyde associated reactions with the tissue proteins. Such formalin induced chemical modifications with mass shifts corresponding to endogenous PTMs are not possible to compensate for in search software. A thoughtful selection of experimental material should thus be considered prior the investigational mass spectral analysis.

Moreover, the amino acid sequence of histone proteins is highly rich in lysine and arginine, especially at the terminal domains. The digestion of proteins with trypsin, which cleaves peptide chains mainly at the carboxyl side of lysine and arginine, may yield very short peptides that are not possible to analyze [60]. Due to such loss of peptides originating from the terminal domain of histone proteins, the discovery of novel disease related PTMs, using the classical bottom up MS approach, remains unrealizable.

Nevertheless, modifications reported in paper III, considered as epigenetic marks of biological origin, including methylation of H1.2K34, H3K80 and acetylation of H3K24, were also identified in clinical samples and may be of relevance for further investigation.

Prognostic value of histone variant H1.3 in PDAC

The results in paper IV indicated that H1.3 expression in PDAC tumors is associated with poor survival and may thus be considered as a tissue specific biomarker for the prognosis of PDAC.

Presently, the function of histone H1.3 subtype in pancreatic cancer has not been elucidated.

As previously described, stress induced H1.3 may be involved in epigenetic silencing by inverse modulation of DNA and histone methylation and histone acetylation. Nucleosome incorporated H1.3 interact with DNA methyltransferases, promoting establishment and maintenance of extensive methylated of CpG regions of DNA. On the contrary, the interference of H1.3 with specific histone-lysine N-methyltransferase and histone acetyltransferase inhibits methylation of H3K4, as well as acetylation of H3, which may further favor transcriptional repression [61, 62, 63]. In cancer, hypermethylated gene promotors were reported to correlate with epigenetic downregulation of tumor suppressors [64] or silencing of genes essential for the sensitivity to chemotherapy [65]. In addition, decreased expression of epigenetic marks H3K4Me2, H3K9Me2 and H3K18Ac, as well as methylation status of CpG islands, were individually reported as significant predictors of poor survival in PDAC [66, 67].

Based on the subcellular distribution of H1.3 revealed by IHC staining, it is reasonable to assume that the role of H1.3 in PDAC biology may expand beyond the nuclear function. As stated in several reports, histone proteins may, in response to the environmental stress, relocate to the cell surface where they interact with negatively charged molecules, such as proteoglycans, and regulate various cellular processes, including cell proliferation and matrix remodeling [68, 69, 70].

Taken together, it remains possible that intra-tumor expression of H1.3 may contribute to the aggressiveness of the disease and poor prognosis in PDAC. H1.3 could thus be used as a functional tissue biomarker for the classification of patients with PDAC into prognostic subgroups associated with poor survival, requiring more radical treatment.

Future Perspectives

The discovery of biomarkers intended for the clinical management of pancreatic adenocarcinoma is a rapidly expanding field. This research trend is continuously generating a remarkable amount of new candidates. The major challenge today is probably to identify biomarkers that actually add a substantial value in diagnosis, prognosis or risk assessment in clinical practice.

NuQ® assay associated with PDAC specific histone variant H1.3

Epigenetics plays an important role in the phenotypical changes linked to the malignant transformation and to the complex biology of pancreatic cancer. The diversity of epigenetic marks may offer specific and easy assessable biomarkers with strong potential for assay development.

The upcoming assignment is directly connected to the studies presented in this thesis and aimed to develop and validate NuQ® immuno-assays for a quantification of nucleosome-bound histone H1.3. Thereafter, a pilot study will be conducted, to analyze the possible diagnostic performance of H1.3 associated NuQ® assay alone or as a member of a panel.

Histone variant H1.3 expression in TILs

The evident immune infiltrate involving expression profiles related to tumor infiltrating B and T cells is associated with the newly classified immunogenic subtype of PDAC presented in Nature [71]. The role of TILs in PDAC was also previously described in correlation with clinical outcome.

In the final study presented in this thesis, TILs highly expressing H1.3 were identified in all tumor specimen. It would be interesting to evaluate the relevance of such TILs, as an immunologic biomarker. A topic, not covered in this thesis.

The initial study could involve a re-evaluation of PDAC tissue sections, currently stained for H1.3. Firstly, the proportion of the H1.3 stained lymphocytes in the tumor section could be estimated. Secondly, quantification of H1.3 positive TILs, in the stromal or intratumor areas could be performed using digital image analysis. Thirdly, subtyping of H1.3 positive TILs may provide a biological relevant information regarding the composition of immune cells and anti-tumor activity.

Information obtained from the analyses could be then hopefully used for prognostic or predictive estimations.

Selection of control samples for biomarker discovery

Control groups comprising healthy individuals and patients with benign disease in pancreas are commonly included in the discovery phase of new biomarkers for the management of PDAC to minimize a biased selection of biomarker candidates for further evaluation studies.

It is worth to consider, that patients suspected with PDAC are commonly suffering from severe medical conditions related to the age or life style. The underlying health issues as such, or the pharmacological response to ingested pharmaceuticals may result in significant molecular changes, often detectable in the blood. Without corresponding controls, it will remain uncertain whether the discriminating biomarkers reflect an underlying medical condition or the presence of pancreatic cancer. To avoid redundant results, appropriate controls, which are carefully matched to the PDAC cases, regarding clinicopathological characteristics and other relevant factors, should be included in the evaluation process.

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References

1. Longnecker D. Anatomy and Histology of the Pancreas. Pancreapedia: Exocrine Pancreas Knowledge Base, version 1.0, March 21, 2014.
2. Pandol SJ. The Exocrine Pancreas. San Rafael, CA: Morgan & Claypool Life Sciences; 2010.
3. Poruk KE, Firpo MA, Adler DG, Mulvihill SJ. Screening for pancreatic cancer: why, how, and who? Ann Surg. 2013 Jan;257(1):17-26.
4. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA Cancer J Clin. 2017 Jan;67(1):7-30.
5. Cancerfondsrapporten 2017. Cancerfonden, p. 19 statistik. Retrieved 2017-06-08.
6. Aho U, Zhao X, Löhr M, Andersson R. Cancer Facts & Figures 2013. Atlanta: American Cancer Society (American Cancer Society 2013). Molecular mechanisms of pancreatic cancer and potential targets of treatment. Scand J Gastroenterol. 2007 Mar;42(3):279-96.
7. Yeo TP. Demographics, epidemiology, and inheritance of pancreatic ductal adenocarcinoma. Semin Oncol. 2015 Feb;42(1):8-18.
8. Stark A. Eibl, Guido. Pancreatic Ductal Adenocarcinoma. Pancreapedia: Exocrine Pancreas Knowledge Base, version 1.0 May 23; 2015.
9. Swords DS, Firpo MA, Scaife CL, Mulvihill SJ. Biomarkers in pancreatic adenocarcinoma: current perspectives. Onco Targets Ther. 2016 Dec 9;9:7459-7467.
10. Ducreux M, Cuhna A.Sa, Caramella C, Hollebecque A, Burtin P, Goéré D, Seufferlein T, Haustermans K, Van Laethem JL, Conroy T, Arnold D, on behalf of the ESMO Guidelines Committee. Cancer of the pancreas: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. Annals of Oncology 26 (Supplement 5): v56–v68, 2015.
11. Byrd DR, Edge S, Compton CC, Fritz AG, Greene FL, Trotti A (Editors). AJCC cancer Staging manual. 7th edition New York, Springer, 2010.
12. Young JL Jr, Roffers SD, Ries LAG, Fritz AG, Hurlbut AA (eds). SEER Summary Staging Manual - 2000: Codes and Coding Instructions, National Cancer Institute, NIH Pub. No. 01-4969, Bethesda, MD, 2001.
13. Katz MH, Pisters PW, Evans DB, Sun CC, Lee JE, Fleming JB, Vauthey JN, Abdalla EK, Crane CH, Wolff RA, Varadhachary GR, Hwang RF. Borderline resectable pancreatic cancer: the importance of this emerging stage of disease. J Am Coll Surg. 2008 May;206(5):833-46; discussion 846-8.
14. Hidalgo M. Pancreatic cancer. N Engl J Med. 2010 Apr 29;362(17):1605-17. Erratum in: N Engl J Med. 2010 Jul 15;363(3):298.

15. Hartwig W, Büchler MW. Pancreatic Cancer: Current Options for Diagnosis, Staging and Therapeutic Management. Gastrointest Tumors. 2013 Sep;1(1):41-52

16. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011 Aug 13;378(9791):607-20.

17. Jamieson NB, Chan NI, Foulis AK, Dickson EJ, McKay CJ, Carter CR. The prognostic influence of resection margin clearance following pancreaticoduodenectomy for pancreatic ductal adenocarcinoma. J Gastrointest Surg. 2013 Mar;17(3):511-21.

18. Macías N, Sayagués JM, Esteban C, Iglesias M, González LM, Quiñones-Sampedro J, Gutiérrez ML, Corchete LA, Abad MM, Bengoechea O, Muñoz-Bellvis L. Histologic Tumor Grade and Preoperative Bilary Drainage are the Unique Independent Prognostic Factors of Survival in Pancreatic Ductal Adenocarcinoma Patients After Pancreaticoduodenectomy. J Clin Gastroenterol. 2017 Jan 13.

19. Bilici A. Prognostic factors related with survival in patients with pancreatic adenocarcinoma. World J Gastroenterol. 2014 Aug 21;20(31):10802-12.

20. Chen JW, Bhandari M, Astill DS, Wilson TG, Kow L, Brooke-Smith M, Toouli J, Padbury RT. Predicting patient survival after pancreaticoduodenectomy for malignancy: histopathological criteria based on perineural infiltration and lymphovascular invasion. HPB (Oxford). 2010 Mar;12(2):101-8.

21. Jamieson NB, Mohamed M, Oien KA, Foulis AK, Dickson EJ, Imrie CW, Carter CR, McKay CJ, McMillan DC. The relationship between tumor inflammatory cell infiltrate and outcome in patients with pancreatic ductal adenocarcinoma. Ann Surg Oncol. 2012 Oct;19(11):3581-90.

22. McDonald OG, Li X, Saunders T, Tryggvadottir R, Mentch SJ, Warmoes MO, Word AE, Carrer A, Salz TH, Natsume S, Stauffer KM, Makohon-Moore A, Zhong Y, Wu H, Wellen KE, Locasale JW, Iacobuzio-Donahue CA, Feinberg AP. Epigenomic reprogramming during pancreatic cancer progression links anabolic glucose metabolism to distant metastasis. Nat Genet. 2017 Mar;49(3):367-376.

23. DeVita VT, Lawrence TS, Rosenberg SA et-al. DeVita, Hellman, and Rosenberg's cancer, principles & practice of oncology. Lippincott Williams & Wilkins. (2008) ISBN:0781772079.

24. Chari ST, Kelly K, Hollingsworth MA, Thayer SP, Ahlquist DA, Andersen DK, Batra SK, Brentnall TA, Canto M, Cleeter DF, Firpo MA, Gambhir SS, Go VL, Hines OJ, Kenner BJ, Klimstra DS, Lerch MM, Levy MJ, Maitra A, Mulvihill SJ, Petersen GM, Rhim AD, Simeone DM, Srivastava S, Tanaka M, Vinik AI, Wong D. Early detection of sporadic pancreatic cancer: summative review. Pancreas. 2015 Jul;44(5):693-712.

25. Rombouts SJ, Mungroop TH, Heilmann MN,van Laarhoven HW, Busch OR, Molenaar IQ, Besselink MG,and Wilmink JW. FOLFIRINOX in Locally Advanced and Metastatic Pancreatic Cancer: A Single Centre Cohort Study. J Cancer. 2016; 7(13): 1861–1866.

26. Kalimuthu SN, Notta F. Molecular pathogenesis of pancreatic ductal adenocarcinoma. Diagnostic Histopathology. 2016 Jun:22(6); 226-235.

27. Maitra A, Hruban RH. Pancreatic cancer. Annu Rev Pathol. 2008;3:157-88.

28. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008 Sep 26;321(5897):1801-6.

29. Yachida S, Jones S, Bozic I, Antal T, Leary R, Fu B, Kamiyama M, Hruban RH, Eshleman JR, Nowak MA, Velculescu VE, Kinzler KW, Vogelstein B, Iacobuzio-Donahue CA. Distant metastasis occurs late during the genetic evolution of pancreatic cancer. Nature. 2010 Oct 28;467(7319):1114-7.

30. Notta F, Chan-Seng-Yue M, Lemire M, Li Y, Wilson GW, Connor AA, Denroche RE, Liang SB, Brown AM, Kim JC, Wang T, Simpson JT, Beck T, Borgida A, Buchner N, Chadwick D, Hafezi-Bakhtiari S, Dick JE, Heisler L, Hollingsworth MA, Ibrahimov E, Jang GH, Johns J, Jorgensen LG, Law C, Ludkovski O, Lungu I, Ng K, Pasternack D, Petersen GM, Shlush LI, Timms L, Tsao MS, Wilson JM, Yung CK, Zogopoulos G, Bartlett JM, Alexandrov LB, Real FX, Cleary SP, Roehrl MH, McPherson JD, Stein LD, Hudson TJ, Campbell PJ, Gallinger S. A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. Nature. 2016 Oct 20;538(7625):378-382. Erratum in: Nature. 2017 Feb 2;542(7639):124.

31. Lomberk GA. Epigenetic silencing of tumor suppressor genes in pancreatic cancer. J Gastrointest Cancer. 2011 Jun;42(2):93-9.

32. Dwivedi RS, Herman JG, McCaffrey TA, Raj DS. Beyond genetics: epigenetic code in chronic kidney disease. Kidney Int. 2011 Jan;79(1):23-32.

33. Silverman BR, Shi J. Alterations of Epigenetic Regulators in Pancreatic Cancer and Their Clinical Implications. Int J Mol Sci. 2016 Dec 19;17(12).

34. Guo M, Jia Y, Yu Z, House MG, Esteller M, Brock MV, Herman JG. Epigenetic changes associated with neoplasms of the exocrine and endocrine pancreas. Discov Med. 2014 Feb;17(92):67-73.

35. Makohon-Moore A, Iacobuzio-Donahue CA. Pancreatic cancer biology and genetics from an evolutionary perspective. Nat Rev Cancer. 2016 Sep;16(9):553-65.

36. Füllgrabe J, Kavanagh E, Joseph B. Histone onco-modifications. Oncogene. 2011 Aug 4;30(31):3391-403.

37. Neureiter D, Jager T, Ocker M, Kiesslich T. Epigenetics and pancreatic cancer: pathophysiology and novel treatment aspects. World J Gastroenterol. 2014;20(24):7830-48.

38. McCleary-Wheeler AL, Lomberk GA, Weiss FU, Schneider G, Fabbri M, Poshusta TL, Dusetti NJ, Baumgart S, Iovanna JL, Ellenrieder V, Urrutia R, Fernandez-Zapico ME. Insights into the epigenetic mechanisms controlling pancreatic carcinogenesis. Cancer Lett. 2013 Jan 28;328(2):212-21. Erratum in: Cancer Lett. 2013 Aug 28;337(1):143-4.

39. Schneider R, Grosschedl R. Dynamics and interplay of nuclear architecture, genome organization, and gene expression. Genes Dev. 2007 Dec 1; 21(23):3027-43.

40. Mishra A, Verma M. Cancer biomarkers: are we ready for the prime time? Cancers (Basel). 2010 Mar 22;2(1):190-208.

41. Winter JM, Yeo CJ, Brody JR. Diagnostic, prognostic, and predictive biomarkers in pancreatic cancer. J Surg Oncol. 2013 Jan;107(1):15-22.

42. Rhea JM, Molinaro RJ. Cancer biomarkers: surviving the journey from bench to bedside. MLO Med Lab Obs. 2011 Mar;43(3):10-2, 16, 18; quiz 20, 22

43. Overview of the SEER Program. National Cancer Institute. Retrieved 2017-06-09.

44. Vandenbroucke JP, von Elm E, Altman DG, Gøtzsche PC, Mulrow CD, Pocock SJ, Poole C, Schlesselman JJ, Egger M; STROBE Initiative. Strengthening the Reporting of Observational Studies in Epidemiology (STROBE): explanation and elaboration. Epidemiology. 2007 Nov;18(6):805-35.

45. Overview of Immunohistochemistry http://www.thermofisher.com: Thermo Fisher Scientific inc.; 2015. Available from: https://www.thermofisher.com/no/en/home/life-science/protein-biology/proteinbiology-learning-center/protein-biology-resource-library/pierce-proteinmethods/overview-immunohistochemistry.html. Retrieved 2016-11-15

46. Edge S, Byrd DR, Compton CC, Fritz AG, Greene FL, Trotti A. AJCC Cancer Staging Manual. Springer-Verlag New York, 2010.

47. Sobin LH, Gospodarowicz MK, Wittekind C. The TNM classification of malignant tumours. Wiley-Blackwell, 2009.

48. Fortner JG, Klimstra DS, Senie RT, Maclean BJ. Tumor size is the primary prognosticator for pancreatic cancer after regional pancreatectomy. Ann Surg 1996; 223: 147-153.

49. Agarwal B, Correa AM, Ho L. Survival in pancreatic carcinoma based on tumor size. Pancreas 2008; 36: e15-20.

50. Egawa S, Takeda K, Fukuyama S, Motoi F, Sunamura M, Matsuno S. Clinicopathological aspects of small pancreatic cancer. Pancreas 2004; 28: 235-240.

51. Pongprasobchai S, Pannala R, Smyrk TC, Bamlet W, Pitchumoni S, Ougolkov A et al. Long-term survival and prognostic indicators in small (<or=2 cm) pancreatic cancer. Pancreatology: official journal of the International Association of Pancreatology 2008; 8: 587-592.

52. Klein CA. Parallel progression of primary tumours and metastases. Nat Rev Cancer. 2009 Apr;9(4):302-12.

53. Pantel K, Brakenhoff RH. Dissecting the metastatic cascade. Nat Rev Cancer. 2004 Jun;4(6):448-56

54. Christians KK, Heimler JW, George B, Ritch PS, Erickson BA, Johnston F et al. Survival of patients with resectable pancreatic cancer who received neoadjuvant therapy. Surgery 2016; 159: 893-900.

55. Bönisch C, Hake SB. Histone H2A variants in nucleosomes and chromatin: more or less stable? Nucleic Acids Res. 2012 Nov;40(21):10719-41.

56. Vissers JH, Nicassio F, van Lohuizen M, Di Fiore PP, Citterio E.The many faces of ubiquitinated histone H2A: insights from the DUBs. Cell Div. 2008 Apr 22;3:8.

57. Ballehaninna UK, Chamberlain RS.The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. J Gastrointest Oncol. 2012 Jun;3(2):105-19.

58. Noberini R, Uggetti A, Pruneri G, et al. Pathology Tissue-quantitative Mass Spectrometry Analysis to Profile Histone Post-translational Modification Patterns in Patient Samples. Mol Cell Proteomics 2016;15:866-877.

59. Shi SR, Taylor CR, Fowler CB, Mason JT. Complete solubilization of formalin-fixed, paraffin-embedded tissue may improve proteomic studies. Proteomics Clin Appl. 2013 Apr;7(3-4):264-72.

60. Garcia BA, Mollah S, Ueberheide BM, et al. Chemical derivatization of histones for facilitated analysis by mass spectrometry. *Nat Protoc* 2007;2:933-938.

61. Rutowicz K, Puzio M, Halibart-Puzio J, Lirski M, Kotliński M, Kroteń MA, Knizewski L, Lange B, Muszewska A, Śniegowska-Świerk K, Kościelniak J, Iwanicka-Nowicka R, Buza K, Janowiak F, Żmuda K, Jõesaar I, Laskowska-Kaszub K, Fogtman A, Kollist H, Zielenkiewicz P, Tiuryn J, Siedlecki P, Swiezewski S, Ginalski K, Koblowska M, Archacki R, Wilczynski B, Rapacz M, Jerzmanowski A. A Specialized Histone H1 Variant Is Required for Adaptive Responses to Complex Abiotic Stress and Related DNA Methylation in Arabidopsis. Plant Physiol. 2015 Nov;169(3):2080-101.

62. Yang SM, Kim BJ, Norwood Toro L, Skoultchi AI. H1 linker histone promotes epigenetic silencing by regulating both DNA methylation and histone H3 methylation. Proc Natl Acad Sci U S A. 2013;110(5):1708-13.

63. Julio E. Herrera,[1,*] Katherine L. West,[1] R. Louis Schiltz,[2] Yoshihiro Nakatani,[2] and Michael Bustin[1]. Histone H1 Is a Specific Repressor of Core Histone Acetylation in Chromatin. Mol Cell Biol. 2000 Jan; 20(2): 523–529.

64. Herman JG, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. N Engl J Med. 2003;349(21):2042-54.

65. Tan AC, Jimeno A, Lin SH, Wheelhouse J, Chan F, Solomon A, et al. Characterizing DNA methylation patterns in pancreatic cancer genome. Mol Oncol. 2009;3(5-6):425-38.

66. Manuyakorn A, Paulus R, Farrell J, Dawson NA, Tze S, Cheung-Lau G, et al. Cellular histone modification patterns predict prognosis and treatment response in resectable pancreatic adenocarcinoma: results from RTOG 9704. J Clin Oncol. 2010;28(8):1358-65.

67. Thompson MJ, Rubbi L, Dawson DW, Donahue TR, Pellegrini M. Pancreatic cancer patient survival correlates with DNA methylation of pancreas development genes. PLoS One. 2015;10(6):e0128814.

68. Chen R, Kang R, Fan XG, Tang D. Release and activity of histone in diseases. Cell Death Dis. 2014;5:e1370.

69. Hampton RY, Golenbock DT, Raetz CR. Lipid A binding sites in membranes of macrophage tumor cells. J Biol Chem. 1988;263(29):14802-7.

70. Henriquez JP, Casar JC, Fuentealba L, Carey DJ, Brandan E. Extracellular matrix histone H1 binds to perlecan, is present in regenerating skeletal muscle and stimulates myoblast proliferation. J Cell Sci. 2002;115(Pt 10):2041-51.

71. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, Miller DK, Christ AN, Bruxner TJ, Quinn MC, Nourse C, Murtaugh LC, Harliwong I, Idrisoglu S, Manning S, Nourbakhsh E, Wani S, Fink L, Holmes O, Chin V, Anderson MJ, Kazakoff S, Leonard C, Newell F, Waddell N, Wood S, Xu Q, Wilson PJ, Cloonan N, Kassahn KS, Taylor D, Quek K, Robertson A, Pantano L, Mincarelli L, Sanchez LN, Evers L, Wu J, Pinese M, Cowley MJ, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chantrill LA, Mawson A, Humphris J, Chou A, Pajic M, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Lovell JA, Merrett ND, Toon CW, Epari K, Nguyen NQ, Barbour A, Zeps N, Moran-Jones K, Jamieson NB, Graham JS, Duthie F, Oien K, Hair J, Grützmann R, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Rusev B, Capelli P, Salvia R, Tortora G, Mukhopadhyay D, Petersen GM; Australian Pancreatic Cancer Genome Initiative, Munzy DM, Fisher WE, Karim SA, Eshleman JR, Hruban RH, Pilarsky C, Morton JP, Sansom OJ, Scarpa A, Musgrove EA, Bailey UM, Hofmann O, Sutherland RL, Wheeler DA, Gill AJ, Gibbs RA, Pearson JV, Waddell N, Biankin AV, Grimmond SM. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature. 2016 Mar 3;531(7592):47-52.

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Clinical Management of Pancreatic Cancer Aided by Histone Signatures

Pancreatic cancer is one of the most difficult cancers to detect and treat. The survival rate is as dismal today as it was 50 years ago. There is an unmet need to identify new diagnostic and prognostic biomarkers that could contribute to earlier diagnosis and aid in the choice of treatment.

Epigenetic modifications are implicated the pathogenesis of pancreatic cancer and may function as biomarkers. This thesis re-evaluates the current staging system for pancreatic cancer at the population level and aims to identify and develop nucleosome associated epigenetic biomarkers in serum and tissue.

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