Universidade de Lisboa

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Epigenetic Regulation of Signalling Pathways in Pancreatic Cancer

Inês Correia da Silva Pires Faleiro

Dissertação orientada pelo Professor Doutor Pedro Castelo-Branco e pela Professora Doutora Elsa Rodrigues.

Mestrado em Ciências Biofarmacêuticas

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De acordo com o disposto no ponto 1 do artigo nº 45 do Regulamento de Estudos Pós Graduados da Universidade de Lisboa, deliberação nº 4624/2012, publicada em Diário da República – II Série nº 65 – 30 de Março de 2012, o Autor desta dissertação declara que participou na concepção e execução do trabalho experimental, interpretação dos resultados obtidos e redação dos manuscritos.

The studies presented in this thesis were performed at the Center for Biomedical Research (CBMR), Algarve University, under the scientific supervision of Professor Pedro Castelo-Branco, and at the Faculty of Pharmacy, University of Lisbon, under the scientific supervision of Professor Elsa Rodrigues.

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Abstract

Pancreatic cancer is one of the most lethal malignancies worldwide. Deregulation of epigenetic marks is known to alter the expression of crucial genes for cancer development. Replicative immortality, achieved mainly through telomerase reactivation, and aberrant activation of the PI3K/Akt, Wnt, Notch and Hedgehog pathways triggers transduction cascades that potentiate cell proliferation.

The aim of this study is to evaluate epigenetic alterations of genes related with these biological processes as potential pancreatic cancer biomarkers.

Gene selection of the PI3K/Akt, Wnt, Notch and Hedgehog pathways was based on differential expression between normal and malignant tissue using the pancreatic expression database and the miRDB database was used to uncover miRNAs targeting the selected genes. Methylation and miRNA analysis was performed using The Cancer Genome Atlas (TCGA) data on the cohort of pancreatic cancer and the impact of DNA methylation and miRNA expression on patient's outcome was also analysed.

Most of the selected genes presented higher expression in tumour tissue. Our results reveal that the majority of the CpGs sites analysed were hypermethylated in tumour tissue. Methylation levels of the selected genes allowed the distinction between normal and malignant tissue even in initial stages of the disease revealing its potential as a diagnostic tool for pancreatic cancer.

The methylation levels of the *TERT*, *ITGA4*, *SFN, ITGA2*, *PIK3R1* and *SFRP2* genes could act as independent prognostic indicators of patients' survival with higher sensitivity and specificity than the currently implemented biomarker. Additionally, differential methylation of the *TERT, SFN* and *PIK3R1* genes were also associated with recurrence of the patients.

Moreover, analysis of the expression of miRNAs involved in the regulation of the *TGFBR1, PTEN, EIF4EBP1, AKT3, JAG1* and *CSNK1A1* have demonstrated the ability to discriminate between groups of patients with different outcomes when comparing the patients with highest and lowest expression of each miRNAs.

Despite the promising results in this area no epigenetic biomarkers have reached the clinic yet. Our results reveal that the methylation levels of genes involved in pancreatic carcinogenesis could be used to predict the outcome of pancreatic cancer patients with high sensitivity and specificity.

These results provide new evidences of the potential of epigenetic alterations as pancreatic cancer biomarkers for disease screening and management.

Keywords

Pancreatic Cancer, epigenetics, DNA methylation, miRNAs, biomarker

Resumo

O cancro do pâncreas é um dos mais letais em todo o mundo e a desregulação do padrão epigenético das células influencia a expressão de genes cruciais para o desenvolvimento e progressão da doença. A capacidade ilimitada de autorrenovação celular resultante principalmente da reativação da enzima telomerase e a ativação das vias de sinalização PI3K/Akt, Wnt, Notch e Hedgehog desencadeia cascatas de transdução de sinal que potenciam a proliferação celular contribuindo para o processo carcinogénico.

O principal objetivo deste estudo é avaliar alterações epigenéticas de genes relacionados com estes processos biológicos como potenciais biomarcadores para o cancro do pâncreas.

A selecção dos genes envolvidos nas vias de sinalização PI3K/Akt, Wnt, Notch e Hedgehog foi baseada na expressão diferencial dos genes entre tecido normal e maligno usando a base de dados Pancreatic Expression Database e para a selecção de miRNAs envolvidos na regulação dos genes de interesse recorremos à base de dados miRDB. Os níveis de metilação e de expressão dos miRNAs foram analisados usando os dados do The Cancer Genome Atlas (TCGA) relativos à coorte de pacientes com cancro do pâncreas e o seu potencial para discriminar entre pacientes com diferentes desfechos clínicos e para identificar os pacientes que se encontram em maior risco de progressão da doença assim como uma menor sobrevivência dos pacientes foi avaliado.

A maioria dos genes selecionados apresentou maior expressão no tecido tumoral. Os nossos resultados revelam que a maioria das regiões genómicas analisadas estão hipermetiladas no tecido tumoral e que os níveis de metilação dos genes selecionados permitem a distinção entre tecido normal e maligno mesmo nos estadios iniciais da doença, revelando seu potencial como biomarcador de diagnóstico para o cancro de pâncreas.

A metilação dos genes *TERT, ITGA4, SFN, ITGA2, PIK3R1* e *SFRP2* permitiram discriminar entre grupos de pacientes com diferentes desfechos clínicos considerando o seu tempo de sobrevida com maior sensibilidade e especificidade que o biomarcador atualmente implementado na clinica para este tipo de cancro. Adicionalmente, a metilação diferencial dos genes TERT, SFN and PIK3R1 revelou estar também associada com a recorrência dos pacientes.

Além disso, a análise de expressão de miRNAs envolvidos na regulação dos genes *TGFBR1, PTEN, EIF4EBP1, AKT3, JAG1* and *CSNK1A1* permitiu diferenciar grupos de pacientes com diferentes tempos de sobrevivência comparando os pacientes com maior e menor expressão de cada miRNA.

Os resultados obtidos demonstram o potencial da análise de metilação do DNA e dos níveis de miRNAs como indicadores de prognóstico com elevada sensibilidade e especificidade. Este estudo revela novas evidências sobre o potencial das alterações epigenéticas como biomarcadores de diagnóstico e prognóstico para o cancro do pâncreas de modo a contribuir para uma melhoria da qualidade e esperança de vida de pacientes com esta doença.

Palavras-chave

Cancro pancreático, epigenética, metilação de DNA, miRNAs, biomarcadores

Index

List of Tables

List of Figures

Abbreviations

5hmC – 5-hydroxymethylcytosine

5mC – 5- methylcytosine

ADM – Acinar to ductal metaplasia

ALT – Alternative lengthening of telomeres mechanism

AUC – Area under curve

CA19-9 – Cancer antigen 19-9

DNMT – DNA methyltransferase

EMT – Epithelial to mesenchymal transition

H3K27ac – Acetylation of histone 3 at lysine 27

H3K27me – Methylation of histone 3 at lysine 27

H3K36me – Methylation of histone 3 at lysine 36

H3K4me – Methylation of histone 3 at lysine 4

H3K4me2 – Di-methylation of histone 3 at lysine 4

H3K4me3 – Tri-methylation of histone 3 at lysine 4

H3K9ac – Acetylation of histone 3 at lysine 9

H3K9me – Methylation of histone 3 at lysine 9

H3K9me3 – Tri-methylation of histone 3 at lysine 9

HAT – Histone acetyltransferase

HDAC – Histone deacetylase

HDM – Histone demethylase

HTM – Histone methyltransferase

IPMN – Intraductal papillary-mucinous neoplasm

OS – Overall survival

PanIN – Pancreatic intraepithelial neoplasia

PanNETs – Pancreatic neuroendocrine tumours

PCA – Pancreatic cancer

PDAC – Pancreatic ductal adenocarcinoma

PED – Pancreatic expression database

PI3K – Class I phosphoinositidine 3- kinases

RFS – Recurrence-free survival

ROC – Receiver operator curve

TCGA – The Cancer Genome Atlas

TERT – Telomerase reverse transcriptase gene

TET – Ten-eleven translocation enzymes

THOR – TERT Hypermethylated oncologic region

CHAPTER 1 - GENERAL INTRODUCTION

1. CHAPTER 1 - GENERAL INTRODUCTION

1.1 Pancreatic Cancer

Cancer is defined as a group of diseases characterized by an abnormal and uncontrolled division of cells that can ultimately proliferate and invade other tissues [1,2]. Hananan and Weinberg have proposed six biological processes as the hallmarks of cancer: sustained proliferative signalling, replicative immortality, bypass of growth suppressors regulation, resistance to cell death, induction of angiogenesis and ability to metastasize and invade other tissues (Fig. 1A) [2]. Recently, two more processes were proposed to the list as critical events to tumour development: cell reprogramming of energy metabolism and the ability to evade the control mediated by our immune system (Fig. 1B) [2].

It is a complex disease marked by aberrant expression of tumor promoting genes, named oncogenes, and tumor suppressor genes [2]. Altered transcriptional patterns confer cells specific capabilities that allow them to escape the regulatory mechanisms responsible for maintaining cell homeostasis.

The main cause of death by cancer is recurrence after treatment. The tumour mass is composed by a heterogeneous group of cells with distinct sensibility to therapeutic agents making the treatment of this disease more difficult [3]. Therapeutic resistance is related to the existence of cancer stem cells and several mechanisms of resistance have already been described affecting multiple cellular pathways [2,3].

An additional layer of complexity must be considered as an increasingly number of studies have highlighted the importance of the group of cells present in the tumor microenvironment including cancer-associated fibroblasts, endothelial cells, pericytes and infiltrating cells of the immune system [2]. This special subgroup of cells is involved in cellular processes that enable tumor growth and development.

All these factors result in a highly complicated disease to treat being the second most common cause of death [4].

Pancreatic cancer (PCA) is one of the most mortal malignancies worldwide with a 5-year survival rate of 7% [4]. Known risk factors associated with pancreatic cancer include family history, age, cigarette smoking, and history of pancreatitis or diabetes mellitus [5,6].

The early symptoms of pancreatic cancer can be vague and unrecognized including weight loss, abdominal and mid back pain, jaundice, indigestion and loss of appetite. Frequently, these symptoms are not enough to make a clear diagnose of pancreatic cancer as they can also be associated with other diseases as gallstones, gastritis, irritable bowel syndrome, gastroenteritis, indigestion or liver disease leading to misdiagnosis [6]. A late diagnosis and a poor response rate to treatment make the discovery and validation of novel biomarkers for screening and management of PCA an absolute necessity [5].

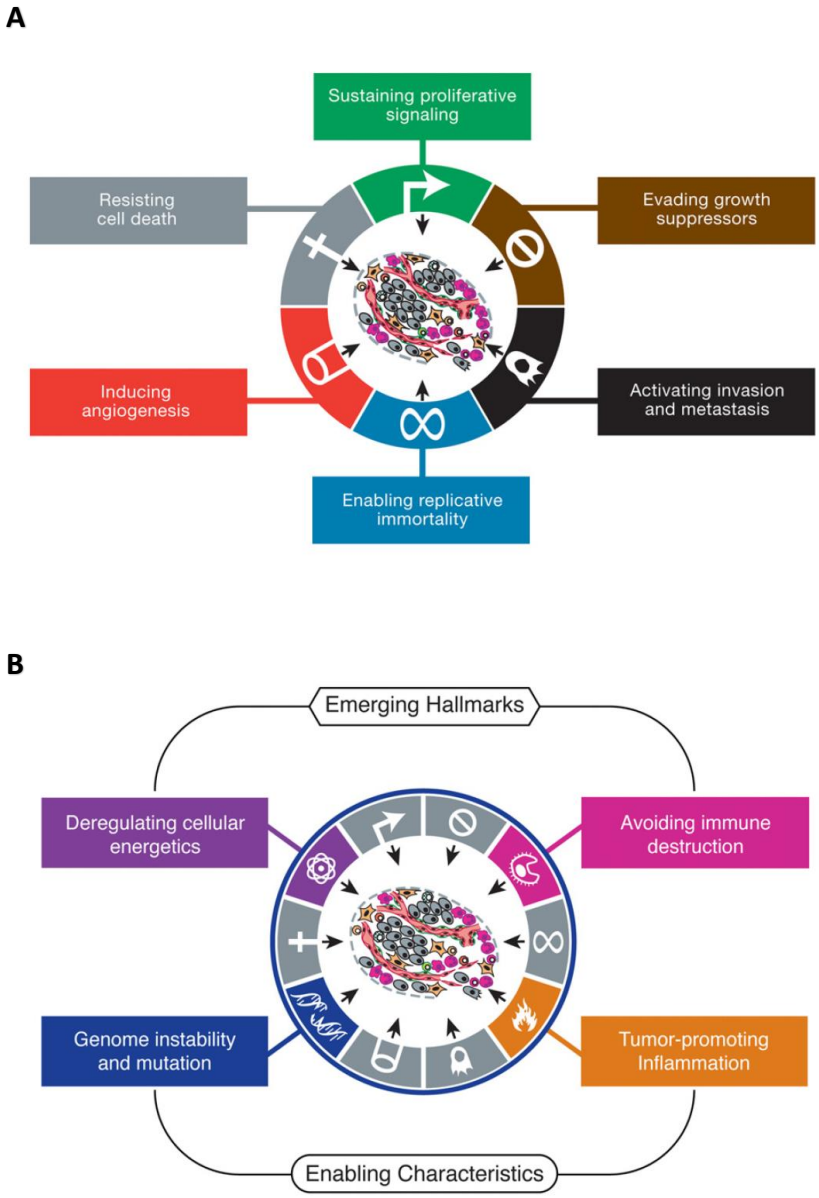


Figure 1. Hallmarks of Cancer. A. Illustration of the six first established hallmarks of cancer. **B.** Recently proposed emerging hallmarks and characteristics that contribute to the malignant transformation of cells. From Hananan *et al,* 2011.

1.2 Disease subtypes

PCA can be divided into two main groups, depending from which pancreatic cell type it arises. Almost all PCAs are classified as exocrine tumours since the affected cells are exocrine cells (responsible for enzyme production) and can arise from acinar cells, connective tissue or from the ductal epithelium where pancreatic ductal adenocarcinomas (PDAC) represent about 80% of all pancreatic cancers [5].

About 60-70% of all PCAs originate from cells localized in the head of the pancreas while 20-25% arise in the body and the tail of the pancreas [7]. The remaining minority of the

cases diffusely affect different pancreatic cells [7]. Tumours in the head of the pancreas normally present obstruction of the common bile duct and/or the pancreatic duct and develop specific symptoms that allow a more rapid diagnose compared with other pancreatic cancer [7].

Only about 5% of all cases develop from pancreatic endocrine cells, the islet cells, which are responsible for hormone production [8]. These tumours tend to be less aggressive than the exocrine ones [8].

Pancreatic neuroendocrine tumours (PanNETs) can be classified as functional or non-functional tumours. Non-functional PanNETs represent the majority of neuroendocrine tumours of the pancreas and are associated with a poorer prognosis as the disease is generally diagnosed at an advanced stage [8]. Functional PanNETs present hormone excess syndrome which leads to specific clinical symptoms due to increased hormone production facilitating the diagnosis of the disease [8,9].

The islets of Langerhans are formed by the endocrine cells of the pancreas: β-cells, α-cells, γ-cells and δ-cells. These cells are responsible for the production of several hormones such as insulin, glucagon, pancreatic polypeptide and somastotatin, respectively. Accordingly, functional tumours of the pancreas are defined by the hormone secreted being named insulinomas, glucagonomas, VIPomas and somastinomas, [8,10].

1.3 Therapeutic Strategies

Currently the therapeutic options available are surgical resection, radiotherapy and chemotherapy. However, only 15-20% of the patients are eligible for surgery since by the time of diagnosis the majority of patients present the disease at advanced stage [5].

While surgery remains the principal therapeutic strategy for pancreatic cancer, medical treatment of patients with advanced disease differs according to the distinct disease subtypes [7,11].

For resectable PDAC the standard of care is surgery followed by adjuvant chemotherapy with fluorouracil and leucovorin or gemcitabine. In borderline cases, resectable tumours are treated with neoadjuvant therapy and/or radiotherapy followed by surgery and posterior administration of adjuvant therapy or chemotherapy depending on the surgery success [6,7].

In locally advanced and metastatic PDAC surgical removal of the tumour is no longer a viable option. For these patients, the administrated regimens consist in FOLFIRINOX (5-fluorouracil, leucovorin, irinotecan and oxaliplatin) and gemcitabine plus nab-paclitaxel or gemcitabine alone for patients with reduced tolerance to the treatment and best supportive care [6,7].

For PanNETs patients with advanced disease, the pharmacological agents available include somastotin analogs, chemotherapy and targeted therapy using everolimus (a mammalian target of rapamycin (mTOR) inhibitor) and sutinib (a multikinase inhibitor) [11].

Despite efforts of the scientific community to develop new therapeutic approaches for pancreatic cancer no effective therapies have reach the clinic and the mortality rates for this type of cancer have remain almost unaltered with tendency to increase [4,6].

Most patients present progressive disease and recurrence after treatment with a response rate to chemotherapy inferior to 20% making it necessary the discovery and validation of novel biomarkers for diagnosis, prognosis and prediction of therapeutic response [5].

Currently, the only FDA approved biomarker for PCA is the Cancer-Antigen 19-9 (CA19-9, a modified Lewis(a) blood group antigen) with a sensitivity of 60-70% and a specificity of 70-85% [12]. However, this biomarker is not useful as a diagnostic tool since other diseases can also cause an increase in the plasma levels of this protein. Additionally, in a subset of pancreatic cancer patients not harbouring a functional Lewis enzyme required for CA19-9 production the levels of this protein are very low or undetectable leading to false negatives [13]. Despite not being useful as a diagnostic biomarker, CA19-9 presents significant value as a prognostic tool and can be used to monitor patients' response to treatment, helping the clinicians to adopt the best therapeutic approach [12]. Until now, no biomarker revealed to significantly outperform CA19-9 in the clinic.

Our study will be focused mainly on pancreatic ductal adenocarcinomas however, other histological subtypes will also be included and therefore the molecular pathogenesis of both PDAC and PanNETs will be addressed.

1.4 Molecular Pathogenesis of Pancreatic Cancer

The development and progression of pancreatic cancer is driven by successive accumulation of somatic mutations and epigenetic alterations [6,14,15].

1.4.1 Genetics of pancreatic ductal adenocarcinoma

The most important genetic events in PDAC, the most common type of PCA, are the activation of the oncogene *KRAS* and the inactivation of the tumour suppressor genes cyclin dependent kinase inhibitor 2A (*CDKN2A)*, tumour protein p53 (*TP53)* and *SMAD4* [16].

Several lesions such as intraductal papillary-mucinous neoplasm (IPMN), pancreatic intraepithelial neoplasia (PanIN) and mucinous cystic neoplasm can progress and originate PDACs [16]. The precursor lesions mostly associated with tumour development are PanINs which consist of epithelial neoplasms that occur in pancreatic ducts [16].

One of the cellular processes implicated in PCA is the process of acinar to ductal metaplasia (ADM). ADM is a cellular mechanism required for pancreatic tissue regeneration after inflammation or injuries [17]. These ADM lesions can progress to PanIn lesion and eventually progress to pancreatic ductal adenocarcinoma in response to oncogenic signalling [18].

Alterations in the most important PDAC driver genes are also observed in precursor lesions such as PanIN with activation of the oncogene KRAS as an early event of lesion development, which then leads to inactivation of CDKN2A, characteristic of PanIN2 stage. With the progression of the lesion and the establishment of a PanIN3 lesion, TP53 and SMAD4 are inactivated, which are the most commonly observed genetic alterations in PDAC. This is consistent with the progression of these lesions to a malignant state [16,19].

Cancer heterogeneity is one of the main reasons of therapeutic failure comprising inter and intra-individual changes between malignant cells. Gene mutations differ from individual to individual and the initial alteration responsible for the induction of a malignant state was not identified yet. However, global genomic analysis revealed that the deregulation of some signalling pathways including the phosphatidylinositol 3-kinase (PI3K)/Akt, Wnt, Notch and Hedgehog pathways is deeply involved in PDAC development [20,21]. A detailed description of these signalling pathways can be found in Section 3.2.1 (a simplified representation of those signalling pathways is illustrated in Fig. 2).

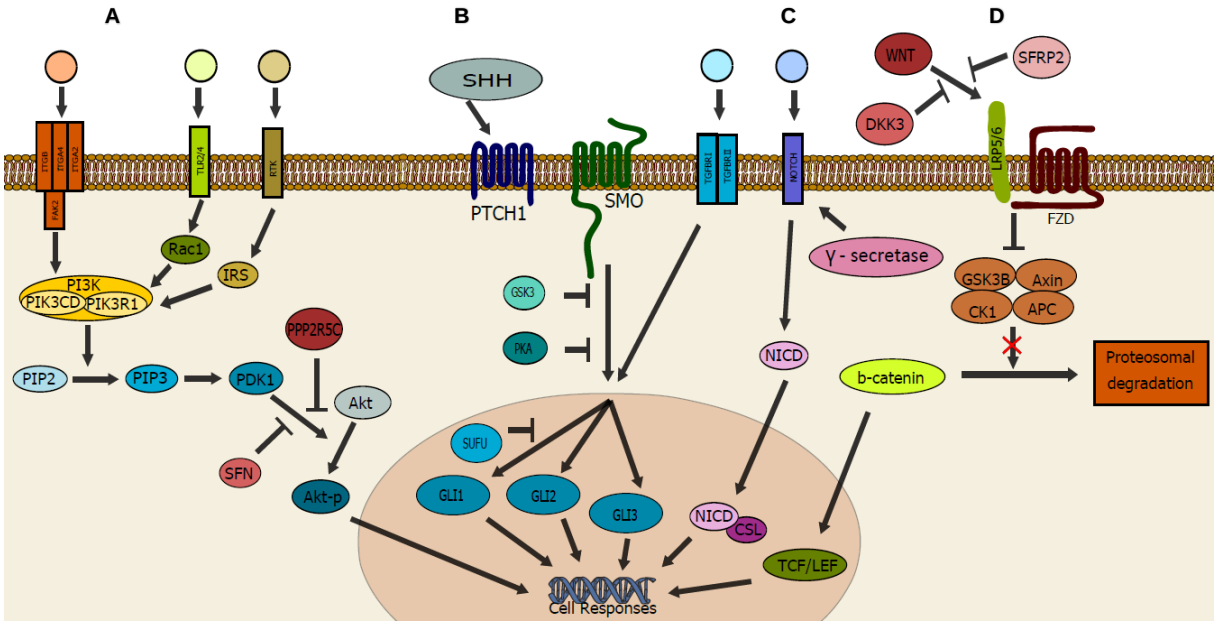


Figure 2 – Representation of the signalling pathways involved in pancreatic cancer. A. PI3K/Akt pathway: In the PI3K/Akt pathway, extracellular ligands bind to cell surface receptors leading to class I phosphoinositidine 3- kinases (PI3Ks) activation. When activated, PI3K generates PIP3 that activates phosphoinositide-dependent protein kinase 1 (PDK1) and leads to subsequent Akt activation. Upon activation, Akt phosphorylates several proteins that control multiple cellular processes that contribute to cancer development including cell survival and proliferation. **B. Hedgehog pathway:** Binding of Hedgehog ligands to Patched receptor (PTCH1) disrupts the repression of SMO proteins that are then capable of activating downstream proteins such as GLI proteins that act as transcriptional activators inducing the expression of several genes that can contribute to cancer progression and development. **C. Notch pathway:** Binding of Delta and Jagged Notch ligands induces receptors cleavage by γ-secretase. Release and translocation to the nucleus of the cytoplasmic domain allows its association with transcription factors promoting expression of genes associated with cell growth and proliferation. **D. Wnt pathway:** In the canonical Wnt pathway, β-catenin is phosphorylated and targeted for degradation by the proteasome (off state). Binding of the Wnt, a secreted protein, to its receptor, Frizzled, leads to stabilization of β-catenin by downstream proteins of the pathway (on state). This leads to β-catenin translocation to the nucleus where it forms a complex with transcription factors (TCF-Lef) to induce gene expression. Activation of the pathway is represented by an arrow (→) and repression of the pathway is represented by a blocked-line (⊢).

1.4.2 Genetics of pancreatic neuroendocrine tumours

The genetic alterations behind PanNETS development and progression affect distinct genes in comparison to PDAC [22]. The most affected genes in PanNETs are the menin 1 (*MEN1)* gene, Death Domain Associated Protein (*DAXX*) gene, ATRX chromatin remodeler

(*ATRX*) gene and *Mtor* gene [22,23]. Indeed, a study of whole-genome sequencing using PanNETs samples revealed four pathways commonly altered in this type of pancreatic: DNA damage repair, chromatin remodelling, telomere maintenance and mTOR signalling [23].

Resistance to therapeutic agents is one of the major factors that contribute to the discouraging survival rates of the patients. One of the causes of treatment failure is poor diffusion of drugs which is influenced by the formation of a desmoplastic reaction creating a unique and protective microenvironment surrounding cancer cells [18]. This microenvironment is composed of extracellular matrix proteins, fibroblasts and immune cells as well as its secreted factors affecting cells behaviour and its ability to metastasize and resist to the effects of chemotherapeutic agents [18].

1.5 Epigenetics

Epigenetic modifications are somatically heritable and reversible changes that contribute to gene expression regulation without altering the DNA sequence. Among these are histone post-translational modifications, DNA methylation and non-coding RNAs, especially miRNAs [24]. All cells in the human body have essentially the same genome, however cells from different tissues have distinct structures and functions as the result of differential gene expression. The process of cell differentiation during the embryonic development is due to epigenetic reprogramming of the cells allowing each cell to express only certain genes conferring them specific functions [25,26]. Also, the epigenome can be influenced by various factors including diet, exercise or medication mediating the interplay between the environment and gene expression [26].

Cancer is one of the leading causes of death worldwide and cancer development and progression comprises wide alterations in gene expression that result not only from genetic but also from epigenetic modifications.

Epigenetic regulation is implicated in the normal development and function of the pancreas and deregulation of these mechanisms can result in the development of pancreatic diseases including pancreatic cancer (Figure 3) [27].

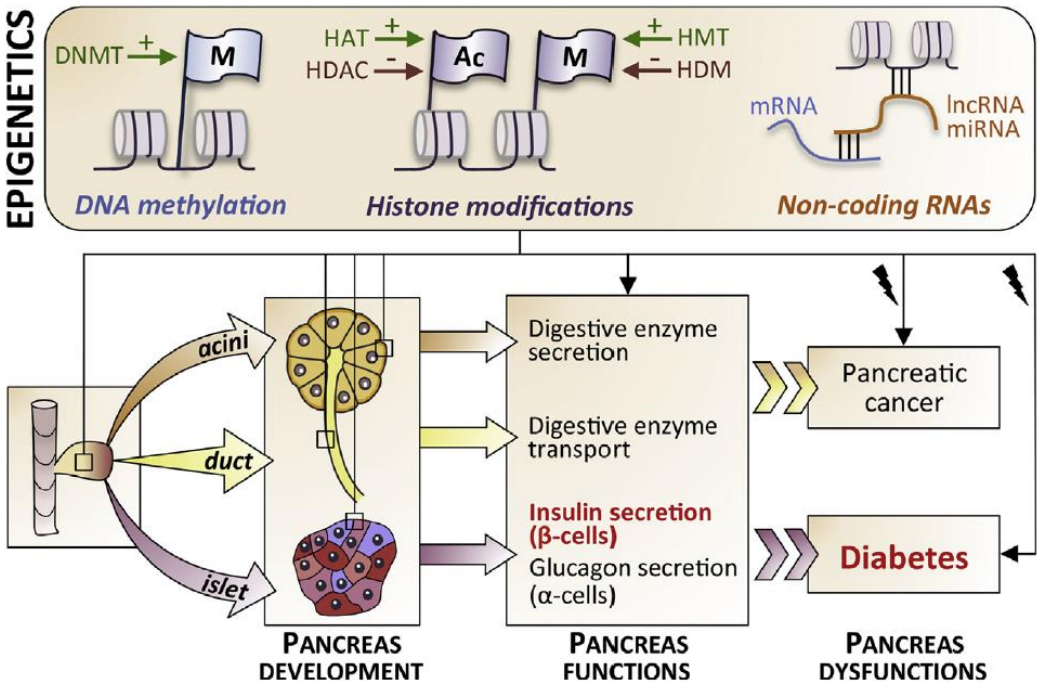


Figure 3. Implication of epigenetics in pancreas development, function and disease. DNA methylation, histone modifications and the action of non-coding RNAs are crucial for pancreas normal development and are deeply involved in the regulation of pancreas functions. Deregulation of these mechanisms leads to an impairment in pancreas functions resulting in the development of several pancreatic diseases. From Quilichini *et al,* 2015. **DNMT:** DNA methyltransferase; **HAT:** histone acetyltransferase; **HDAC:** histone deacetyltransferase; **HMT:** histone methyltransferase; **HDM:** histone demethyltransferase.

1.5.1 Histone Modifications

There are several histone post-translational modifications that can affect the compaction state of chromatin, which influences the folding, position and organization of DNA, thereby affecting gene expression. Some of these alterations include histone methylation, acetylation, phosphorylation, ubiquitylation and sumoylation [28,29]. Among these, the best described histone modifications with important roles in epigenetic deregulation in cancer are histone acetylation and methylation [24].

Histone acetylation can promote disruption of the electrostatic interactions between histones and DNA by reducing the positive charge of histone tails, which leads to a more uncondensed chromatin state. Addition of the acetyl group is carried out by histone acetyltransferases (HATs), and its removal is catalysed by histone deacetylases (HDACs) [30].

In addition to influencing DNA-histone interactions, histone acetylation can promote recruitment of the chromatin remodeling complex SWI/SNF (SWItch/Sucrose Non-Fermentable) which activity is associated with transcription activation [31,32].

Histone methylation can occur on lysine or arginine residues and, depending on the target, it can lead to activation or repression of gene expression [33]. Unlike acetylation and phosphorylation, however, histone methylation does not alter the charge of the histone protein. Furthermore, there is an added level of complexity when considering this modification; lysines

may be mono-, di- or tri-methylated, whereas arginines may be mono-, symmetrically or asymmetrically di-methylated [33].

The regulation of histone methylation levels is achieved by the activity of histone demethylases (HDMs) and histone methyltransferases (HTMs) [33].

As previously mentioned these post-translational modifications in histones can be associated with active or repressive chromatin. Modifications such as histone 3 methylation at lysine 4 (H3K4me), histone 3 di-methylation at lysine 4 (H3K4me2), histone 3 tri-methylation at lysine 4 (H3K4me3), histone 3 acetylation at lysine 9 (H3K9ac), histone 3 methylation at lysine 9 (H3K9me) or histone 3 acetylation at lysine 27 (H3K27ac) are associated with active chromatin whereas histone 3 tri-methylation at lysine 36 (H3K36me3), histone 3 tri-methylation at lysine 9 (H3K9me3) or histone 3 methylation at lysine 27 (H3K27me) are associated with repressive chromatin [34].

1.5.2 DNA methylation

DNA methylation results from the addition of a methyl group on the 5-carbon of a cytosine residue in a CpG site by a DNA methyltransferase (DNMT) [35]. The enzyme forms a complex with the DNA molecule to allow the transfer of the methyl group to the cytosine residue of CpG dinucleotides [35]. DNA methylation is involved in processes such as genomic imprinting, inactivation of the X chromosome and silencing of repetitive DNA sequences [25].

There are 3 active DNMTs in eukaryotes: DNMT1 is responsible for the maintenance of methylation patterns after DNA replication whereas *de novo* methylation is carried out by DNMT3A and DNMT3B [30].

The mechanisms of *de novo* methylation and how DNMTs target specific DNA sequences remain unknown. However, it is known that DNMTs can bind to DNA through a conserved PWWP domain, named after a conserved Pro-Trp-Trp-Pro motif, and interact between them or with methyl-binding domain proteins (MBDs) and transcription factors (TFs) to reinforce DNA methylation and influence gene transcription [36–39].

The idea that DNA methylation is a more dynamic process than previously believed is starting to arise with the possibility of directed DNA methylation and the recent discovery of enzymes capable to erase DNA methylation [40,41]. While the mechanism of DNA methylation and the enzymes involved have been well characterized, the mechanism of DNA demethylation remains controversial [42]. DNA demethylation is the removal or modification of the methyl group from 5-methylcytosine (5mC) [42]. The discovery of the ability of the ten-eleven translocation (TET) family enzymes to oxidize 5mC to 5-hydroxymethylcytosine (5hmC) using molecular oxygen as substrate has revolutionized this area [42]. In fact, overexpression of these enzymes resulted in a decreased of genomic 5mC levels [42].

If these modifications could directly influence DNA-protein interactions or if the biological effect is only due through disruption of 5mC patterns has not yet been scrutinized but it is believed that 5hmC is a key intermediate in active demethylation pathways [42].

Several pathways have been proposed following 5mC oxidation: the oxidized base can be passively removed during replication or actively through enzymatic reactions by enzymes involved in DNA repair mechanisms [42].

Considering the CG content of the human genome the abundance of CpG sites across the genome is relative lower than the expected [43]. However, the human genome contains sequences in most gene promoters with high content of CpG sites known as CpG islands [44].

In these regions, DNA methylation can contribute to changes in chromatin conformation influencing gene expression by affecting DNA exposure to transcription factor binding [45].

Generally, gene promoter DNA methylation is recognized as an event leading to transcriptional repression. However, there are several exceptions to this classical view of epigenetics related to DNA methylation, with increased gene expression associated with promoter DNA hypermethylation [46–48]. Gene activation through DNA methylation can be due to activation of an alternative TSS or to inhibition of repressive protein binding [49].

Increased gene expression associated with promoter CpG hypermethylation is also associated with an increase in H3K4me3, a histone mark characteristic of gene activation, reinforcing the interplay between these epigenetic mechanisms in transcriptional regulation [49]. Indeed, the interplay between proteins involved in both histone modifications and DNA methylation consolidate the effect of these epigenetic mechanisms. Histone acetylation and H3K4me are marks associated with active gene transcription and are typically associated with unmethylated DNA [50]. Histones marks associated with transcriptional repression as H3K9m and H3k27me are often associated with DNA methylation [50].

Any alteration that affects the activity of epigenetic-modifying enzymes can lead to an imbalance in gene expression regulation and provide the basis, or contribute, to the initiation of carcinogenesis.

1.5.3 MicroRNAs (miRNAs)

miRNAs are small endogenous ncRNAs with 21-25 nucleotides that participate in the regulation of gene expression by targeting specific mRNAs for translation repression or degradation.

miRNA genes can be localized in specific locus or in introns of other genes and are mainly transcribed by RNA polymerase II originating a primary transcript, the pri-miRNA [51], which is then processed by the RNase DROSHA. This enzyme and its cofactor Di George syndrome critical region 8 (DGR8) constitute the microprocessor responsible for the cleavage of the pri-miRNA to generate a pre-miRNA that contains a stem loop structure [51]. This structure is recognized and processed by DICER, after pre-miRNA exportation to the cytoplasm by exportin-5 [51]. DICER cleavage originates a RNA duplex, which will then associate with the RNA-induced silencing complex (RISC). Only the guide strand that consists of the mature miRNA remains incorporated into the complex while the other RNA strand is degraded [51]. The mature miRNA will then guide the RISC complex to the 3’ untranslated region (3’UTR) of a specific mRNA that depending on their complementarity level the mRNA is degraded or its translation blocked [51].

Previous studies have shown that miRNA expression patterns differ in normal and tumour tissue and that miRNAs can act either as tumour suppressors or oncogenes depending on their target [51]. Downregulation of a miRNA that targets an oncogene or upregulation of a miRNA that targets a tumour suppressor gene can promote carcinogenesis [51].

1.6 Epigenetic Alterations in Pancreatic Cancer

Disruption of the normal epigenetic patterns of the cells is associated with the development of PCA [52]. Also, deregulation of epigenetic-modifying enzymes disturbs normal epigenetic patterns and is associated with cancer development and progression.

For instance, in pancreatic cancer, the histone deacetylases HDAC2 and HDAC7 and the histone methyltransferase EZH2 are upregulated [15]. EZH2 upregulation is associated to cell proliferation, invasion and migration and is an indicator of a worst prognosis [15].

Specific histone modifications have also been associated with pancreatic cancer [53]. Analysis of two cohorts of patients revealed that low levels of the histone marks H3K4me2, H3K9me2, or H3K18ac are independent factors associated with poor prognosis and lower survival probability [53].

The DNA methyltransferases DNMT1, DNMT3A, DNMT3B are also upregulated in pancreatic cancer [54]. In fact, increased expression of DNMT1 was associated with disease progression suggesting the role of epigenetic regulators in PCA development [54]. Interestingly, several groups have performed genome-wide DNA methylation analysis of pancreatic cancer [55–57]. Those studies revealed aberrant patterns of DNA methylation of genes involved in important signalling pathways implicated in pancreatic carcinogenesis including transforming growth factor beta (TGF-β), Wnt, integrin signalling, cell adhesion, stellate cell activation, axon guidance and genes involved in stem cell pluripotency [55–57]. Recently, Mishra and coworkers have analysed the data of The Cancer Genome Atlas (TCGA) and found three distinct subtypes considering the genome-wide methylation patterns [58].

Epigenetic changes such as hypermethylation of CpG islands in several genes have been found in pancreatic cancer and in cancer precursor lesions such as PanINs. The methylation levels increase during the progression of these lesions. So, a higher level of methylation is probably associated with the progression of these lesions to cancer [59]. It has been shown that methylation levels can be detected through the analysis of pancreatic juice samples therefore, it has the potential to become a biomarker for the early detection of premalignant lesions [59].

Finally, miRNA levels are also altered in pancreatic cancer, affecting the expression of genes involved in cell cycle control, apoptosis, migration, and invasion and have the potential to be used as biomarkers for diagnosis and prognosis [15].

Several authors have described combinatory panels of miRNAs that can potentially be used for pancreatic cancer diagnosis [15]. Importantly, those panels were evaluated using serum samples from the patients proving the feasibility of miRNA profiling as a non-invasive method for PCA diagnosis. Results showed that several miRNAs have altered expression and were associated with disease progression and with worse prognosis. Of these miRNAs, miR-155 was also upregulated in pancreatic juice samples of patients with IPMN, a PCA precursor lesion [15].

1.6.1 Epigenetic therapy

Deregulation of epigenetic marks leads to changes in gene expression that, in cancer cells, can result in activation of oncogenes or inactivation of tumour suppressor genes, both of

which can contribute to cancer. Unlike genetic mutations, however, epigenetic changes are reversible. Therefore, the development of drugs capable of restoring the normal epigenetic patterns of cells has great therapeutic potential.

Two strategies for epigenetic therapy are currently in use: small molecules that inhibit epigenetic-modifying enzymes and manipulation of miRNA expression.

Amongst the small molecule inhibitors are HDAC inhibitors and DNMT inhibitors. HDAC inhibitors (HDACi) are classified into 4 groups according to their chemical structures: hydroxamates (SB393, Vorinostat, Panobinostat), cyclic peptides (Romidepsin), benzamides (Entinostat and Mocetinostat) and aliphatic fatty acids (Valproic Acid) [60]. The majority of HDACi inhibit zinc-dependent HDACs by interacting with the zinc ion. In cancer cells, the inhibition of histone deacetylation restores expression of tumour suppressor genes that were previously silenced by epigenetic mechanisms [60,61].

DNMT inhibitors are divided into nucleoside analogues and non-nucleoside analogs [30]. Nucleoside analogues, such as Azacitidine, Decitabine and FdCyd, are cytosine analogues modified at the C5 position. Inside the cell these compounds are metabolized and incorporated into DNA [30]. DNA methyltransferases can bind to these modified nucleotides but their modification at C5 prevents their methylation. It also prevents the dissociation of the enzyme thereby reducing DNMT activity at other sites [30]. Non-nucleoside analogues, such as Hydralazine, Procainamide and MG98, inhibit methylation by binding to the catalytic region of the enzyme [30].

Another focus of epigenetic therapy is the manipulation of miRNA expression and activity. Several strategies have been employed to silence miRNAs that are overexpressed in cancer. These include anti-miRNA oligonucleotides (AMOs), peptide nucleic acids (PNAS), miRNA-masking antisense oligonucleotides (miR-mask) and miRNA sponges [62]. Restoration of miRNA expression that has been downregulated in cancer is achieved by administration of synthetic miRNAs or by induced expression of miRNA coding genes using viral constructs, such as adenovirus-associated vectors [62].

To investigate the efficacy of this new therapeutic approach in cancer, our group has performed a comprehensive review where we analysed the results of clinical trials testing epigenetic therapies in urologic cancers [63] (Annex 1).

The best results were achieved when using epigenetic drugs as part of multidrug therapy regimens. Despite the obvious importance of epigenetics in the development of cancer, few epigenetic therapies have thus far reached advanced clinical testing. As our study demonstrates, pre-clinical data has not translated into the hoped-for clinical responses. This is likely secondary to the nonspecific actions of epigenetics drugs and the consequent toxicities associated with their administration. Many of the epigenetic therapies being tested have global epigenetic effects on both cancerous and non-cancerous tissues. Moreover, some of them have additional non-epigenetic effects that limit their efficacy.

A better knowledge of the specific mechanism of action of these agents is essential to overcoming their clinical limitations and improving therapeutic success.

As for other cancers, epigenetic therapy can be a new therapeutic option for pancreatic cancer patients as inhibition of epigenetic enzymes showed promising results in preclinical trials using PCA models [64,65]. Specifically, inhibition of HDAC1 using PDAC cell lines resulted in an increase in apoptosis and inhibition of HMTs presented synergistic effects when combined with gemcitabine [64].

Some clinical trials are currently evaluating the potential of epigenetic therapy in PCA but so far, most of them failed to reproduce the results observed in pre-clinical studies [64,65].

However, studies using research models of the disease continue to present promising results and hopefully, epigenetic therapies will be used to treat patients with pancreatic cancer in the near future.

1.6.2. Epigenetic alterations as potential biomarkers

Development of efficient biomarkers for disease screening and management represents a major challenge in PCA. A biomarker is defined as any substance, structure or process that can be measured in the body and evaluated as an indicator of presence/absence of a pathological state, outcome of the disease or pharmacologic response to therapy [66].

The development of a biomarker with clinical applicability is the result of several phases of scientific research [67]. First, a set of samples is analysed to identify changes between patients' samples and controls that can potentially be used as a disease biomarker (discovery cohort) [67]. The alterations that revealed to be the most relevant in predicting the presence or absence of disease or that can distinguish patients according to different clinical parameters are then validated in a larger group of samples (validation cohort) [67]. The candidate alterations validated in this cohort are subsequently evaluated in independent cohorts of patients to confirm the sensitivity and specificity of the test in different populations. The optimization of the test in a clinical context and its applicability regarding socio-economic terms is then evaluated [67].

Recent efforts and investments in this area resulted in the development of new and innovative technologies with the potential to be used in clinical laboratories for epigenetic profiling [68,69].

Epigenetic biomarkers hold some advantages over genetic and protein based biomarkers. Contrarily to genetic studies that require the analysis of all gene length for mutations profiling, examination of DNA methylation is generally focused on specific CpG sites covering a smaller region [68]. Additionally, DNA methylation profiling might contribute to increased sensitivity as generally this epigenetic alteration in observed in a higher percentage of tumours [68]. The establishment of assays for the analysis of histone alterations in the clinic is more challenging since those epigenetic modifications present lower stability compared with DNA methylation [68].

Profiling of miRNAs expression levels is another promising alternative to incorporate epigenetic analysis in clinical tests. miRNAs present high stability in tissues and its possible its detection using low amounts of biological samples [70].

Understanding the epigenetic alterations associated with cancer progression can lead to the development of novel biomarkers for an early diagnosis, improving the chances of survival for many patients.

1.7 Objectives

The main goal of this work is to discovery novel PCA biomarkers, focusing on epigenetic alterations, namely DNA methylation and miRNAs.

Our study focused essentially on two hallmarks of cancer: the competence of cells to replicate indefinitely and the ability to activate signalling pathways that promote cell proliferation [2].

Firstly, we have analysed the methylation pattern of a specific region of the telomerase (*TERT*) gene promoter and its correlation with gene expression and patients' outcome. This gene has been shown to be involved in replicative immortality and was previously established by our group as differentially methylated in several types of cancer with the potential to be a pan-cancer biomarker. Additionally, TERT is involved in multiple other mechanisms beyond telomere maintenance that allow cells to acquire a malignant phenotype [2,71]. Specifically, TERT is a cofactor implicated in Wnt signalling pathway activation [2,71].

This reveals the importance of TERT in pancreatic carcinogenesis and with this initial approach, we intended to validate our methodology and to improve the study pipeline for subsequent analysis.

Secondly, we explored alterations in DNA methylation in genes related with the pancreatic cancer signalling pathways: PI3K/Akt, Notch, Wnt and Hedgehog. Moreover, we aimed at uncovering miRNAs that target genes involved in the former signalling pathways and understand if those miRNAs can themselves be potential pancreatic cancer biomarkers.

To achieve that goal, we propose an *in silico* approach to evaluate epigenetic changes characteristic of pancreatic cancer cells that could be used as biomarkers and/or therapeutic targets. The *in silico* analysis was based on a discovery cohort with patient data publicly available through the TCGA data for pancreatic cancer, and on the analysis of DNA methylation, gene and miRNA expression of pancreatic cancer patients using that database.

Finally, to assess the potential of the most relevant genes as novel alternative biomarkers for pancreatic cancer we have combine gene expression/methylation changes with clinical outcomes including disease progression and tumour recurrence. The ultimate goal of our work is to develop to a personalized medicine approach in which treatment is projected to the specific needs of each patient to obtain better outcomes.

CHAPTER 2 - METHODS

2. CHAPTER 2 - METHODS

In order to evaluate epigenetic alterations involved in PCA we performed multi-dimensional analysis of data from publicly available datasets. The bioinformatic tools and resources used in this project are described below.

2.1 Bioinformatic Resources

2.1.1 Pancreatic Expression Database

The Pancreatic Expression Database (PED) is a public repository of genomic, transcriptomic and epigenomic data obtain from biopsies and body fluids collected from patients and healthy individuals freely accessible at www.pancreasexpression.org [72].

PED contains data not only derived from pancreatic cancer patients and pancreatic cancer cell lines but also from patients with benign disease and cancer precursor lesions such as IPMNs, PanINs and mucinous cystic neoplasms [72].

The Pancreatic Expression Landscape integrated in the PED database is the result of a comprehensive meta-analysis of pancreatic gene expression data extracted from numerous published studies [72]. PED allows the analysis of differential gene expression considering different samples comparison (e.g. Pancreatic Adenocarcinoma vs Healthy donor), Log Fold-change, p-value or cell pathway to identify gene deregulation considering specific biological functions [72,73].

2.1.2 The Cancer Genome Atlas

The Cancer Genome Atlas (TCGA) project results from a collaboration between the National Cancer Institute (NCI) from the National Institute of Health (NIH) and the National Human Genome Research Institute (NHGRI). [74]. This extensive and collaborative effort have generated a wide variety of genomic and epigenomic data in large cohorts of 33 distinct human cancers, including pancreatic cancer [74].

The TCGA is a publicly available database (https://cancergenome.nih.gov) with the purpose to stimulate the scientific investigation by the cancer research community in order to improve the methods of diagnosis and the therapeutic strategies of cancer [74].

2.1.3 miRDB

To date, around thousands of human miRNAs have been discovered and several databases with miRNA annotations have been created allowing researchers to predict miRNA regulation of multiple genes [75,76]. However, some of the miRNAs identified from high-throughput studies may not have any functional relevant role. Most of the publicly available databases do not distinguish between functional and non-functional miRNAs which may result in false positive associations and eventually hide real biological findings [75,76].

The miRDB is an open-access database for microRNA data analysis accessible at http://mirdb.org that allows miRNA target prediction and contains miRNA functional annotations [75,76]. The users can search by individual or multiple target genes or by specific miRNAs [75,76]. Additionally, miRDB also allows the users to query by miRNAs involved in specific biological pathways [75,76]. The results page displayed after the query includes information about the miRNA and the targeted gene with all the targets having a prediction

score ranging between 50 and 100, highlight of the target sites and links to other miRNAs related databases including the TarBase [75,76].

In miRDB, the criteria for the selection of functional miRNA is based on four parameters: literature search, sequence conservation, expression profile and functional annotations by miRbase. For each miRNA is attributed a score according to the number of associated Pubmed records, level of conservation and number of orthologous, number of normalized read counts from RNA-seq experiments and previously classification by miRbase [75,76]. The final combination of the scores designated for each parameter yields the functional score for a specific miRNA. miRNAs with higher scores present more evidence of having a relevant biological and functional role.

2.2 Gene Selection

In this study, we included only the genes related with the PI3K/Akt, Wnt, Notch and Hedgehog pathways that present differential expression levels between malignant and healthy pancreatic tissue. To perform gene selection we relied on the data available on the Pancreatic Expression Landscape integrated in the PED database [72].

We performed a query using the online web resource available at https://pancreasexpression.org/includes/PancreaticCancerLandscape.html. A fold-change equal or bigger than 1.5 and a p-value lower than 0.05 were established as including criteria in our study. The expression data available in this database results from the Affymetrix HG U133 Plus 2.0 array platform were one gene can be represented by multiple probes and gene-pathway association is based on Ingenuity Pathway Analysis (www.ingenuity.com) version 8.5 [72].

2.3 Epigenetic Alterations Analysis

After gene selection we assessed epigenetic changes in the genes of interested considering its methylation status and the expression of regulatory miRNAs using the data from the TCGA cohort of pancreatic cancer.

2.3.1 Data collection

Patient data was retrieved from the TCGA data portal via the UCSC Cancer Genome Browser (http://xena.ucsc.edu/). Data was specifically extracted from the pancreatic cancer cohort with data for 196 samples. Incomplete information was available for some patients and thus the sample sizes differ between analyses. For that reason, the sample size is explicitly stated for each analysis in the respective figure and figure legend. Moreover, we only included data from patients with no history of neoadjuvant therapy (n=193), as this parameter could independently influence methylation levels.

Normal tissue in the TCGA pancreatic cancer collection is derived from uninvolved tissue surrounding the pancreas including adipose, omentum, subcutaneous tissue or small intestine. We will henceforth refer to those samples collectively as “normal tissue”. Data processing was conducted according to the TCGA data access policies.

To investigate if epigenetic alterations could be potential biomarkers in pancreatic cancer we have analysed the methylation status of genes related with signalling pathways as well as alterations in associated miRNAs and compared it with several clinical parameters.

In Table 1 are represented the characteristics of the patients from the TCGA pancreatic cancer cohort considered for this study: approximately 55% of the patients are male against 45% female, patients present a mean age of 64.94 years ranging from 35 to 88 years. Many of the patients revealed a positive family history of cancer while few had previously history of chronic pancreatitis and diabetes, known risk factors for PCA development (Table 1).

Considering the patients' outcome after primary therapy administration, 60 patients presented complete remission, 9 revealed a partial response to the treatment while 8 patients had stable disease and 53 patients presented progressive.

Regarding the histological classification of the tumour, 142 were classified as pancreatic ductal adenocarcinomas, the most common type of pancreatic cancer with 8 tumours being classified as neuroendocrine tumours (Table 1). The remaining samples presented discrepancies in the classification or were classified as distinct types of pancreatic cancer. However, the number of samples for each type did not reach a significant value to include the different subtypes separately.

Most of the patients in this cohort that were considered in our analysis are representative of early pathological stages of the disease with the majority (n=140) belonging to the stage II of disease. This will allow us to search for epigenetic biomarkers for diagnosis and prognosis of initial stages of the disease.

Table 1 – Characteristics of the patients from the TCGA pancreatic cancer cohort.

Parameter		Number
Gender	Female	75
	Male	94
	NA	14
Age mean (range)		64.94(35-88)
Family history of cancer	No	46
	Yes	64
	NA	73
History of chronic pancreatitis	No	117
	Yes	13
	NA	53
History of diabetes	No	99
	Yes	36
	NA	48
Primary therapy outcome	Complete remission	60
	Partial response	9
	Progressive disease	52
	Stable disease	8
	NA	54
Histological subtype	Pancreatic ductal adenocarcinoma	141
	Neuroendocrine Tumour	8
	Other	18
	NA	16
Pathological stages	I	20
	II	140
	III	5
	IV	4
	NA	14

2.3.2 Methylation analysis

In order to evaluate the methylation status of the selected genes, level 3 methylation data derived from the Illumina Infinium HumanMethylation450K array was analysed for the pancreatic cancer cohort (normal tissue (n=8) and primary tumour (n=170)). The methylation score ranges from unmethylated (0) to completely methylated DNA (1). Differences between the two groups were evaluated by statistical analysis. We considered as differentially methylated the CpGs sites with a methylation delta beta absolute value (|Δβ|) equal or bigger than 0.2 and a p-value lower than 0.05.

The effect of DNA methylation in gene expression regulation is influenced by CpG location thus, characterization of the CpGs differentially methylated according to gene region was also performed. CpGs were annotated according to the manifest file for the Infinium HumanMethylation450 version 1.2 CSV format (23/05/2013) available at https://support.illumina.com/downloads.html.

To investigate the relationship between DNA methylation and gene expression we have assessed the correlation between these two parameters. Gene expression data (level 3 data, RNA-seq Version 2 Illumina; gene-level transcription estimates, as in log2(x+1) transformed RSEM normalized count) and clinical data from the pancreatic cancer cohort was retrieved from the TCGA data portal and mapped to corresponding THOR methylation status using the unique TCGA identifier barcodes. Due to the lack of expression data from control samples in the TCGA cohort, the DNA methylation/gene expression correlation analysis was performed considering only data from pancreatic tumour tissue samples.

2.3.3 miRNA expression analysis

miRNA data for the TCGA pancreatic cancer cohort (level 3 data, IlluminaHiSeq_miRNASeq, log2(RPM+1)) was analysed to investigate differences of miRNA expression levels between patients and controls.

Since the regulation of gene expression mediated by miRNA is an epigenetic mechanism not directly dependent on DNA methylation we have considered for this analysis all the genes that presented differential expression levels (n=35), independently of the methylation differences between the two groups. The following inclusion criteria were established: the miRNA targets a gene related with the pathways previously selected, presents a target score superior to 90 (the higher the score the higher the statistical confidence in the mRNA-miRNA complementarity result), and has functional annotation according to the miRDB database [75,76]. Due to the lack of information regarding miRNA expression levels in normal samples, we were only able to investigate the correlation between miRNA and gene expression with the data derived from malignant tissue.

Additionally, the absence of data from normal pancreatic tissue did not allow us to perform the receiver operator (ROC) curve to establish the cut-off value for the analysis of the impact of miRNA expression levels on patient's outcome.

Alternatively, we assessed the survival time of the patients comparing the groups of patients with the highest and lowest levels of miRNA expression. Unfortunately, without the ROC curve results we were not able to assess the sensitivity and specificity values of the test.

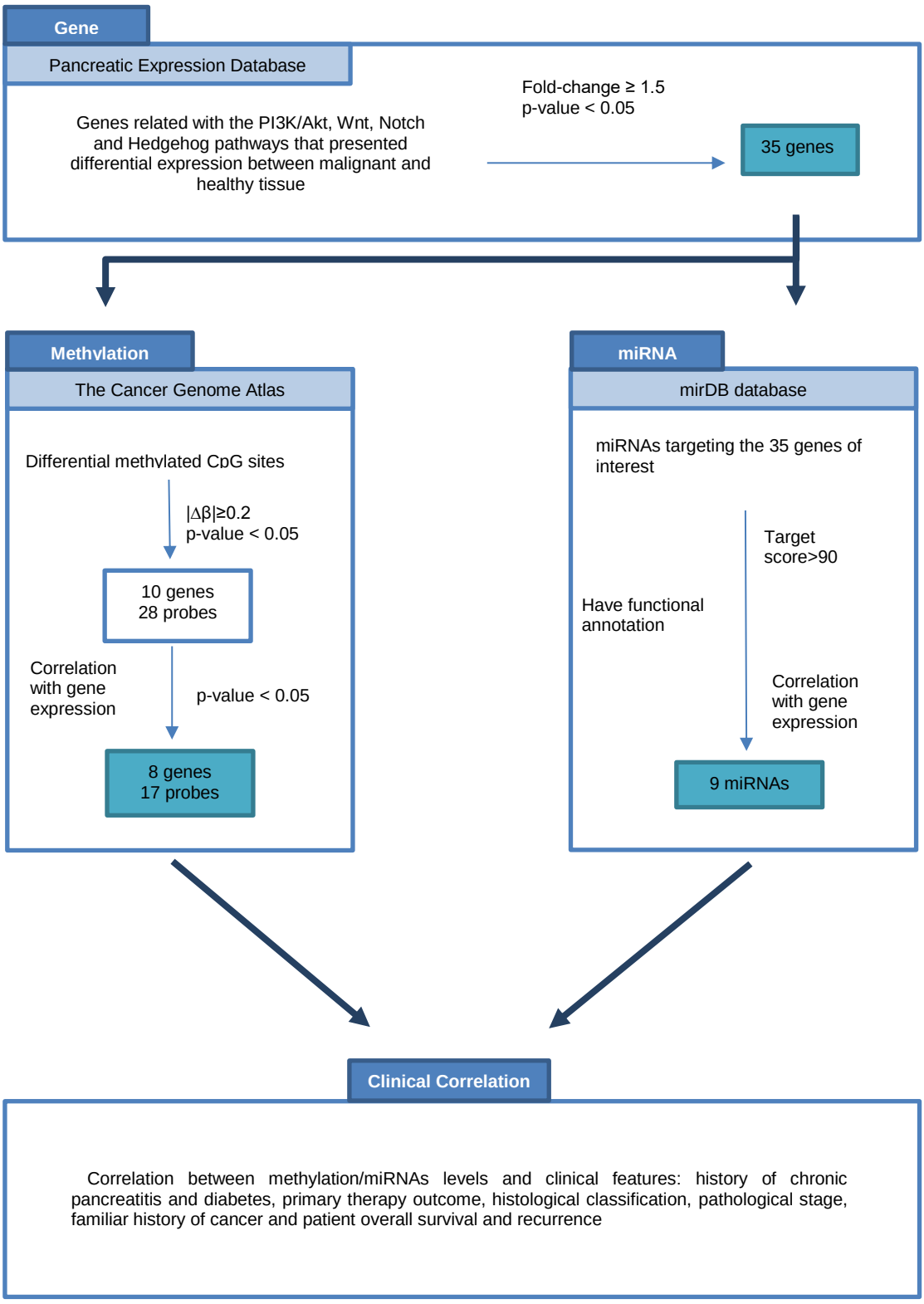
2.3.4 Clinical data analysis

The clinical and pathological parameters evaluated were the following: history of chronic pancreatitis and diabetes, primary therapy outcome, histological classification, pathological stage and familiar history of cancer. Histological classification and pathological stage were used to investigate the impact of DNA methylation of the selected genes on disease prognosis by crossing the data from HumanMethylation450K array regarding DNA methylation status with the clinical information for each patient. Patient overall survival and recurrence were also analysed to determine the clinical significance of the observed epigenetic alterations and their potential as a biomarker and therapeutic target. Methylation cut-offs for each probe were established by performing ROC curve analysis considering an area under the ROC curve (AUC) with a minimum value of 0.8 to distinguish between healthy and malignant tissue. Only the cut-offs values that presented sensitivity and specificity values comparable or higher to the values of the CA19.9, the current biomarker for pancreatic cancer management, were selected for analysis. The patients with methylation values below and above the cut-off value are defined as lowly methylated and highly methylated, respectively.

2.3.5 Statistical analysis

The statistical analysis was performed using the unpaired t-test for data from a normal distribution. Otherwise the two-tailed Mann-Whitney test was applied, with a confidence interval of 95% for two groups comparisons. Correlation analysis was performed using the Spearman correlation coefficient. To analyse the differences between more than two groups we used one-way ANOVA, followed by Kruskal-Wallis test and Dunn's Multiple Comparison Test. Overall survival (OS) and recurrence-free survival (RFS) was determined by Kaplan-Meier Survival curves and comparisons were done with the log-rank test. All statistical analysis was performed using GraphPad Prism5.0.

2.4 Study Pipeline



CHAPTER 3 - RESULTS AND DISCUSSION

3.1 The TERT hypermethylated Oncologic Region (THOR) predicts recurrence and survival in pancreatic cancer

Faleiro, I, Apolónio, JD, Price, AJ, Andrade de Mello, R, Roberto, VP, Tabori, U, Castelo-Branco, P (2017). Future Oncology. In press.

3.1.1 Introduction

The defining feature of cancer cells replicative immortality is attained by telomere maintenance [77,78]. Ordinarily, telomere attrition occurs with each round of cell division due to the end replication problem. In non-malignant tissues, this phenomenon of telomere shortening imposes a ceiling on proliferative capacity in any given cell lineage. With the exception of the early developmental period, and select stem cell populations, telomeres are not reconstructed after shortening. In order to restore telomere length, 85-90% of cancers reactivate the telomerase reverse transcriptase enzyme while only about 10% are dependent on the alternative lengthening of telomeres (ALT) mechanism [79]. Telomerase reactivation in cancer is intimately related with expression of the telomerase reverse transcriptase (*TERT*) gene, which also serves as a prognostic factor [80,81].

Both genetic and epigenetic events have been found to deregulate *TERT* expression in cancer [79,82]. In this regard, point mutations and DNA methylation have gained special attention. The mechanisms underlying mutational *TERT* activation are unambiguous. Indeed, mutations (C228T and C250T) in the *TERT* core promoter [83] are known to generate binding motifs for E-twenty-six (ETS) transcription factors [83,84], and thereby upregulate *TERT* expression.

DNA methylation of the *TERT* promoter, on the other hand, has generated conflicting mechanistic hypotheses. This controversy is likely due to inconsistencies in the precise definition of the regions that constitute the epigenetically vulnerable portions of the *TERT* promoter [85]. The general consensus at the moment is that methylation of the *TERT* core promoter as a whole decreases *TERT* expression while methylation of a specific region upstream of the core promoter increases *TERT* expression [46,86–90]. The notion of epigenetic upregulation of an oncogene is counterintuitive given the traditional silencing effect exerted by hypermethylation. This region, termed TERT Hypermethylated Oncologic Region (THOR), was shown to be associated with *TERT* expression and disease progression in childhood brain tumours and biochemical recurrence in prostate cancer [46,91]. Studies from international groups have corroborated this correlation in brain tumours, hepatocellular, gastric and medullary thyroid carcinomas, to name a few [86–90]. To our knowledge, promoter mutations were not found in pancreatic adenocarcinoma until now [92].

To investigate if THOR could be a potential biomarker in pancreatic cancer we have analysed THOR methylation status and compared it with several clinical parameters.

3.1.2 THOR is hypermethylated in pancreatic cancer

To investigate if THOR is methylated in pancreatic cancer the CpG site targeted by the probe cg11625005 (chr5:1,295,737; position -575 in relation to the transcription start site (TSS)), localized within the THOR region (-591/-159), was analysed (Fig. 4). Pancreatic cancer revealed differential methylation at cg11625005 (a surrogate for the THOR region) with increased methylation levels in primary tumour tissue compared to normal tissue with mean values of 0.5579 and 0.3588, respectively ($p<0.0001$, Fig. 5A). THOR hypermethylation in pancreatic cancer is in concordance with previous results observed for other types of cancer such as prostate cancer [91].

Since THOR was shown to associate with *TERT* expression in other types of cancer [46], we performed a correlation analysis between *TERT* expression and THOR methylation.

Our analysis showed that *TERT* expression has a positive correlation with THOR methylation levels in pancreatic cancer (Fig. 5B, r=0.3580, p<0.0001).

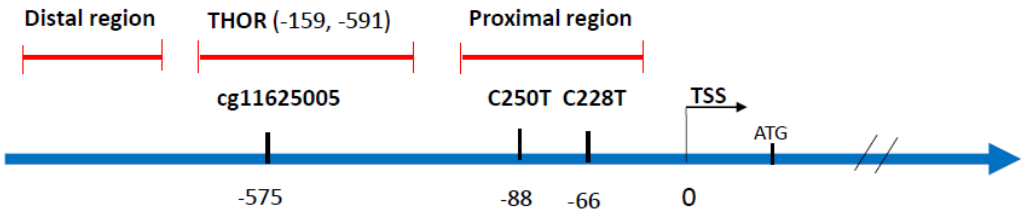


Figure 4. Schematic representation of the *TERT* promoter. THOR, localizes between -159 and -591 base pairs from the transcription start site (TSS), between the proximal and distal regions of the *TERT* promoter. The position of the probe cg11625005 used to evaluate THOR methylation status is shown (chr5:1295737, GRCh37/hg19 assembly). Known *TERT* promoter mutations C250T and C228 (chr5:1295250 and chr5:1295228, respectively, GRCh37/hg19 assembly) are also indicated. The scheme is not scaled.

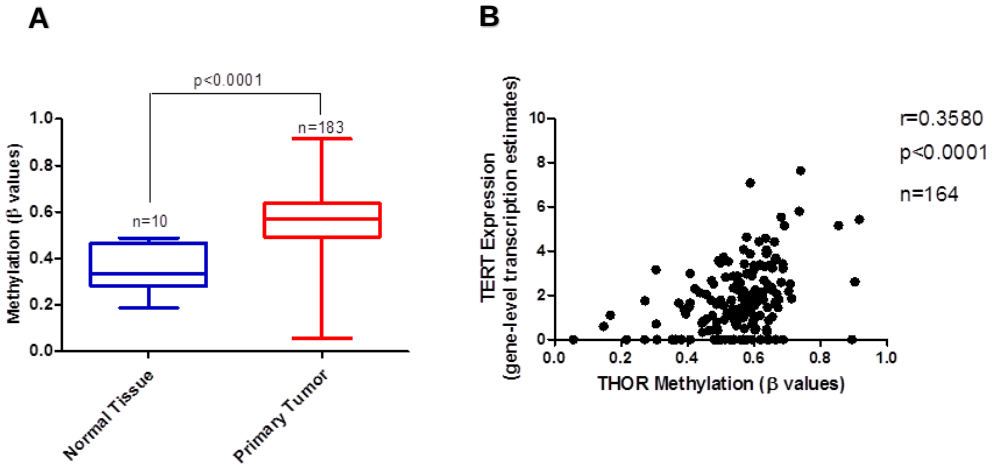


Figure 5. Hypermethylation of THOR in pancreatic cancer. THOR methylation was assessed using the cg11625005 probe in (**A**) normal (n=10) and primary tumour tissue (n=183) in TCGA pancreatic cancer cohort. Methylation was expressed as β-value. Data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value. Statistical analysis was performed using the Mann-Whitney test (**B)** Correlation between cg11625005 methylation (expressed as β-value, X-axis) and *TERT* expression (as gene expression estimates, RSEM, Y-axis) in pancreatic cancer was examined by Spearman's correlation analysis.

3.1.3 THOR methylation distinguishes normal from tumour tissue in pancreatic cancer

To assess if THOR could be a useful tool for predicting disease progression, methylation levels were analysed across disease stages (I-IV) and histological subtypes. In pancreatic cancer, THOR could differentiate normal tissue from all disease stages, including stage I (normal *vs* stage I, p=0.0140; normal *vs* stage II, p<0.0001; normal *vs* stage III, p=0.0024 and normal *vs* stage IV, p=0.0360 Fig.6A). Notably, these results suggested that THOR has potential to be used as a disease biomarker even in early stages.

The ability of THOR to distinguish normal tissue from pancreatic cancer extended to all histological subtypes of ductal and colloid pancreatic carcinomas but no significant differences were observed in the neuroendocrine variety, the less aggressive form of pancreatic cancer (Fig. 6B). However, it has been documented that this specific type of pancreatic cancer relies on the ALT mechanism to maintain telomere integrity [79].

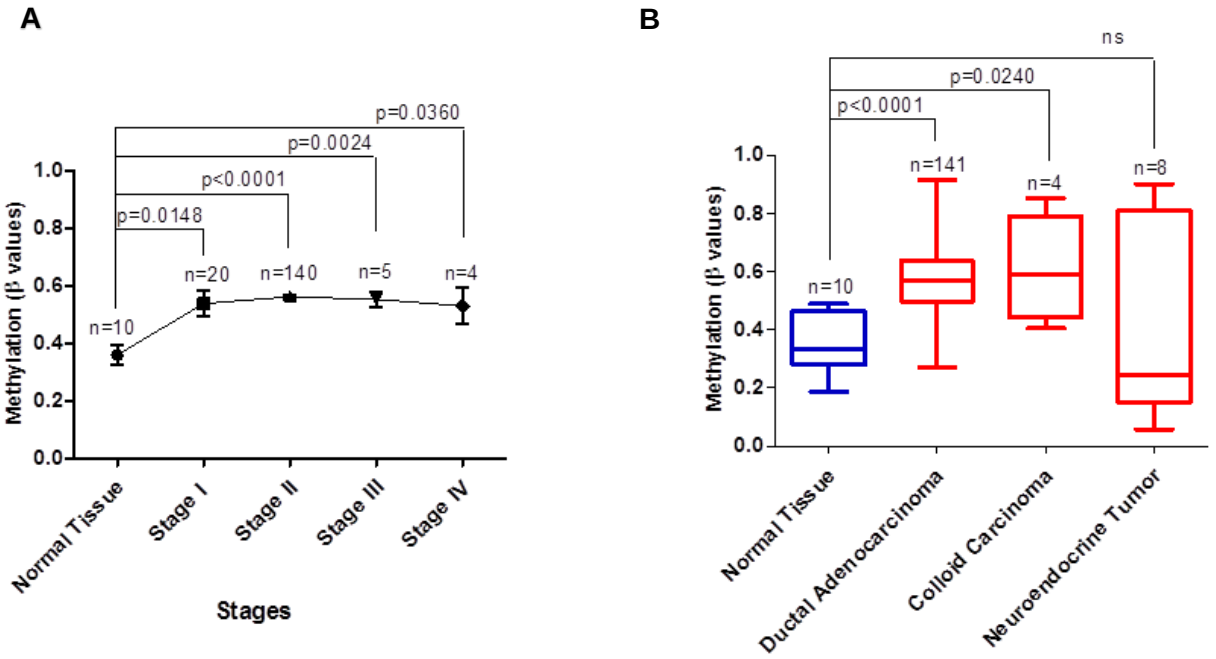
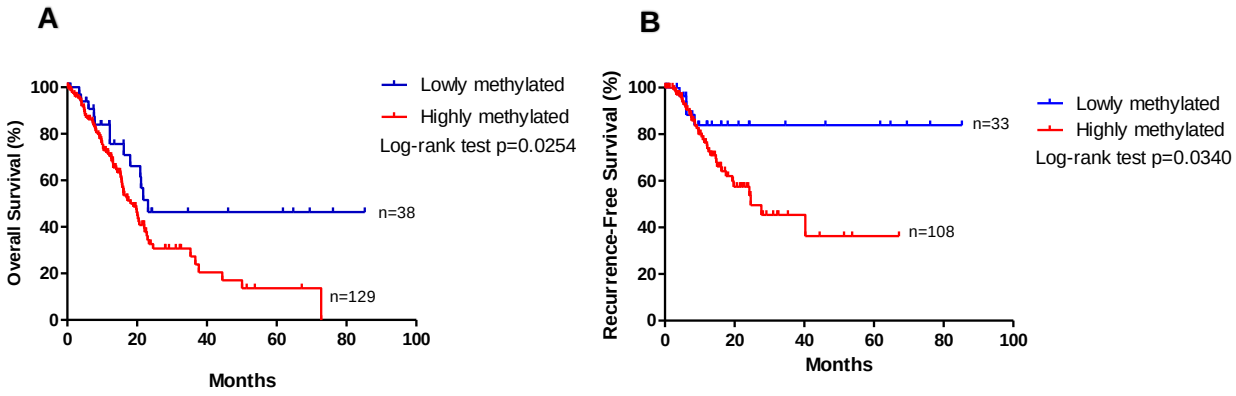


Figure 6. THOR discriminates between normal tissue and malignant pancreatic tissue. Comparison between methylation levels of THOR using the cg11625005 probe according with **(A**) the tumour stage (mean ± SEM**);** and (**B**) the different histological subtypes of pancreatic cancer (data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value). Statistical analysis of the data was performed using the Mann-Whitney test.

3.1.4 THOR methylation predicts outcome in patients with pancreatic cancer

To find out if THOR methylation levels could predict patient outcome and risk of relapse after treatment, the overall survival (OS) and recurrence-free survival (RFS) of the patients was calculated. Pancreatic cancer patients with higher levels of THOR methylation had a shorter OS and thus a poorer prognosis (p=0.0254, Fig. 7A).

Furthermore, RFS of patients with highly methylated THOR is less than half of those with lowly methylated THOR (p=0.0340, Fig. 7B). These results showed that THOR's potential as a prognostic biomarker can be extended to still more tumour types (i.e pancreatic) than previously believed [46,91].



Cut-off Value	Sensitivity (%)	Specificity (%)
0.4893	76	100

Figure 7. THOR can predict survival and recurrence in pancreatic cancer. Kaplan-Meier curve for (**A**) overall survival and (**B**) recurrence-free survival of pancreatic cancer patients. For this analysis, we establish the cut-off value of 0.4893, with a sensitivity of 76% and a specificity of 100% for an AUC of 0.9049 (p<0.0001) in the analysed population (malignant versus benign tissues). Patients with methylation levels inferior to 0.4893 are considered as lowly methylated (represented in blue) and patients with methylation levels superior to 0.4893 are considered highly methylated (represented in red).

3.1.5 Discussion

In this study, we explored the biomarker potential of the THOR methylation signature in pancreatic cancer. To that end, we mined the data made publicly available by the TCGA for their pancreatic cancer cohort, and analysed tumour progression, pathological features and patient outcome focusing on THOR behavior.

We found that THOR is hypermethylated in pancreatic tumour tissue when compared to normal tissue and that THOR methylation correlates with *TERT* expression in tumour samples. Thus, it is possible that THOR is required to activate *TERT* expression in pancreatic cancer.

Previous studies of these tumour types did not detect the ALT mechanism (with the exception of the neuroendocrine subtype of pancreatic cancer), reinforcing the notion that telomerase reactivation is the dominant form of telomere maintenance in these cancers [79,93]. Our data were consistent with these findings as they showed that the methylation status of THOR was higher in tumour tissue from pancreatic patients when compared to the control. Previous studies supporting our findings proved that telomerase reactivation is critical for replicative immortality, and by extension, with highly proliferative tumours, such as pancreatic cancer [77,78].

To further explore THOR as a possible biomarker for pancreatic cancer we quantified THOR methylation throughout disease progression. We found that THOR hypermethylation is an early event in pancreatic cancer and is maintained until stage IV. These findings also indicate that THOR could be used to help distinguish normal tissue from early stage pancreatic cancer, which has posed a major clinical challenge [5]. In fact, delayed diagnosis is largely responsible for the dismal prognosis of pancreatic cancer. Furthermore, THOR could discriminate some histological subtypes of pancreatic cancer. Additionally, THOR was associated with reduced OS and RFS in pancreatic cancer.

These findings suggest that THOR could be a potential diagnostic and prognostic tool in this type of cancer, and future studies should aim to further confirm THOR as a *bona fide* biomarker. Pancreatic cancer is a highly proliferative cancer, so it is possible that pancreatic cancer cells might be more dependent on THOR methylation and subsequent telomerase reactivation, as supported by others [46,77,78].

In conclusion, this proof of principle study supports the potential of THOR methylation to be a specific cancer biomarker in pancreatic cancer, since it could distinguish between normal and malignant tissue and is related with patient survival and recurrence. Nevertheless, further studies are needed to generalize these findings beyond the TCGA cohort.

CHAPTER 3 - RESULTS AND DISCUSSION

3.2 Epigenetic alterations of PI3K/Akt, Wnt, Hedgehog and Notch signalling pathways are associated with survival of pancreatic cancer patients

3.2.1. Introduction

Sustained proliferative signalling is one of the principal characteristics of cancer cells. Aberrant activation of the PI3K/Akt, Wnt, Notch and Hedgehog pathways due to gene expression deregulation contributes to the process of tumourigenesis (Fig. 2) [20]. These pathways have been described as deregulated in PCA and associated with tumour development and progression.

3.2.1.1 Signalling Pathways

3.2.1.1.1 PI3K/Akt Pathway

In the PI3K/Akt pathway, extracellular ligands bind to cell surface receptors leading to PI3Ks activation [21]. Upon activation, PI3K generates PIP3 that recruits both phosphoinositide-dependent protein kinase 1 (PDK1) and Akt to the plasma membrane. This leads to Akt activation that when activated, phosphorylates several proteins that control multiple cellular processes including cell survival and motility [21].

PI3K/Akt signalling pathway is activated in both PDACs and PanNETs [94,95] and impacts important mechanisms that contribute to pancreatic carcinogenesis such as the processes of ADM and the formation of the desmoplastic reaction involved in drug resistance [18].

Despite PI3K/Akt signalling being deeply involved in pancreatic carcinogenesis, the therapeutic strategies targeting proteins involved in this pathway have failed to demonstrate efficiency in clinical trials [18]. PI3K inhibition in combination with other therapeutic agents might improve treatment and lead to a better response of the patients. In fact, combined therapy using a PI3K/mTOR inhibitor and panobinostat, a histone deacetylase inhibitor, have demonstrated an additive effect in cell growth inhibition by studies *in vitro,* reinforcing the role of epigenetic alterations in PCA development [18].

3.2.1.1.2 Embryonic Signalling – Hedgehog, Notch and Wnt Pathways

Hedgehog signalling is implicated in pancreas development and in differentiated pancreatic tissue [96]. There are three human hedgehog genes, which encode for proteins that act in different steps of the embryonic development. These ligands are synthesized as precursor proteins that after several post-translational modifications as well as an autocatalytic step result in the secreted protein capable of producing the biologic effect [96]. Binding of the secreted protein to Patched receptor (PTCH1) disrupts the repression of Smoothened, Frizzled Class Receptor (SMO) proteins. SMO proteins are then capable of activating downstream proteins such as glioma-associated oncogene (GLI) proteins that act as transcriptional activators inducing the expression of several genes that can contribute to cancer progression and development [21,97]. *GLI1* is the principal pathway activator while *GLI2* can act as an activator or a repressor [97]. *GLI3* constitutes the principal pathway repressor [97].

Pancreatic cancer is characterized by aberrant hedgehog ligands expression [97]. However, ligand-dependent hedgehog canonical signalling is not activated in pancreatic tumour epithelium [97]. Hedgehog ligands appear to activate this pathway in stromal cells stimulating the production of factors that influence tumour growth whereas GLI activity in epithelial cells is mediated through alternative pathways such as the oncogenic KRAS or the TGFB signalling [97].

Activation of hedgehog signalling increases during the progression of precursor lesions as PanINs to pancreatic ductal adenocarcinoma [96,98]. Both *in vitro* and *in vivo* studies showed that pharmacologic inhibition of hedgehog signalling blocked cell growth and proliferation [96,98]. This reflects the role of hedgehog pathway in pancreatic tumourigenesis and the potential of investigating alterations in genes related with this pathway as biomarkers and therapeutic targets.

Notch genes encode for transmembrane receptors that mediate organ development and tissue proliferation during the embryonic development. Binding of its ligands, Delta and Jagged induces receptors cleavage mediated by γ-secretase [21]. Release and translocation to the nucleus of the cytoplasmic domain allows its association with transcription factors promoting expression of genes associated with cell growth and proliferation [21].

The role of Notch signalling in cancer is still controversial as there is evidence of Notch activation leading to both cancer development and suppression [99,100]. In pancreatic cancer, this signalling pathway can act as both oncogenic and tumour suppressor in different cellular contexts [99,101]. The oncogenic role of Notch is associated with the most aggressive form of pancreatic cancer, PDAC while in PanNETs this pathway seems to act as tumour suppressor [99,101]. In PDAC, acquisition of epithelial to mesenchymal transition (EMT) phenotype by cancer cells can be induced by Notch signalling activation [97]. Inhibition of this pathway leads to inhibition of cell growth and suppresses the metastatic capacity of pancreatic cancer cells [97]. This pathway is known to be activated in response to pancreatic inflammation and promotes the ADM process [17].

Notch pathway pharmacologic inhibition using γ-secretase inhibitors in PCA biological models resulted in EMT blockage and suppressed pancreatic cells proliferation and invasion [97,100]. However, these pharmacological agents present high toxicity with multiple side effects associated with its administration and thus are not an effective therapeutic option for PCA treatment [100]. Alternatively, abrogation of this pathway with other agents with less adverse effects can be a viable option for PCA treatment. Some studies reported that genistein could inhibit Notch expression through upregulation of miR-34a [100]. Additionally, administration of agents with ability to modify the epigenome of cells such curcumin and sulforaphane were also capable of decrease Notch expression and suppress cancer progression [100].

Wnt signalling is also involved in pancreas development but absent in the mature pancreatic tissue [102]. There are three different Wnt-signalling transduction cascades: the canonical Wnt- β -catenin, the Wnt-Ca2+ and the planar-cell polarity [21].

In the canonical pathway, when off state, β-catenin is phosphorylated and targeted for degradation by the proteasome. Binding of the secreted protein Wnt to its receptor, Frizzled, activates this pathway and leads to stabilization of β-catenin by downstream proteins of the pathway. This leads to β-catenin translocation to the nucleus where it forms a complex with transcription factors (TCF-Lef) to induce gene expression [21].

Canonical Wnt signalling is activated in PDAC and PanIN samples but contrarily to other gastrointestinal cancers, mutations in *APC* or β-catenin gene are rare in this type of cancer [102]. The role of this pathway in PanNETs is not fully understood but it appears that Wnt activation mediated by Menin can inhibit islet tumour cell proliferation [103].

These pathways play key roles in pancreatic carcinogenesis and mechanisms involved in the transcriptional regulation of pathway control genes hold enormous potential as biomarkers and therapeutic targets for PCA.

Here, we will focus on DNA methylation and miRNAs to investigate the epigenetic regulation of genes related with those pathways and we will integrate these data with the clinical data from the patients to scrutinize the potential of epigenetic alterations as PCA biomarkers.

3.2.2 Results

3.2.2.1 Genes involved in PI3K/Akt and embryonic signalling pathways are deregulated in pancreatic cancer

In order to get more insights into the relevance of the PI3K/Akt, Notch, Hedgehog and Wnt signalling pathways in pancreatic cancer, we started by assessing which genes involved in these pathways are differentially expressed between normal and malignant tissue. Pathway analysis is dependent on functional annotation of genes and different databases can retrieve different sets of genes associated with specific pathways, making gene selection challenging. In this study, the selection of the genes of interest was based on the differential gene expression between normal and malignant tissue using the Pancreatic Expression Landscape resource integrated in PED database [72,73]. The data information obtained from this online platform is the result of a meta-analysis performed by Gadaleta and coworkers that combines the data from 309 pancreatic cancer data files including data from 4 samples of healthy pancreas, 53 samples of normal pancreatic tissue adjacent to cancer and 96 PCA samples [73].

In this first analysis, the inclusion criteria to unveil genes differentially expressed between healthy and PCA patient samples were a fold-change ≥ 1.5 and a p-value <0.05. With these inclusion criteria, we obtained a total of 35 genes that were differentially expressed. The genes representative of each pathway and the respective alterations are represented in Table 2. From those 35 genes, 15 genes were related with the PI3K/Akt pathway, 1 gene associated with the Notch pathway, 5 and 14 genes involved in the Hedgehod and Wnt embryonic signalling pathways, respectively (Table 2).

Our analysis revealed that the vast majority of the genes differentially expressed are upregulated in tumour tissue except for the *MAP2K2* and *EIEF4EBP1* genes of the PI3K/Akt pathway and the *SFRP5* gene, related with the Wnt pathway. These genes present higher levels of expression in samples of normal pancreatic tissue (Table 2).

Of the PI3K/Akt pathway set of genes, the most altered one was the *SFN* gene with a maximum of 5.65 fold increase in tumours (Table 2).

The only gene related with the Notch pathway that presented differential expression between malignant and normal tissue was the *JAG1* with the tumour samples expressing almost two times more than the control samples.

Considering the Hedgehog and the Wnt pathway, the genes that presented major increases were the *GLI3* and the *SFRP4* genes, being the latest the one that presented the highest difference with the malignant tissue, being expressed nearly seven times more than the healthy tissue (Table 2). As expected, most genes upregulated in the tumour samples are associated with pathway activation.

These results reflect the deregulation of those signalling pathways in patients with pancreatic cancer reinforcing its importance in pancreatic carcinogenesis.

Table 2 - Differentially expressed genes in pancreatic cancer. Genes related with the PI3K/Akt, Notch, Hedgehog and Wnt pathways that present differential expression between malignant and healthy pancreatic tissue in the data available on the Pancreatic Expression Landscape integrated in the PED database.

Pathway	Gene	Name	Pathway activation (↑) or repression (↓)	Probe	Fold-Change	p-value	Expression Higher
PI3K/Akt	*ITGA4*	Integrin subunit alfa 4	↑	205884_at	2.26	5.98E-05	Tumour
				205885_s_at	2.21	9.64E-05	
	SFN	Stratifin	↑	209260_at	2.64	1.54E-04	Tumour
				33322_i_at	5.65	4.61E-11	
				33323_r_at	5.47	2.82E-09	
	PPP2R5C	Protein phosphatase 2 regulatory subunit B'gamma	↓	1557718_at	2.47	<0.05	Tumour
	PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta	↑	203879_at	2.08	9.38E-04	Tumour
	ITGA2	Integrin subunit alfa 2	↑	205032_at	3.07	2.75E-05	Tumour
				227314_at	4.77	1.08E-10	
	PIK3R1	Phosphoinositide-3-kinase regulatory subunit 1	↑	212239_at	1.92	<0.05	Tumour
	AKT3	AKT serine/threonine kinase 3	↑	212609_s_at	1,90	4.00E-05	Tumour
				222880_at	1,58	6.55E-04	
	EIF4EBP1	Eukaryotic translation initiation factor 4E binding protein 1	↑	221539_at	-2.46	3.11E-08	Normal Tissue
	INPP5D	inositol polyphosphate-5-phosphatase D	↓	203332_s_at	2.11	9.17E-04	Tumour
	JAK2	Janus kinase 2	↑	205842_s_at	2.05	3.71E-05	Tumour
	MRAS	Muscle RAS oncogene homolog	↑	225185_at	1.84	3.16E-06	Tumour
	MAP2K2	Mitogen-activated protein kinase kinase 2	↑	213490_s_at	-2.03	5.22E-06	Normal Tissue
	MAP3K8	Mitogen-activated protein kinase kinase kinase 8	↑	205027_s_at	3.48	4.37E-08	Tumour
	RAC2	Rac family small GTPase 2	↑	213603_s_at	3.51	1.78E-07	Tumour
	PTEN	Phosphatase and tensin homolog	↓	1556006_s_at	3.88	<0.05	Tumour
				242482_at	2.14	<0.05	
				233314_at	2.24	0.003	
Notch	*JAG1*	Jagged 1	↑	209098_s_at	1.34	0.032	Tumour
				209099_x_at	1.71	1.36E-04	Tumour
				216268_s_at	1.94	1.83E-05	Tumour
Hedgehog	*TGFBR1*	Transforming growth factor beta receptor 1	↑	224793_s_at	1.90	1.19E-04	Tumour
	GLI3	GLI family zink finger 3	↓	227376_at	2.40	2.32E-04	Tumour
	TGFB1	Transforming growth factor beta 1	↑	203085_s_at	2.23	3.6E-04	Tumour
	CCNB1	Cyclin B1	↑	214710_s_at	2.13	0.002	Tumour
				228729_at	2.03	0.001	
	PRKAR1A	protein kinase cAMP-dependent type I regulatory subunit alpha	↑	242482_at	2.14	<0.05	Tumour

Table 2 (continued)

Pathway	Gene	Function	Pathway activation(↑) or repression (↓)	Probe	Fold-change	p-value	Expression Higher
Wnt	*DKK3*	Dickkopf WNT signalling pathway inhibitor 3	↓	202196_s_at	4.10	2.48E-07	Tumour
				214247_s_at	3.48	2.11E-04	
				230508_at	1.19	4.33E-02	
	SFRP2	Secreted frizzled related protein 2	↓	223121_s_at	5.36	3.74E-09	Tumour
				223122_s_at	4.85	5.39E-10	
	CDH3	Cadherin 3	↑	228729_at	2.03	0.001	Tumour
	CTNNB1	Catenin beta 1	↑	203256_at	2.37	0.001	Tumour
	FRZB	Frizzled related protein	↓	203697_at	2.46	3.11E-04	Tumour
				203698_s_at	3.29	3.61E-06	
	FZD7	Frizzled class receptor 7	↑	203705_s_at	2.48	1.19E-04	Tumour
				203706_s_at	3.56	1.23E-06	
	LEF1	Lymphoid enhancer binding factor 1	↑	221558_s_at	5.41	8.95E-16	Tumour
	SFRP4	Secreted frizzled related protein 4	↓	204051_s_at	6.76	1.06E-14	Tumour
	SFRP5	Secreted frizzled related protein 5	↓	207468_s_at	-3.79	6.33E-06	Normal Tissue
	TCF4	Transcription factor 4	↑	203753_at	1.82	7.88E-04	Tumour
				212387_at	2.29	1.04E-05	
				213891_s_at	2.04	1.86E-04	
				222146_s_at	2.43	1.50E-06	
				228837_at	1.35	5.85E-05	
	WNT2	Wnt family member 2	↑	205648_at	2.27	1.45E-04	Tumour
	WNT5A	Wnt family member 5A	↑	205990_s_at	1.83	0.007	Tumour
	PPARD	Peroxisome proliferator activated receptor delta	↑	37152_at	3.21	4.43E-11	Tumour
	CSNK1A1	Casein kinase 1 alpha 1	↓	243338_at	2.41	<0.05	Tumour

3.2.2.2 Differential DNA methylation of pathway related genes correlates with gene expression

Since gene expression is highly regulated through epigenetic mechanisms, we asked if the differential expression of the genes identified above could be due to such mechanisms.

Hence, we investigated the methylation status of the 35 differentially expressed genes by analysing the DNA methylation data available at the TCGA for the pancreatic cancer cohort. For that, each of the 35 genes identified were analysed individually regarding the methylation status for each of the probes covering the entire gene. Of notice, in this database, different genomic locations have different coverage regarding DNA methylation, meaning that different genes are covered by a different number of probes.

Here, we have analysed the methylation status of each of the probes covering the 35 genes identified above. As previously mentioned, in the methylation analysis, only the probes with a methylation delta beta absolute value (|Δβ|) equal or bigger than 0.2 and a p-value lower than 0.05 were considered as differentially methylated and selected for further analysis.

In a general view, our analysis revealed that from the 35 genes in study only 9 genes were differentially methylated, meaning that only those presented probes meeting our inclusion criteria, corresponding to a total of 27 probes (Table 3). From these, about 64% of the probes were hypermethylated while 36% were hypomethylated in primary tumour tissue when compared with control samples (Fig. 8A). However, it is known that the effect of DNA methylation on gene expression is dependent of the CpGs genomic locations within a gene.

With that in mind, we investigated which regions of the selected genes were differentially methylated (Table 3 and Fig. 8B). We found that the location of the CpG probes analysed in our study were not evenly distributed along the entire gene region and that 70% of the CpG probes differentially methylated were located in important regulatory regions: the promoter region (TSS1500 and TSS200) and the 5'UTR (Fig. 8B). These regions are deeply involved in transcriptional regulation and alteration of their normal methylation patterns can lead to altered gene expression and protein production [104]. None of the CpG sites significantly altered was at the 3'UTR of the genes.

A closer view of the differentially methylated genes revealed that more than half were related with the PI3K/Akt pathway suggesting that DNA methylation can be an important mechanism for the deregulation of this pathway in pancreatic carcinogenesis (Table 3). In fact, for the PI3K/Akt pathway, one third of the genes analysed (five of fifteen initial genes) fulfilled the established parameters to be considered differentially methylated: *ITGA4*, *SFN*, *PIK3CD*, *ITGA2* and *PIK3R1*. From these, we could only identify one probe differentially methylated in *PIK3CD*, *ITGA2* and *PIK3R1* genes while *ITGA4* and *SFN* presented four and five probes, respectively.

Regarding the other four differentially methylated genes, two were related with the Hedgehog signalling pathway, namely *TGFBR1* and *GLI3,* and the other two (*DKK3* and the *SFRP2*) with the Wnt pathway (Table 3). From these, *TGFBR1 and DKK3* presented only one probe with |Δβ|>0.2, while *GLI3 and SFRP2* had four and nine differentially methylated probes.

JAG1, the only gene related with the Notch signalling, did not present a |Δβ|>0.2 and thus it was not considered for further analysis.

Since DNA methylation is known to affect gene expression, we performed correlation analysis between these two parameters. From the 9 genes differentially methylated only 7 had at least one CpG site which methylation correlated with gene expression with a p-value<0.05 (Table 4). In total, the methylation levels of 16 probes covering 7 different genes presented significant correlation with gene expression (Table 4).

To our knowledge, only the *PIK3R1, SFN* and the *ITGA2* genes have been previously reported as differentially methylated in PCA compared to normal pancreatic tissue [55,57,105]. In a study carried out by Ramirez and colaborators, *SFN* hypermethylation was associated with patients' sensitivity to therapy with cisplatin and gemcitabine in lung cancer [68,106].

To further explore the biomarker potential of the epigenetic alterations here identified, only the genes that presented alterations in the methylation levels, and correlated changes in expression levels were selected for the analysis considering the clinical parameters of the patients.

Table 3 – Differential methylated genes in the TCGA pancreatic cancer cohort.

Pathway	Gene	Probe	CpG location	Mean methylation Normal tissue	Mean methylation Tumour tissue	|Δβ|	Methylation higher	p-value
PI3K/Akt	*ITGA4*	cg25652029	5'UTR;1stExon	0.05121	0.263	0.21179	Tumour	0.0004
		cg06952671	5'UTR;1stExon	0.02106	0.2828	0.26174	Tumour	<0.0001
		cg21995919	5'UTR;1stExon	0.04834	0.2738	0.22546	Tumour	0.0003
		cg25024074	1stExon	0.07445	0.3427	0.26825	Tumour	0.0003
	SFN	cg17330303	5'UTR;1stExon	0.8891	0.6121	0.277	Normal Tissue	0.0002
		cg13466284	5'UTR;1stExon	0.7461	0.5177	0.2284	Normal Tissue	0.0001
		cg07786675	1stExon	0.8341	0.5408	0.2933	Normal Tissue	0.0001
		cg13374701	1stExon	0.9243	0.677	0.2473	Normal Tissue	0.0001
		cg12583970	1stExon	0.8847	0.6573	0.2274	Normal Tissue	<0.0001
	PIK3CD	cg07805542	Body	0.6719	0.3175	0.3544	Normal Tissue	0.0005
	ITGA2	cg08446038	Body	0.547	0.2715	0.2755	Normal Tissue	0.0023
	PIK3R1	cg15021292	TSS1500	0.7572	0.5152	0.242	Normal Tissue	0.0002
Hedgehog	*TGFBR1*	cg13443911	TSS1500	0.7153	0.5034	0.2119	Normal Tissue	0.0013
	GLI3	cg17390350	5'UTR	0.08603	0.3863	0.30027	Tumour	0.0003
		cg06310816	5'UTR	0.08255	0.3941	0.31155	Tumour	0.0004
		cg11366849	5'UTR	0.1762	0.4582	0.282	Tumour	0.0002
		cg25726664	TSS1500	0.0804	0.3018	0.2214	Tumour	0.0017
Wnt	*DKK3*	cg26446832	Body	0.8128	0.5586	0.2542	Normal Tissue	0.0002
	SFRP2	cg20881942	TSS1500	0.1358	0.3823	0.2465	Tumour	0.0007
		cg00082664	TSS1500	0.08895	0.3402	0.25125	Tumour	0.0002
		cg25645268	TSS1500	0.0347	0.2554	0.2207	Tumour	<0.0001
		cg23207990	TSS1500	0.07334	0.328	0.25466	Tumour	0.0002
		cg22178613	TSS1500	0.09554	0.3297	0.23416	Tumour	0.0002
		cg23121156	TSS200	0.114	0.3387	0.2247	Tumour	0.0002
		cg05164933	TSS200	0.08375	0.3545	0.27075	Tumour	0.0002
		cg03202804	TSS200	0.0791	0.284	0.2049	Tumour	0.0002
		cg05874561	1stExon	0.07394	0.3025	0.22856	Tumour	<0.0001

Table 4 – Correlation between DNA methylation and gene expression levels in the TCGA pancreatic cancer cohort.

Pathway	Gene	Probe	Spearman r	p-value	Correlation
PI3K/Akt	*ITGA4*	cg25652029	-0.3868	<0.0001	Negative
		cg06952671	-0.3615	<0.0001	Negative
		cg21995919	-0.2992	<0.0001	Negative
		cg25024074	-0.3396	<0.0001	Negative
	SFN	cg17330303	-0.5648	<0.0001	Negative
		cg13466284	-0.5833	<0.0001	Negative
		cg07786675	-0.5519	<0.0001	Negative
		cg13374701	-0.6049	<0.0001	Negative
		cg12583970	-0.5921	<0.0001	Negative
	PIK3CD	cg07805542	0.2534	0.0011	Positive
	ITGA2	cg08446038	-0.4785	<0.0001	Negative
	PIK3R1	cg15021292	0.2214	0.0045	Positive
Wnt	*SFRP2*	cg20881942	-0.2976	0.0001	Negative
		cg23207990	-0.161	0.0395	Negative
		cg22178613	-0.1907	0.0144	Negative
Hedgehog	*GLI3*	cg25726664	-0.216	0.0055	Negative

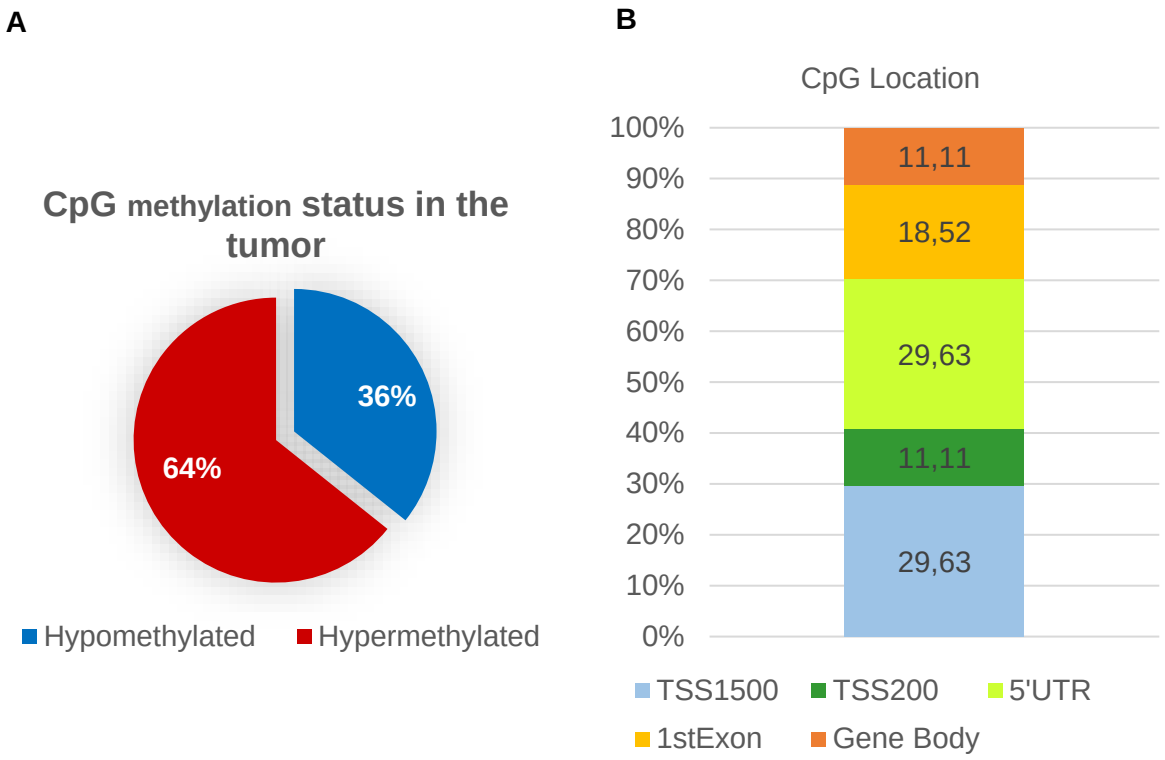


Figure 8 - CpGs differentially methylated in pancreatic cancer. A. CpGs hypomethylated (% represented in blue) and hypermethylated (% represented in red) in tumour samples compared to the control. **B.** Distribution of the probes for the Infinium HumanMethylation450. **TSS1500 and TSS200:** probes located within 1500 and 200 base pairs from the transcription start site, respectively; **5'UTR:** 5' untranslated region.

3.2.2.3 miRNAs alterations are correlated with gene expression

Since only 9 out of the 35 differentially expressed genes present distinct levels of methylation between healthy and tumour samples, we asked if other epigenetic mechanism could be regulating those genes. In addition to DNA methylation, miRNAs are also involved in epigenetic regulation [24] and are known to be deregulated in cancer, including pancreatic cancer, acting as either tumour repressors or oncomiRs [62].

In that sense, we first searched for miRNAs targeting the 35 genes in this study using the miRDB database and the previously established criteria. Only 30 genes were predicted to be targets of miRNAs, according to our selection criteria. Next, since miRNAs can impact on gene expression we assessed the correlation between miRNA expression and expression of the mRNA targets. For that, we used the miRNA and gene expression data available at the TCGA. Unfortunately, we were not able to compare miRNA expression levels between normal and malignant tissue due to the scarcity of data from normal tissue. Thus, the correlation between miRNA and target mRNA expression was only performed on tumour samples and there was no available data for all miRNAs. Of the miRNAs that fulfilled the selection criteria, a total of 28 miRNAs presented a significant correlation with gene expression levels, targeting

a total of 9 different genes (Table 5). As expected, most of the miRNAs presented a negative correlation with gene expression levels since miRNAs are known to downregulate gene expression through the induction of translational repression or degradation of specific mRNAs [62,107].

However, the idea that gene regulation mediated by miRNAs leads undoubtedly to translational repression has been recently challenged [107]. An increasing number of studies have been reporting that miRNA can positively regulate gene expression under specific conditions [107]. Intriguingly, 5 of the miRNAs analysed in this study showed a positive correlation with gene expression (Table 5).

To assess the potential of miRNAs expression as PCA biomarkers, we have considered the 28 miRNAs that significantly correlated with gene expression for the correlation analysis with the survival of the patients.

Table 5 – Correlation between miRNA and gene expression levels in the TCGA pancreatic cancer cohort.

Pathway	Gene	Human mature miRNA name	Spearman R	p-value
PIK3/Akt	*TGFBR1*	hsa-miR-590-3p	-0.3210	<0.0001
		hsa-miR-200a-3p	-0.2180	0.0050
		hsa-let-7c-5p	0.1989	0.0107
		hsa-let-7g-5p	-0.2481	0.0002
	INPP5D	hsa-miR-126-5p	0,2909	p<0.0001
	PTEN	hsa-miR-142-5p	0,3341	p<0.0001
		hsa-miR-29a-3p	-0,1648	0,0284
		hsa-miR-19a-3p	-0,3278	p<0.0001
		hsa-miR-29b-3p	-0,4141	p<0.0001
		hsa-miR-19b-3p	-0,3884	p<0.0001
	EIF4EBP1	hsa-miR-874-3p	-0,2989	p<0.0001
	MRAS	hsa-miR-654-5p	0,2409	0,0013
		hsa-miR-1271-5p	0,1548	0,0397
		hsa-miR-96-5p	-0,6703	p<0.0001
	AKT3	hsa-miR-15b-5p	-0,164	0,0292
		hsa-miR-16-5p	-0,1538	0,041
		hsa-miR-30e-3p	0,1533	0,0416
		hsa-miR-181b-5p	-0,2615	0,0004
		hsa-miR-17-3p	-0,1998	0,0077
		hsa-miR-181a-5p	-0,2417	0,0012
		hsa-miR-22-3p	0,2977	p<0.0001
Hedgehog	*JAG1*	hsa-miR-153-3p	-0,1956	0,0091
		hsa-miR-186-5p	-0,1627	0,0304
Wnt	*CSNK1A1*	hsa-miR-30a-5p	-0,2055	0,0061
		hsa-miR-30b-5p	-0,2191	0,0034
		hsa-miR-30e-5p	-0,3264	p<0.0001
		hsa-miR-30c-5p	-0,3144	p<0.0001
	FZD7	hsa-miR-338-5p	-0,2028	0,0068

3.2.2.4 Epigenetic alterations predict outcome in patients with pancreatic cancer

3.2.2.4.1 DNA Methylation

To determine the clinical significance and biomarker potential of the observed epigenetic alterations we analysed patient overall survival considering the methylation levels of the most relevant probes and the expression levels of selected miRNAs.

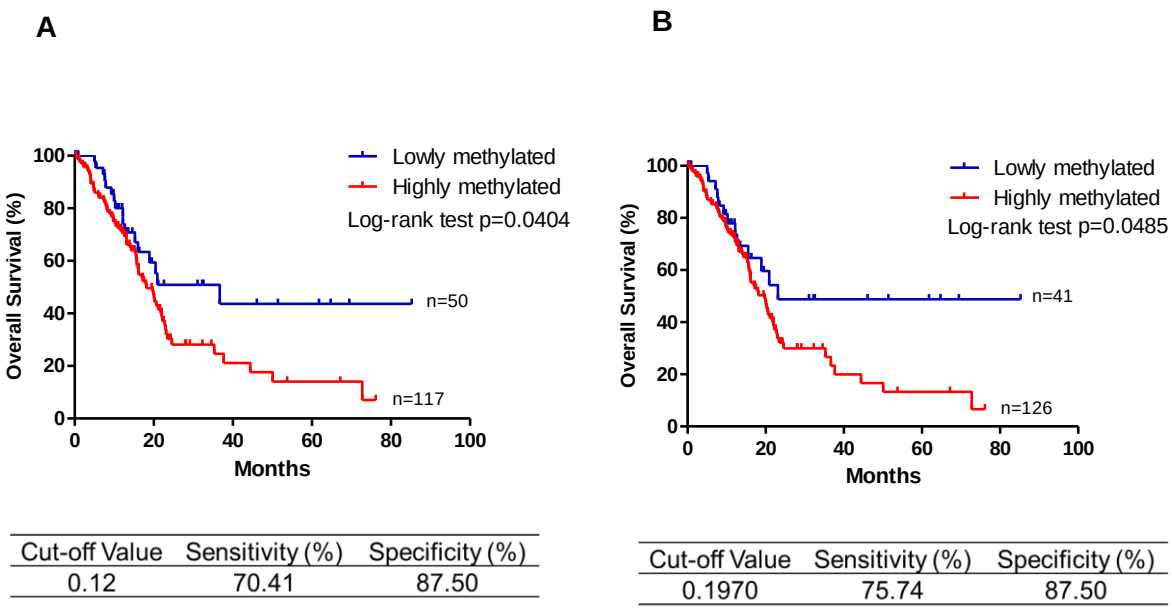
The methylation levels of the *ITGA4*, *SFN*, *ITGA2* and *PIK3R1* genes, related with the PI3K/Akt pathway, were correlated with the survival of the patients (Figs. 9, 10, 12, 13). Interestingly, patients with higher methylated levels in *SFN, ITGA2* and *PIK3R1* presented a better prognosis (Fig. 10, 12, 13) while higher levels of *ITGA4* were indicative of a worst prognosis (Fig. 9).

The methylation levels of the CpG sites targeted by the cg21995919 and cg25024074 probes in the *ITGA4* gene significantly correlated with the survival of the patients. Both probes were present at the 1st exon of the gene and presented negative correlation with gene expression (Table 3, 4). Methylation of this region is usually associated with transcriptional repression [104]. In our analysis, we tested multiple cut-off values for each probe to uncover the value with the higher potential to distinguish subgroups of patients with different outcomes.

For the cg21995919 probe, the most relevant methylation cut-off value was of 0.12 that revealed a sensitivity of 70.41% and specificity of 87.59% (Fig.9A). Regarding the cg25024074 probe, we considered the methylation cut-off value of 0.1970 with sensitivity and specificity values of 75.74% and 87.50, respectively (Fig. 9B).

This gene encodes for the alpha 4 subunit of an integrin protein. When associated with a beta subunit it forms a heterodimeric protein. Integrins are proteins present at the cell membrane that are involved in the activation of cellular pathways that play a role in cell motility and proliferation including the PI3K/Akt pathway [21].

Patients with methylation values superior to the cut-off values presented lower time of survival and higher methylation values appear to be associated with lower expression of the gene. However, the Spearman r values are not supportive of a strong correlation (Table 4). This region represents a potential binding site for both transcriptional activators and repressors. Methylation of this region can potentially impair the binding of activators leading to a decrease in gene expression.



Cut-off Value	Sensitivity (%)	Specificity (%)
0.12	70.41	87.50

Cut-off Value	Sensitivity (%)	Specificity (%)
0.1970	75.74	87.50

Figure 9 – *ITGA4* methylation can predict survival in pancreatic cancer. A. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg2195919. For this analysis, we establish the cut-off value of 0.12 with sensitivity and specificity values of 70.41% and 87.50%, respectively. **B.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg25024074. For this analysis, we establish the cut-off value of 0.1970 with sensitivity and specificity values of 75.74% and 87.50%, respectively. Patients with methylation levels inferior to the cut-off value are considered as lowly methylated (represented in blue) and patients with methylation levels superior to the cut-off value are considered highly methylated (represented in red).

Considering the epigenetic regulation of the *SFN* gene, the methylation levels of five probes were significantly correlated with the survival of the patients: probes cg17330303, cg13466284, cg07786675, cg13374701 and cg12583970. The cg13466284 target a CpG located in the 5'UTR in the gene and the remaining probes target CpGs located in the 1st exon of the gene (Table 3). Methylation of these regions has been shown to be associated with transcriptional repression [104] and methylation in these regions were negatively correlated with gene expression.

For the cg17330303 the most meaningful cut-off value was 0.6973 with sensitivity and specificity values of 75.15% and 87.50%, respectively (Fig.10A). Analysis of the methylation levels of this probe also presented predictive potential regarding RFS of patients (Fig.11A). Comparing the OS of the patients considering the cut-off value of 0.5906 for the cg13466284 probe it is possible to discriminate patients with different outcomes with a sensitivity of 75.15% and specificity of 87.50% (Fig.10B).

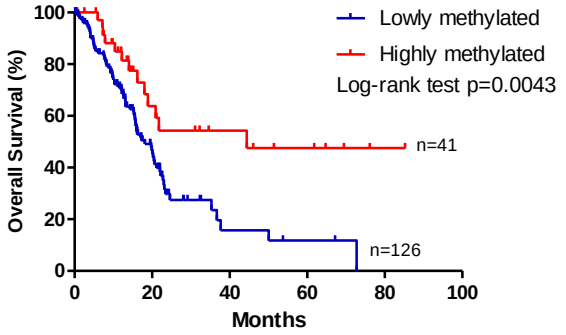
Similarly, the survival analysis considering the methylation levels of the cg07786675 probe establishing the cut-off value of 0.5922 have revealed to be highly sensitive with a test sensitivity of 75.15%. With this cut-off value, we obtained a specificity value of 87.50% (Fig.10C). Nevertheless, methylation levels in these regions could predict RFS.

For the cg13374701, significant differences between highly and lowly methylated groups of patients considering the cut-off value of 0.7923 were observed. The sensitivity and specificity values obtained for this test were 80.47 and 87.50%, respectively (Fig.10D).

Methylation of this region was also correlated with RFS of patients with lower methylation being associated with early recurrence (Fig. 11B).

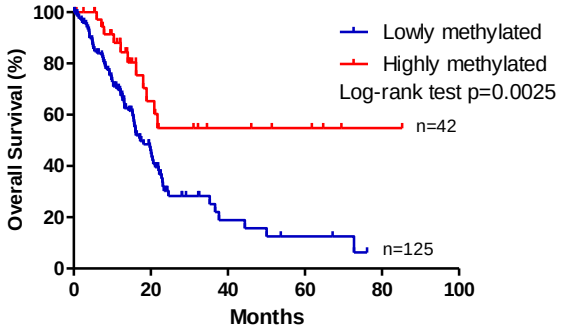
Finally, for the cg12583970 probe the cut-off value of 0.7683 had the potential to predict the OS and RFS of the patients with a sensitivity and specificity of 75.15% and87.50%, respectively (Fig.10E, 11C). Patients with methylation values inferior to the cut-offs presented lower time of survival. Lower methylation levels can lead to increased gene expression and consequent activation of the signalling pathway that can facilitate the progression of the disease. The *SFN* gene encodes for a protein involved in Akt/mTOR pathway activation and overexpression of this gene in PCA cell lines was associated with resistance to cisplatinum treatment [108].

A



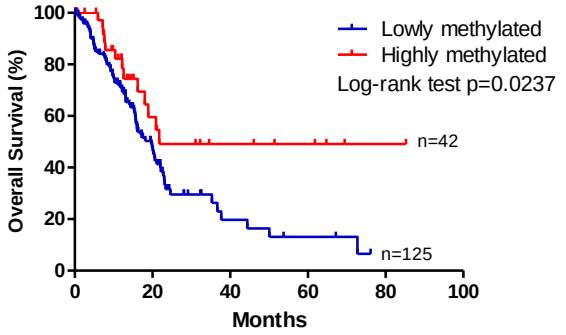
Cut-off Value	Sensitivity (%)	Specificity (%)
0.6973	75.15	87.50

B



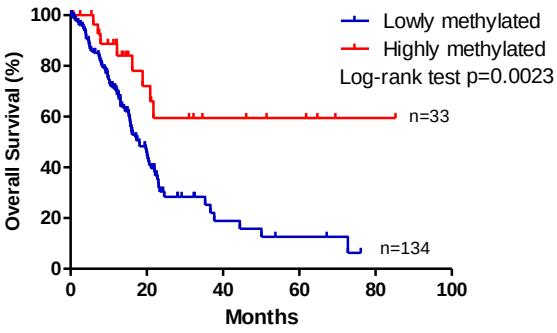
Cut-off Value	Sensitivity (%)	Specificity (%)
0.5906	75.15	87.50

C



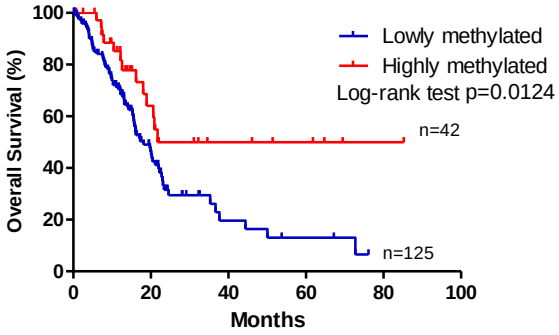
Cut-off Value	Sensitivity (%)	Specificity (%)
0.5922	75.15	87.50

D



Cut-off Value	Sensitivity (%)	Specificity (%)
0.7923	80.47	87.50

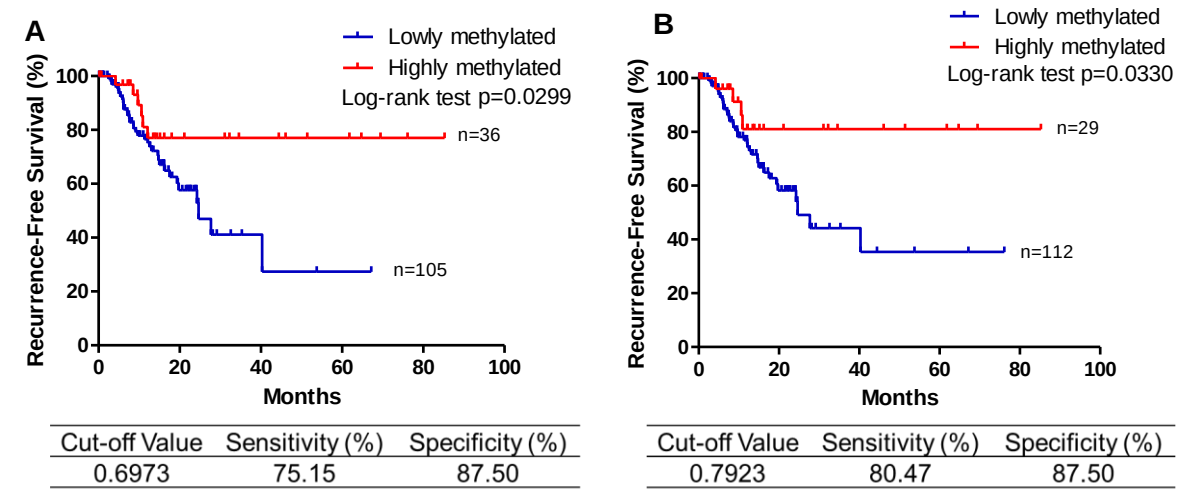
E



Cut-off Value	Sensitivity (%)	Specificity (%)
0.7683	75.15	87.50

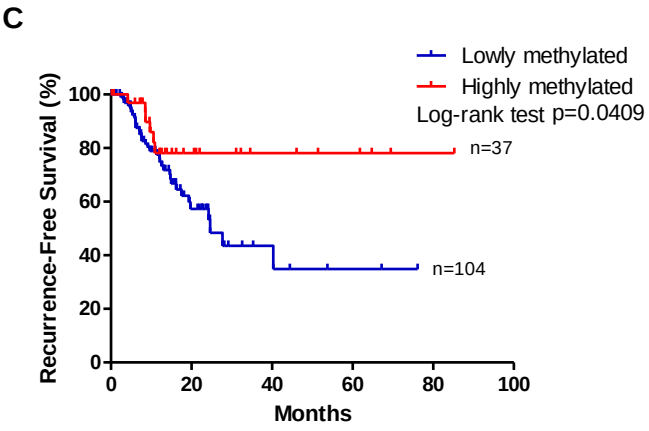
Figure 10 – *SFN* methylation can predict survival in pancreatic cancer. A. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg17330303. For this analysis, we establish the cut-off value of 0.6973 with sensitivity and specificity values of 75.15% and 87.50%, respectively. **B.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg13466284. For this analysis, we establish the cut-off value of 0.5906, with sensitivity and specificity values of 75.15% and 87.50%, respectively. **C.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg07786675. For this analysis, we establish the cut-off value of 0.5922,

with sensitivity and specificity values of 75.15% and 87.50%, respectively. **D.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg13374701. For this analysis, we establish the cut-off value of 0.7923, with sensitivity and specificity values of 80.47% and 87.50%, respectively. **E.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg12583970. For this analysis, we establish the cut-off value of 0.7683, with sensitivity and specificity values of 75.15% and 87.50%, respectively. Patients with methylation levels inferior to the cut-off value are considered as lowly methylated (represented in blue) and patients with methylation levels superior to the cut-off value are considered highly methylated (represented in red).



Cut-off Value	Sensitivity (%)	Specificity (%)
0.6973	75.15	87.50

Cut-off Value	Sensitivity (%)	Specificity (%)
0.7923	80.47	87.50



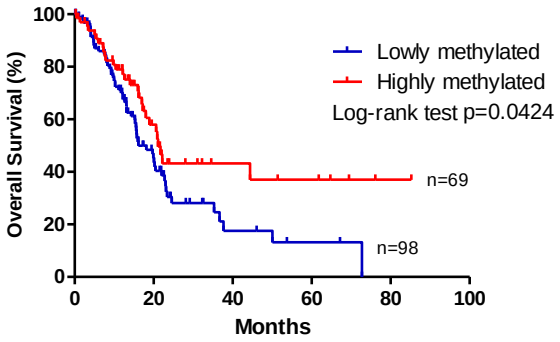
Cut-off Value	Sensitivity (%)	Specificity (%)
0.7683	75.15	87.50

Figure 11 – *SFN* methylation can predict recurrence in pancreatic cancer. A. Kaplan-Meier curve for recurrence-free survival of pancreatic cancer patients considering the methylation levels of the probe cg17330303 considering the cut-off value of 0.6973 with sensitivity and specificity values of 75.15% and 87.50%, respectively. **B.** Kaplan-Meier curve for recurrence-free survival of pancreatic cancer patients considering the methylation levels of the probe cg13374701 considering the cut-off value of 0.7923 with sensitivity and specificity values of 80.47% and 87.50%, respectively. **C.** Kaplan-Meier curve for recurrence-free survival of pancreatic cancer patients considering the methylation levels of the probe cg12583970 considering the cut-off value of 0.7683 with sensitivity and specificity values of 75.15% and 87.50%, respectively.

Regarding the epigenetic regulation of the *ITGA2* gene, that encodes for an alpha subunit of an integrin protein involved in cell adhesion [55], the methylation levels of the cg08446038 probe significantly correlated with the survival of the patients. This probe targets a CpG site located in gene body and presented negative correlation with gene expression.

Using the cut-off value of 0.2796 the methylation of this region predicted the survival of the patients with sensitivity and specificity values of 58.58% and 87.50%, respectively (Fig.12). Patients with methylation values inferior to the cut-off values presented lower time of OS with lower levels of methylation being associated with increased gene expression. Our results are in agreement with a previous study where increased expression of *ITGA2* was correlated with gene hypomethylation and associated with worst prognosis in PCA [55].

Generally, methylation of gene body is associated with transcribed genes and can contribute to cancer causing mutations [104]. Additionally, gene body methylation can lead to the use of alternative promoters and might also influence the process of splicing leading to alternative transcripts production that can ultimately lead to cancer development [104,109]. This may lead to stimulation of this biological pathway required for tumour progression thus being associated with reduced survival of the patients.



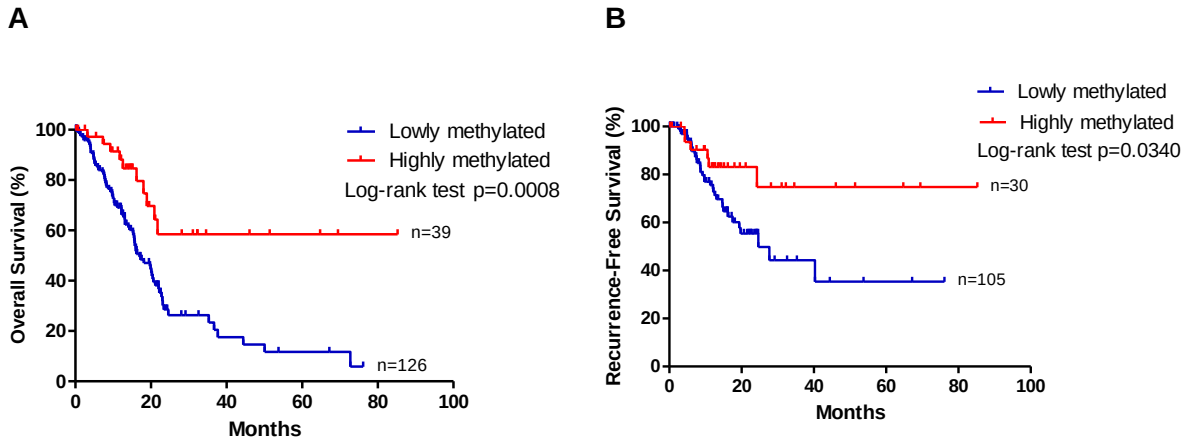
Cut-off Value	Sensitivity (%)	Specificity (%)
0.2796	58.58	87.50

Figure 12 – *ITGA2* methylation can predict survival in pancreatic cancer. A. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg08446038. For this analysis, we establish a cut-off value of 0.2796 with sensitivity and specificity values of 58.58% and 87.50%, respectively. Patients with methylation levels inferior to the cut-off value are considered as lowly methylated (represented in blue) and patients with methylation levels superior to the cut-off value are considered highly methylated (represented in red).

Considering the epigenetic regulation of the *PIK3R1* gene, that encodes for a regulatory subunit of the PI3K enzyme involved in the PI3K/Akt pathway, the methylation of the cg15021292 probe significantly correlated with the survival of the patients. This probe targets a CpG site located in the TSS1500 and presented positive correlation with gene expression.

When establishing the cut-off value of 0.6473, lower methylation levels were associated with reduced OS and RFS of patients (Fig. 13). The test revealed a sensitivity and specificity of 75.60% and 87.50%, respectively (Fig. 13).

The CpG site targeted by this methylation probe is integrated in a genomic sequence that can be recognized by proteins involved in both transcription activation and repression. Methylation of this specific region may facilitate the binding of transcriptional repressors being a reduction in DNA methylation associated with a decreased in gene expression. This gene is upregulated in pancreatic cancer so methylation of this region alone may not be representative of the effect of epigenetic regulation in gene expression, however it appears to have independent prognostic value considering the survival time of the patients.



Cut-off Value	Sensitivity (%)	Specificity (%)
0.6473	75.60	87.50

Figure 13 – *PIK3R1* methylation can predict survival and recurrence in pancreatic cancer. Kaplan-Meier curve for **(A)** overall and **(B)** recurrence-free survival of pancreatic cancer patients considering the methylation levels of the probe cg15021292. For this analysis, we establish the cut-off values of 0.6473, with sensitivity and specificity values of 75.60% and 87.50%, respectively. Patients with methylation levels inferior to the cut-off value are considered as lowly methylated (represented in blue) and patients with methylation levels superior to the cut-off value are considered highly methylated (represented in red).

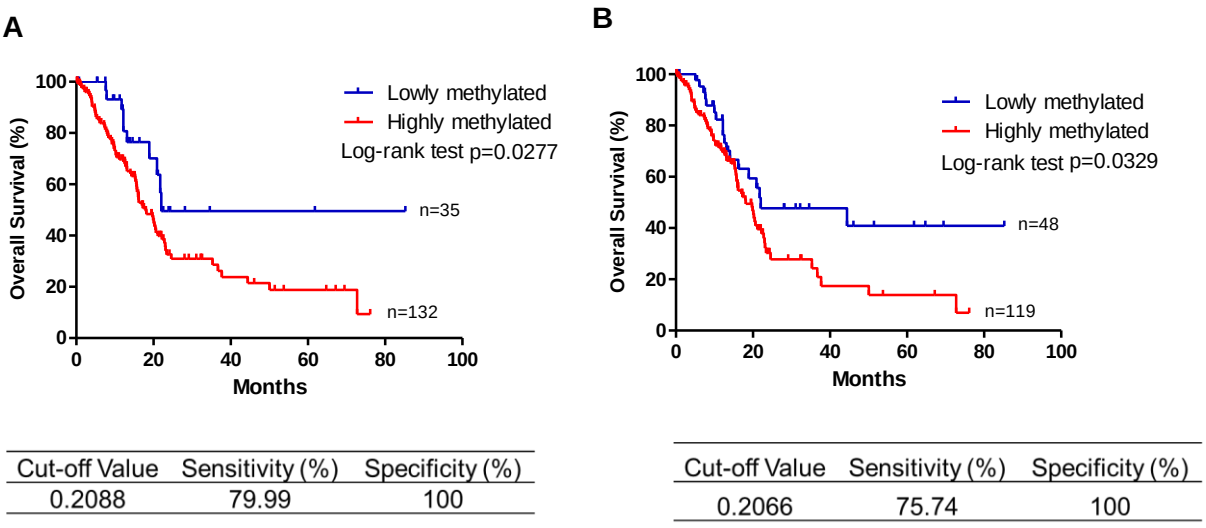
Additionally, methylation of the *SFRP2* gene, involved in the negative Wnt signalling was also associated with the outcome of the patients with CpGs hypermethylation being associated with reduced survival of the patients (Fig. 14). The methylation of two probes significantly correlated with the survival of the patients: cg20881942 and cg23207990. Both probes target CpG sites located at the TSS1500 of the gene and presented negative correlation with gene expression.

Using the cut-off value of 0.2088 for the cg20881942 probe we obtained sensitivity and specificity values of 79.99% and 100%, respectively (Fig.14A). For the cg23207990 we considered the cut-off value of 0.2066 that presented a sensitivity of 75.74% and has revealed to be 100% specific in discriminating patients with different OS (Fig. 14B).

This gene encodes for a protein involved in the negative regulation of the Wnt pathway and patients with methylation values superior to the cut-off values presented lower time of

survival. Increased methylation associated with reduced gene expression might potentiate pathway activation and facilitate tumour progression therefore being associated with worst prognosis of the patients. Methylation of the promoter region of this gene was already described as being associated with decreased gene expression in pancreatic cancer cell lines [110].

However, according to the data available at the PED database, this gene appears upregulated in tumour tissue when compared with normal pancreatic tissue. Methylation of these probes solely may do not illustrate the effect of epigenetic regulation in *SFRP2* expression and further investigation is required to assess the effect of this gene in PCA. Nevertheless, the analysis of the methylation levels of those probes present potential prognostic value considering the survival time of the patients.



Cut-off Value	Sensitivity (%)	Specificity (%)
0.2088	79.99	100

Cut-off Value	Sensitivity (%)	Specificity (%)
0.2066	75.74	100

Figure 14 – *SFRP2* methylation can predict survival in pancreatic cancer. A. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg20881942. For this analysis, we establish the cut-off value of 0.2088 with sensitivity and specificity values of 79.99% and 100%, respectively. **B.** Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the methylation levels of the probe cg23207990. For this analysis, we establish the cut-off value of 0.2066, with sensitivity and specificity values of 75.74% and 100%, respectively. Patients with methylation levels inferior to the cut-off value are considered as lowly methylated (represented in blue) and patients with methylation levels superior to the cut-off value are considered highly methylated (represented in red).

A total of 5 genes and 11 probes were associated with the time of survival of the patients revealing their potential as biomarkers to predict the outcome of the patients.

To understand the biological effect of these alterations that could explain the observed differences in patients' survival we have analysed the methylation of this set of genes and the associated probes considering several clinical and pathological parameters of the patients including history of chronic pancreatitis and diabetes, primary therapy outcome, histological classification, pathological stage and familiar history of cancer.

We did not find significant differences between DNA methylation of the selected genes regarding patients' history of diabetes, primary therapy outcome and familiar history of cancer. Methylation of specific regions of the *SFN* and *SFRP2* genes were significantly altered between patients with and without history of chronic pancreatitis (Fig. 15). Patients with

previous history of chronic pancreatitis, a risk factor for PCA development, presented lower and higher levels of methylation of the *SFN* and *SFRP2* genes, respectively (Fig. 15). These levels of methylation of the *SFN* and *SFRP2* genes were also associated with reduced survival of the patients (Fig. 10, 14).

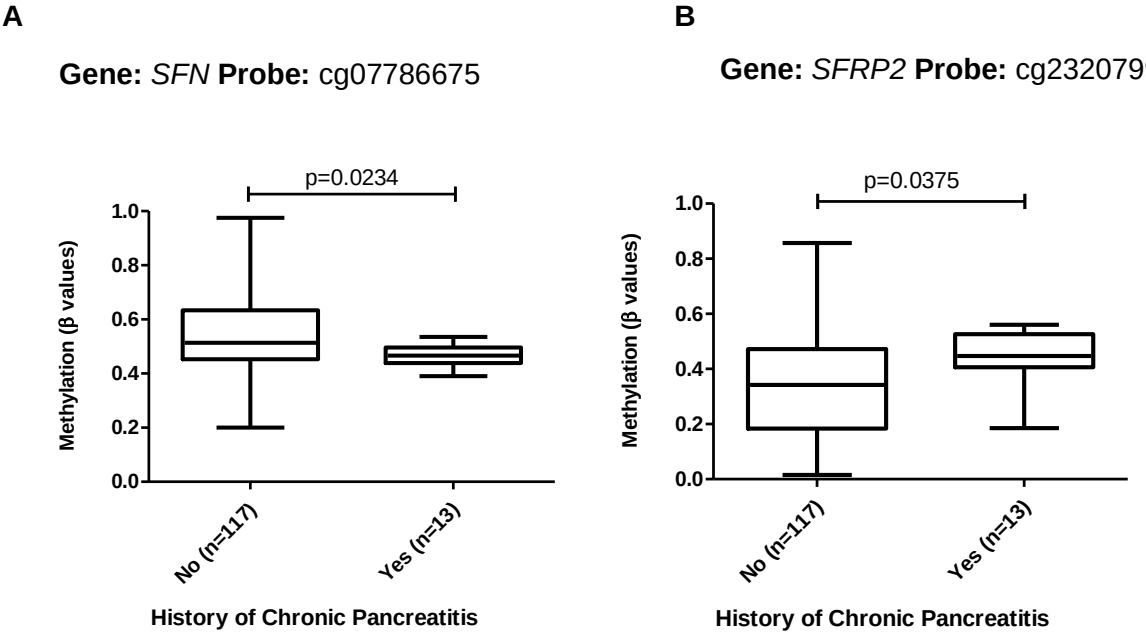
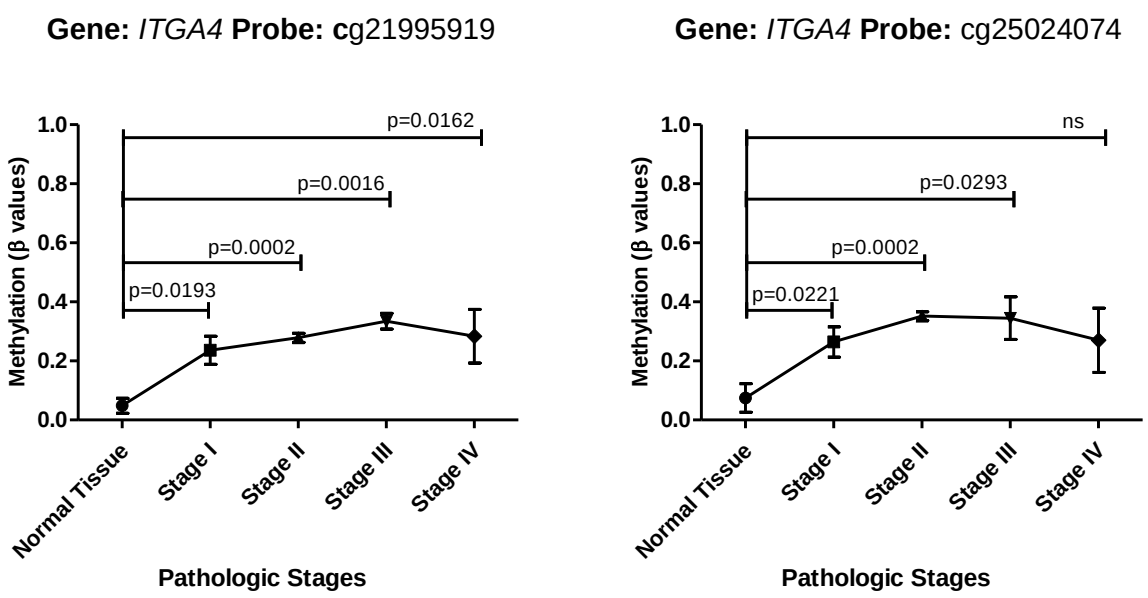


Figure 15 – Methylation of *SFN* and *SFRP2* genes is associated with patients' history of chronic pancreatitis. Methylation of specific regions of the **(A)** *SFN* and **(B)** *SFRP2* genes was assessed using the cg07786675 and cg23207990 probes, respectively. Methylation was expressed as β-value. Data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value. Statistical analysis was performed using the Mann-Whitney test.

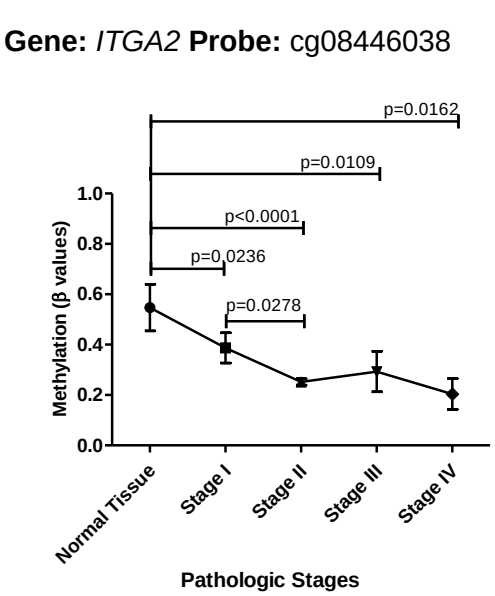
To assess if DNA methylation of the selected genes could be a useful tool for predicting disease progression, methylation levels were analysed across disease stages (I-IV). Analysis of the methylation of 7 probes could differentiate normal tissue from stage I of the disease (Fig. 16, 17, 18). Additionally, methylation of all probes considered for this analysis could distinguish normal tissue from stage II and III of the disease. Significant differences between normal samples and samples from pancreatic cancer of stage IV were observed when evaluating the methylation levels of 8 probes. However, the number of samples representative of stage III and stage IV is too small to be conclusive (Table 1).

Only 6 probes presented levels of methylation able to differentiate between stage I and II of the disease and none of the probes could distinguish between other pathological stages through disease progression, suggesting once again that alteration in the methylation pattern is an early event that is maintained throughout tumour progression. Notably, these results suggested that DNA methylation of the selected genes has potential to be used as a disease biomarker even in initial stages.

A



B



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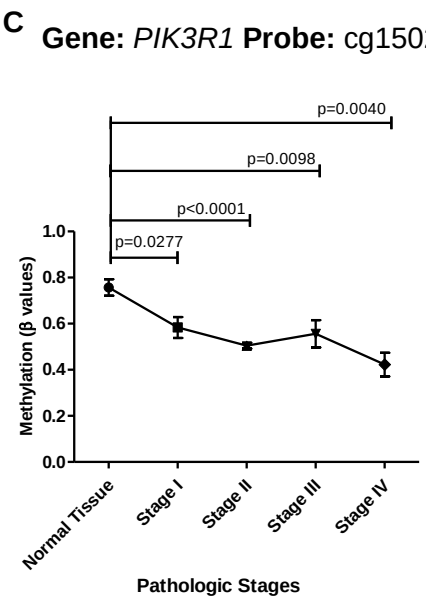
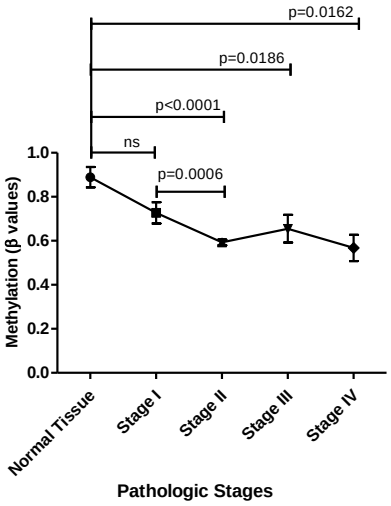
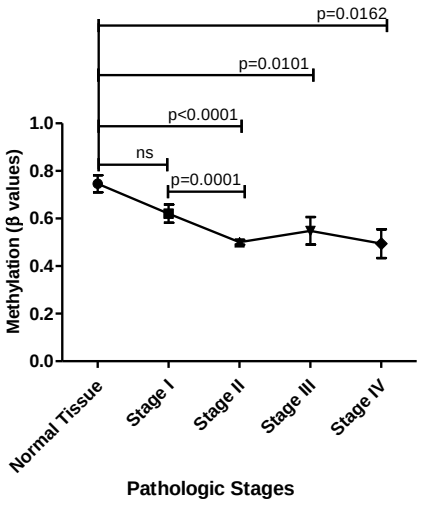


Figure 16 – Methylation of the *ITGA4*, *ITGA2* and *PIK3R1* genes discriminates between normal and malignant tissue of different pathological stages. Comparison between methylation levels of **(A)** *ITGA4,* **(B)** *ITGA2* and **(C)** *PIK3R1* genes according to the tumour pathological stage (mean ± SEM).

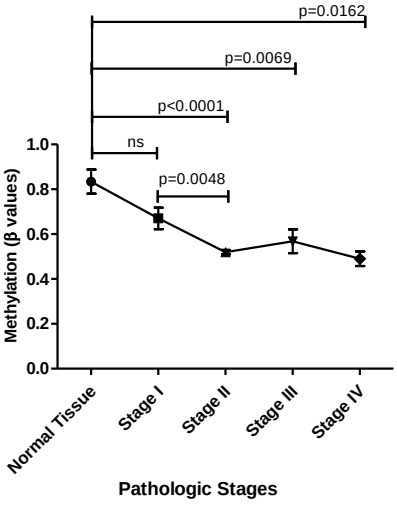
Gene: *SFN* **Probe:** cg17330303



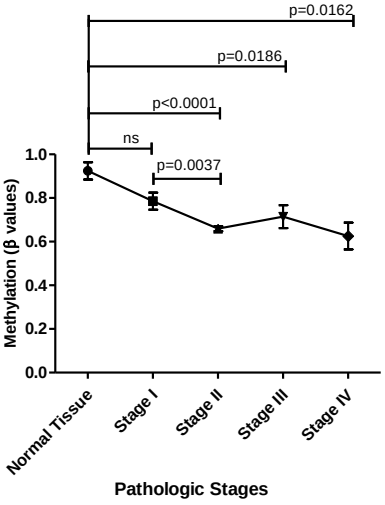
Gene: *SFN* **Probe:** cg13466284



Gene: *SFN* **Probe:** cg07786675



Gene: *SFN* **Probe:** cg13374701



Gene: *SFN* **Probe:** cg12583970

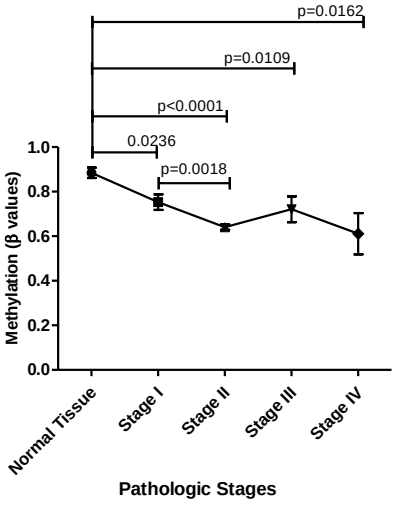


Figure 17 – Methylation of the *SFN* gene discriminates between normal and malignant tissue of different pathological stages. Comparison between methylation levels of *SFN* gene according to the tumour pathological stage (mean ± SEM).

Gene: *SFRP2* **Probe:** cg20881942 **Gene:** *SFRP2* **Probe:** cg23207990

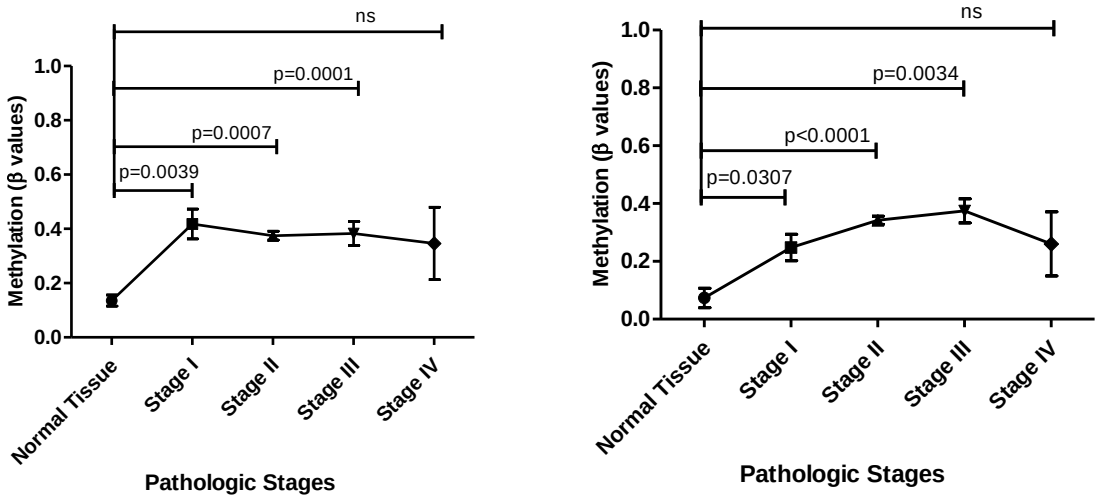


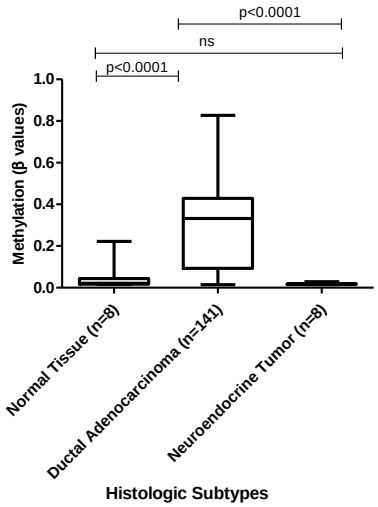
Figure 18 – Methylation of the *SFRP2* gene discriminates between normal and malignant tissue of different pathological stages. Comparison between methylation levels of *SFRP2* gene according to the tumour pathological stage (mean ± SEM).

Afterwards, to assess if methylation of this group of genes differs between histological subtypes of the disease we have compared the methylation levels between normal tissue and samples of ductal adenocarcinomas and neuroendocrine pancreatic tumours.

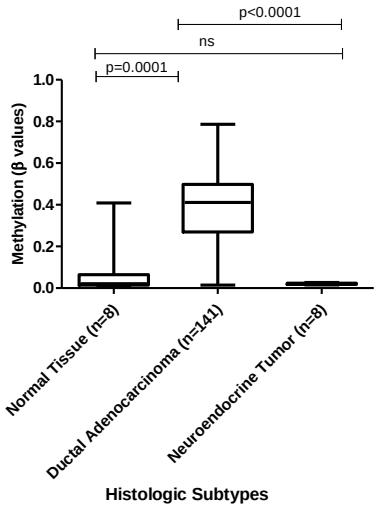
All the probes revealed differential methylation between normal tissue and ductal adenocarcinomas and between the two histological subtypes of pancreatic cancer considered for this study (Fig. 19, 20, 21). The ability to distinguish normal tissue from neuroendocrine pancreatic tumours by DNA methylation alterations was only observed in the case of 2 probes of the *SFN* gene and 1 probe targeting a CpG site of the *SFRP2* gene (Fig. 19, 20, 21). These results reflect the potential of epigenetic alterations as DNA methylation to be used as biomarkers to distinguish normal from malignant tissue and distinct subtypes of the disease.

A

Gene: *ITGA4* **Probe:** cg21995919

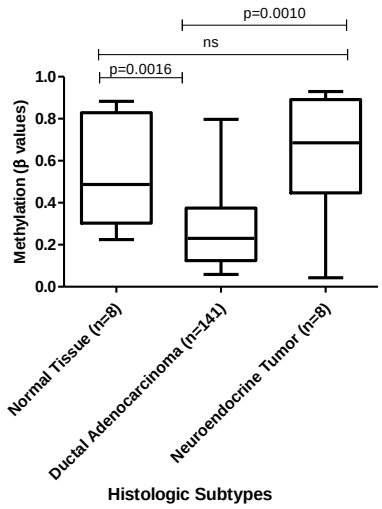


Gene: *ITGA4* **Probe:** cg25024074



B

Gene: *ITGA2* **Probe:** cg08446038



C

Gene: *PIK3R1* **Probe:** cg15021292

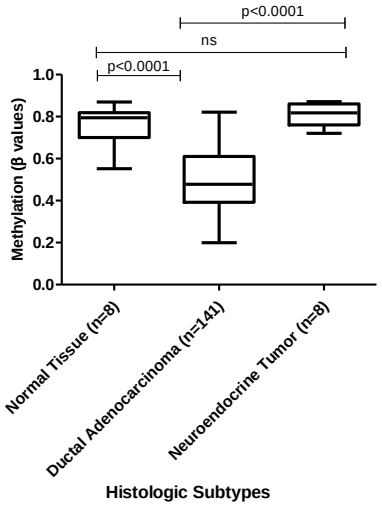
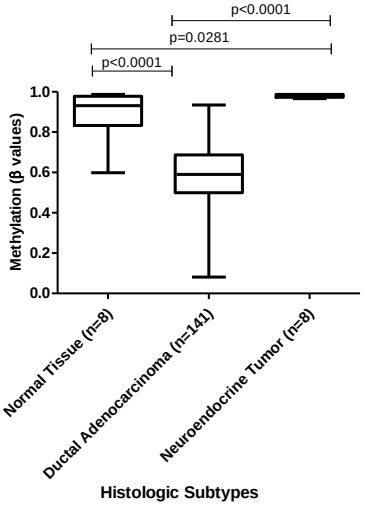
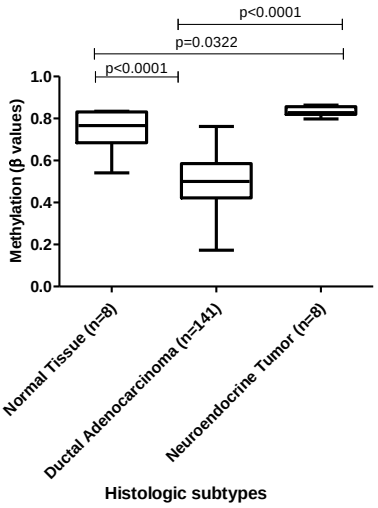


Figure 19 – Methylation of the *ITGA4, ITGA2* and *PIK3R1* genes differs between distinct histological subtypes of pancreatic cancer. Comparison between DNA methylation of **(A)** *ITGA4* **(B)** *ITGA2* and **(C)** *PIK3R1* genes considering different histological subtypes of pancreatic cancer (data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value). Statistical analysis of the data was performed using the Mann-Whitney test.

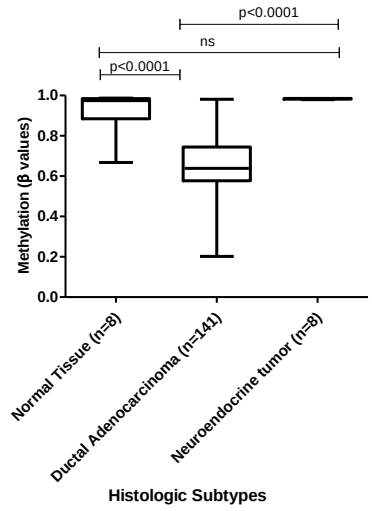
Gene: *SFN* **Probe:** cg17330303



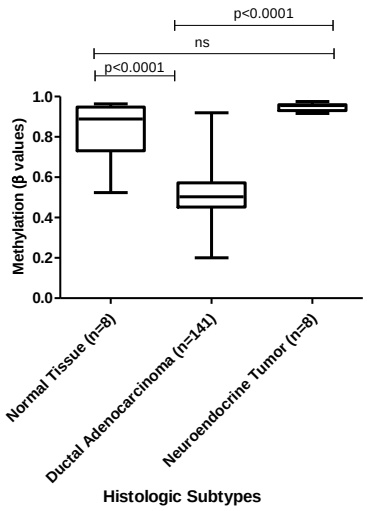
Gene: *SFN* **Probe:** cg13466284



Gene: *SFN* **Probe:** cg13374701



Gene: *SFN* **Probe:** cg07786675



Gene: *SFN* **Probe:** cg12583970

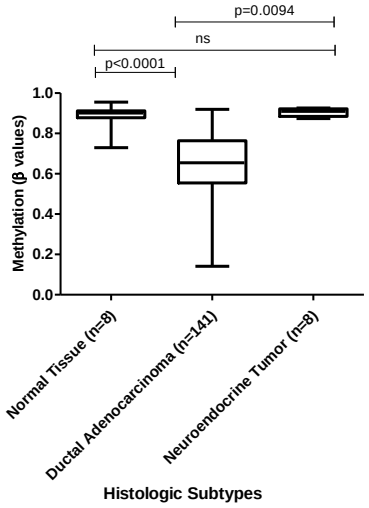


Figure 20 – Methylation of the selected genes differs between distinct histological subtypes of pancreatic cancer. Comparison between DNA methylation of *SFN* genes considering different histological subtypes of pancreatic cancer (data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value). Statistical analysis of the data was performed using the Mann-Whitney test.

Gene: *SFRP2* **Probe:** cg20881942 **Gene:** *SFRP2* **Probe:** cg23207990

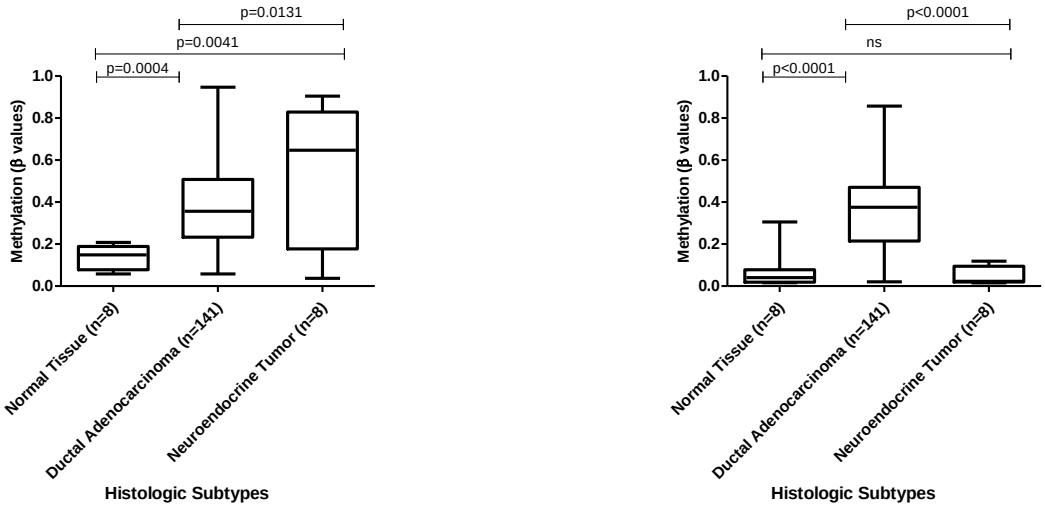


Figure 21 – Methylation of the selected genes differs between distinct histological subtypes of pancreatic cancer. Comparison between DNA methylation of *SFRP2* genes considering different histological subtypes of pancreatic cancer (data is plotted as a box graph with the median (horizontal line inside the box), the 25th to 75th percentiles (upper and lower edges) ± the largest and smallest value). Statistical analysis of the data was performed using the Mann-Whitney test.

3.2.2.4.2 miRNAs

Gene expression regulation by the action of regulatory miRNAs is another epigenetic mechanism with the potential to be used as a pancreatic cancer biomarker [15,24]. Therefore, we assessed the impact of miRNAs expression in the outcome of the patients by comparing the groups of patients with the highest and lowest levels of miRNA expression. Of the 28 miRNAs that correlated with gene expression, the expression levels of 9 could predict survival of the patients (Fig. 22, 23).

Lower expression of the hsa-miR-200a-3p and hsa-let-7g-5p miRNAs targeting the *TGFBR1* gene were associated with worse prognosis (Fig. 22A). The *TGFBR1* gene encodes for a cell membrane kinase protein that forms a complex involved in transduction signal cascades [21]. In PCA, activation of the receptor complex leads to the induction of the hedgehog pathway [97]. As expected, the expression levels of these miRNAs presented negative correlation with gene expression. Lower expression of miRNAs may lead to higher expression of gene and consequent hedgehog pathway activation contributing to cancer development and progression.

Similarly, the patients with lower expression of the miRNAs targeting the *PTEN, EIF4EBP1* and *JAG1* genes had reduced time of survival (Fig. 22B, 22C, 23A).

PTEN is a tumour suppressor gene known to negatively modulate the Akt pathway and is often referred as downregulated in PCA [111]. There is evidence of epigenetic regulation of this gene in PCA. An investigation carried out by Asano and collaborators revealed that PTEN expression was lost in most of PCA cell lines which could be due to gene promoter hypermethylation [111].

Additionally, several other miRNAs upregulated in pancreatic cancer have been described to directly influence *PTEN* expression [112,113]. An example is miR-21, whose higher expression levels are related with cell resistance to 5-fluoroucil treatment [112].

Intriguingly, this gene appears to be upregulated in PCA samples according to our analysis based on the PED data. In the cohort analysed in this study none of the probes covering CpG sites in this gene showed significant differences in methylation levels between tumour and control samples.

However, the expression of the hsa-miR-29b-3p miRNA, known to regulate the expression of the *PTEN* gene, significantly correlated with the survival of the patients (Fig. 22B). The expression level of this miRNA presented negative correlation with gene expression and lower expression of this miRNA was associated with worst survival (Table 5, Fig. 22B). Additional analysis should be done to elucidate the role of *PTEN* in PCA or in specific groups of patients to clarify the obtained results. Moreover, it would be interesting to explore other genes regulated by this miRNA, that also might be involved in pancreatic carcinogenesis, that could help to explain the effect of miRNA expression on patients' survival.

The expression level of one miRNA known to regulate the expression of the *EIF4EBP1* gene significant correlated with the survival of the patients: hsa-miR-874-3p (Fig. 22C). In our analysis, the expression level of this miRNA presented a negative correlation with gene expression and the patients with lower expression of this miRNAs presented decreased survival (Table 3, Fig. 22C).

However, it is described that *EIF4EBP1* appears downregulated in PCA contributing to cancer development and progression [114]. This gene encodes for a protein involved in transcriptional regulation with the ability to hamper or stimulate the expression of specific genes presenting both tumour suppressor and tumour promoting roles [115].

Our results reflect only the data from the patients with highest and lowest expression levels of hsa-miR-874-3p. Nevertheless, we observe a significant difference between the two groups that has encouraged us to continue this analysis in the future with a bigger cohort of patients.

The expression levels of two miRNAs known to regulate the expression of the *JAG1* gene significant correlated with the survival of the patients: hsa-miR-153-3p and hsa-miR-186-5p with lower expression of both miRNAs being associated with worst survival (Fig. 23A). The expression levels of these miRNAs presented a negative correlation with target gene expression (Table 3). The protein encoded by this gene is the ligand for the notch1 receptor and is involved in the activation of the notch pathway [21]. Lower expression of these miRNAs may lead to higher expression of gene and consequent pathway activation contributing to cancer development and progression.

Considering the levels of the hsa-miR-181a-5p, a miRNA targeting the *AKT3* gene, lower levels of expression were associated with a better prognosis and increased time of survival (Fig. 22D). These results were unexpected since *AKT3* is a cancer promoting gene upregulated in PCA and expression of this miRNA was negatively correlated with *AKT3* mRNA levels (Table 5). This gene encodes for a protein member of the AKT kinases family implicated in cell signalling pathways known to influence a variety of biological processes including cell proliferation and migration [21]**.** The obtained results might also be due to the ability of hsa-miR-181a-5p to regulate additional genes with tumour suppressor functions with higher specificity.

Finally, regarding the expression levels of two miRNAs targeting the *CSNK1A1* gene we have obtained contradictory results. Higher expression of the hsa-miR-30a-5p gene was associated with reduced survival of the patients while increased levels of the hsa-miR-30e-5p were indicative of a better prognosis (Fig. 23B). The expression levels of both miRNA presented negative correlation with gene expression (Table 5). The protein encoded by this gene is part of the complex involved in β-catenin degradation [116].

The expression of alternative target genes involved in pancreatic cancer can be influencing these results as each miRNA can regulate a multitude of genes. Therefore, the impact of these miRNA on the survival of the patients can only be speculated. Despite that, our results encourage further studies since the expression of these miRNAs have presented the potential prognostic value considering the survival time of the patients.

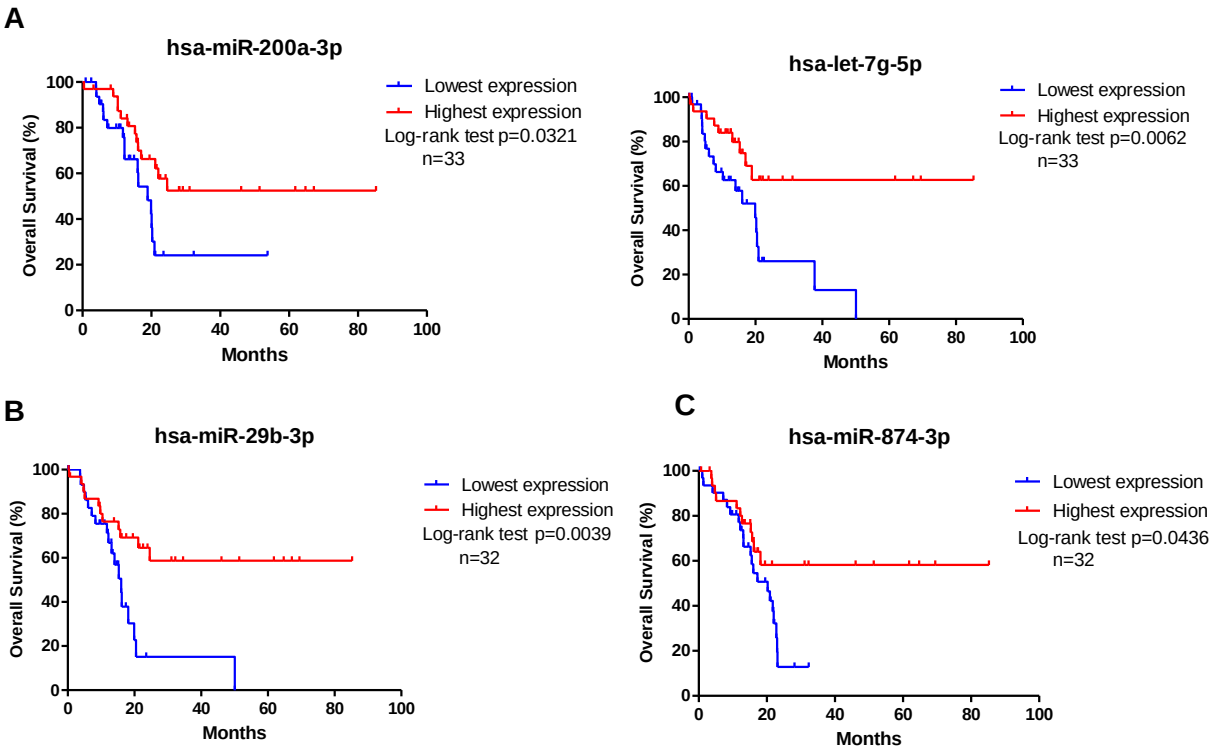


Figure 22 – The expression levels of miRNAs targeting the *TGFBR1, PTEN, EIF4EBP1* and *AKT3* genes can predict survival in pancreatic cancer patients. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the expression values of miRNA targeting the **(A)** *TGFBR1,* **(B)** *PTEN,* **(C)** *EIF4EBP1* and **(D)** *AKT3* genes. The group of the 20% patients with lower levels of expression are represented in blue and the group of the 20% patients with higher levels of expression are represented in red.

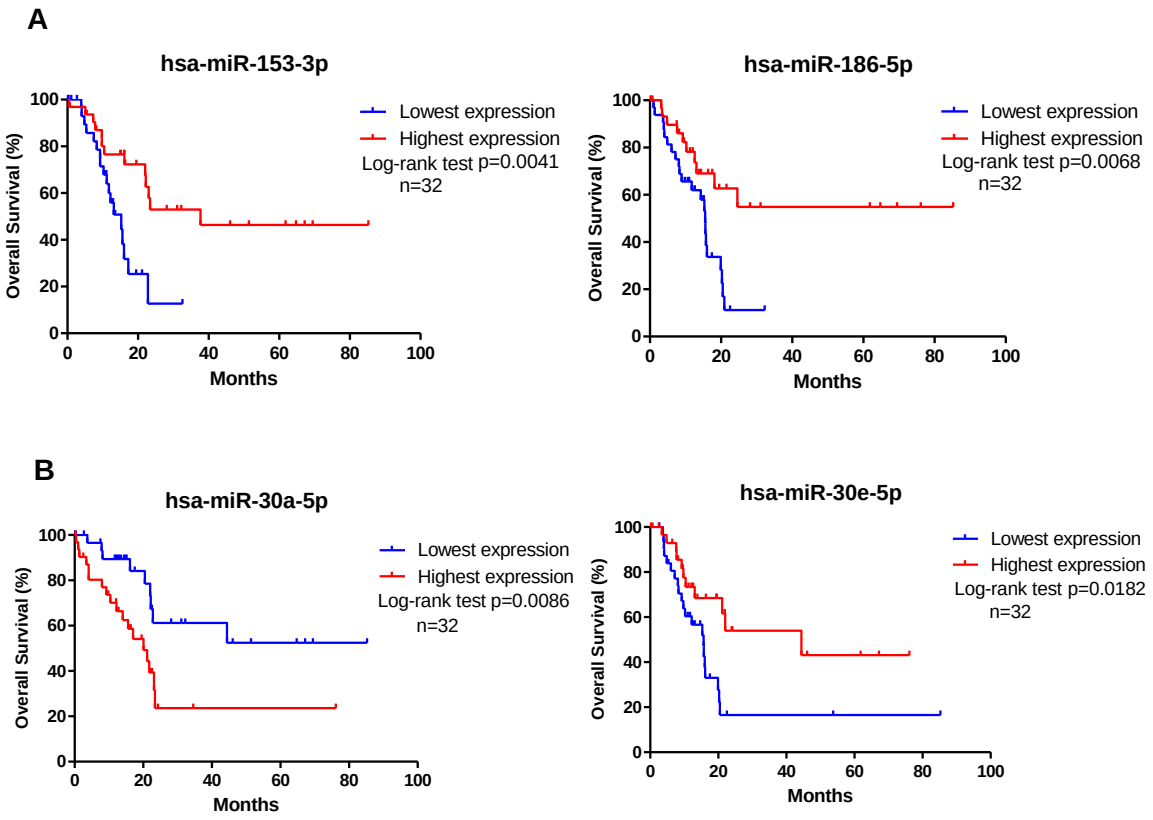


Figure 23 – The expression levels of miRNA targeting the *JAG1* and *CSNK1A1* genes can predict survival in pancreatic cancer patients. Kaplan-Meier curve for overall survival of pancreatic cancer patients considering the expression values of miRNA targeting the **(A)** *JAG1* and **(B)** *CSNK1A1* genes. The group of the 20% patients with lower levels of expression are represented in blue and the group of the 20% patients with higher levels of expression are represented in red.

3.2.3 Discussion

Our results show that epigenetic deregulation of signalling pathways involved in pancreatic carcinogenesis is associated with the survival of the patients and have the potential to be PCA biomarkers with the PI3K/Akt being the most deregulated pathway.

There are still some discrepancies in the literature regarding the expression levels of some genes analysed in our study. Accurate determination of the expression levels of those genes in different subtypes of the disease in larger cohorts may help to explain those divergences. Additionally, further research is required to explain the mechanistic effect of the methylation of specific gene regions and its correlation with gene expression.

Possibly, the analysis of the methylation pattern along the entire gene may be more representative of the effect on gene expression rather than specific CpG sites. Thus, in further studies the methylation status of adjacent CpG sites should be considered.

Eventhough the connection between epigenetic regulation of these genes and the survival of the patients is not yet scrutinized, differential methylation of the *ITGA4, SFN, ITGA2, PIK3R1 and SFRP2* revealed to be independent prognostic factors to predict survival of the patients with higher sensitivity and specificity compared with the currently established biomarker CA19-9. Moreover, alterations in specific miRNAs should also be considered for additional investigation as potential disease biomarkers.

Although our initial results are encouraging, mechanistic studies should be performed to investigate the causality between epigenetic alterations and the outcome of the patients.

CHAPTER 4 - CONCLUSIONS AND FUTURE PERSPECTIVES

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The implementation of biomarkers for PCA is of foremost importance to improve the disappointing survival rates of the patients. Currently, the molecular biomarkers established in the clinic for cancer management are mainly focused on gene and protein expression analysis with few epigenetic biomarkers available [117]. However, profiling of epigenetic alterations holds great potential to improve the molecular evaluation of tumours and help clinicians to adopt the best therapeutic approaches [117].

In this study, we explored the biomarker potential of epigenetic alterations in PCA. We started by investigating the methylation status of the THOR region in TERT promoter and its impact on patients' survival.

Our results show that THOR was significantly hypermethylated in pancreatic tumor tissue when compared to the normal tissue used as control. Also, THOR hypermethylation could distinguish early stage I disease from normal tissue and was associated with worse prognosis. Our preliminary findings support the diagnostic and prognostic values of THOR in pancreatic cancer.

Additionally, we have analysed genes that are involved in signalling pathways that when deregulated contribute to the initiation/progress of the disease including the PI3K/Akt, Wnt, Notch and Hedgehog pathways. As such, we have investigated the epigenetic regulation mediated by DNA methylation and miRNAs of genes differentially expressed in the tumour.

Our results demonstrate the ability to discriminate between groups of patients with different outcomes through the analysis of DNA methylation and miRNA levels.

Differential methylation of 6 genes allowed the discrimination between normal and tumour tissue and were correlated with patient survival. Using specific cut-offs, we could distinguish between two groups of patients regarding their outcome with higher sensitivity and specificity than the currently implemented biomarker for PCA management (CA19-9).

Moreover, analysis of the expression of 9 miRNAs when comparing the data of the patients with highest and lowest expression of each miRNAs could also differentiate between patients with different survival times.

The observed alterations can be more relevant in a subgroup of patients so we intent to further analyse the impact of these changes in the outcome of the patients considering specific clinical/pathological parameters.

Additionally, the development of a combinatory panel of different biomarkers may improve the sensitivity and specificity values compared with single markers thus we want to test the potential of those alterations as part of a panel composed by a combination of distinct genes.

Finally, we aim to evaluate these alterations using samples from PCA patients to assess the potential of the most relevant genes as disease biomarkers and possible therapeutic targets. This will enable us to design patient specific management strategies and therapeutic options for individuals with this disease.

With the increased development of new techniques such as the CRISP-Cas system we are able to modify the epigenome of the cells [118]. With this system, we can specifically alter the methylation status of target CpG sites by coupling epigenetic-modifying enzymes with guide-RNAs to directed the system to specific genomic regions.

This will enable us to compare cells with and without DNA methylation to evaluate the causality between the epigenetic alteration and the cell phenotype and the potential of its reversion as therapeutic strategies for PCA. Additionally, there are multiple strategies to

modulate the miRNA levels within cells that will allow us to test the potential of a miRNA based therapy in PCA [62].

We believe that further research to understand the contribution of these epigenetic alterations on pancreatic carcinogenesis could improve our knowledge about the biological processes inherent to the malignant transformation of pancreatic cells and reveal new therapeutic targets.

In conclusion, this study supports the diagnostic and prognostic value of epigenetic alterations in PCA and encourage further studies to complement the data available at the databases used in this study and to validate the observed alterations as PCA biomarkers.

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SUPPLEMENTARY INFORMATION

Annex 1

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Review

Epigenetic therapy in urologic cancers: an update on clinical trials

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ABSTRACT

Epigenetic dysregulation is one of many factors that contribute to cancer development and progression. Numerous epigenetic alterations have been identified in urologic cancers including histone modifications, DNA methylation changes, and microRNA expression. Since these changes are reversible, efforts are being made to develop epigenetic drugs that restore the normal epigenetic patterns of cells, and many clinical trials are already underway to test their clinical potential. In this review we analyze multiple clinical trials (*n*=51) that test the efficacy of these drugs in patients with urologic cancers. The most frequently used epigenetic drugs were histone deacetylase inhibitors followed by antisense oligonucleotides, DNA methyltransferase inhibitors and histone demethylase inhibitors, the last of which are only being tested in prostate cancer. In more than 50% of the clinical trials considered, epigenetic drugs were used as part of combination therapy, which achieved the best results. The epigenetic regulation of some cancers is still matter of research but will undoubtedly open a window to new therapeutic approaches in the era of personalized medicine. The future of therapy for urological malignancies is likely to include multidrug regimens in which epigenetic modifying drugs will play an important role.

INTRODUCTION

Urologic cancers account for approximately 10% of all cancer deaths in the USA and include bladder, kidney, prostate and testicular cancers [1].

The establishment and progression of malignancy involves broad changes in gene expression that are determined by both genetic and epigenetic events. Genetic events include chromosome rearrangements and duplications as well as translocations, deletions, and single base pair mutations. Epigenetic modifications are somatically heritable changes that modify gene expression without altering the DNA sequence. Among these are histone modifications, DNA methylation, and miRNA expression [2].

HISTONE MODIFICATIONS

Post-translational modification of the histone protein N terminal tails can alter the structure of the nucleosome and change the compaction state of chromatin. Common modifications include methylation, acetylation, phosphorylation, ubiquitylation and sumoylation [2]. Among these, histone acetylation and methylation are best described in cancer epigenetic dysregulation [2].

Histone acetylation neutralizes the positive charge of lysine residues, weakening their electrostatic interactions with DNA [3]. This leads to a more relaxed state of the chromatin and is associated with transcriptional activation. Addition of the acetyl group is carried out by histone acetyltransferases (HATs), and its removal is catalyzed by

histone deacetylases (HDACs) [4]. HDACs are classified into four distinct groups based on their homology to yeast histone deacetylases. Class I HDACs encompass HDAC1, 2, 3 and 8 and, with the exception of HDAC8 that can be located in the nucleus or cytoplasm, are exclusively located in the nucleus. Class II HDACs include HDAC4, 5, 6, 7, 9 and 10 and can be present in the nucleus or the cytoplasm [5, 6]. Class III HDACs are the sirtuins, proteins which require the cofactor NAD+ to be active. Unlike Class I and Class II HDACs, sirtuins are not inhibited by known pharmacologic HDAC inhibitors (HDACi) such as Vorinostat and Trichostatin A (TSA) [5, 6].

Histone methylation occurs at lysine and arginine residues and, depending on the target, can lead to activation or repression of gene expression [7]. Methylation is catalyzed by histone methyltransferases (HTMs) while demethylation is performed by histone demethylases (HDMs). There are currently two histone demethylase families, the lysine specific demethylases (LSD) and the JmjC-domain-containing histone demethylases (JHDMs) [7]. The LSDs comprise LSD1 and LSD2, which are dependent on FAD to be catalytically active [8]. The JHDMs in turn, catalyze the hydroxylation of the lysine methylgroup and require two factors to be catalytically active: Fe(II) and 2-oxoglutarate [7].

In cancer, histone modifications have been associated with both activation and repression of gene expression. Modifications such as histone 3 methylation at lysine 4 (H3K4me), histone 3 di-methylation at lysine 4 (H3K4me2), histone 3 tri-methylation at lysine 4 (H3K4me3), histone 3 acetylation at lysine 9 (H3K9ac), histone 3 methylation at lysine 9 (H3K9me) and histone 3 acetylation at lysine 27 (H3K27ac) are associated with active chromatin whereas histone 3 tri-methylation at lysine 36 (H3K36me3), histone 3 tri-methylation at lysine 9 (H3K9me3) and histone 3 methylation at lysine 27 (H3K27me) are associated with repressive chromatin [9].

DNA METHYLATION

DNA methylation results from addition of a methyl group to the 5-carbon of a cytosine residue by the enzyme DNA methyltransferase (DNMT) [10]. DNMT forms a complex with CpG dinucleotides that allows the transfer of a methyl group to the cytosine residue [10]. Many CpG sites are located in the promoter regions of genes. Collectively they are known as CpG islands. In general, DNA methylation of CpG islands located in gene promoters leads to transcriptional repression. However, there are exceptions to this classical view, in which promoter hypermethylation is associated with increased gene expression [11-14]. This occurs in instances where DNA methylation drives the use of an alternative transcription start site or inhibits the binding of a repressive protein [15]. Increased gene expression in the context of promoter hypermethylation is associated with an increase in H3K4me3, a histone mark characteristic of gene activation [15].

In eukaryotes, DNA methylation is mediated by three DNMTs: DNMT1 is responsible for the maintenance of methylation patterns after DNA replication whereas DNMT3A and DNMT3B carry out *de novo* methylation [4]. Any alteration that affects the activity of these enzymes can lead to an imbalance in methylation that provides the basis, or contributes, to the initiation of carcinogenesis.

MIRNAS

miRNAs are small endogenous non-coding RNAs (ncRNAs), 21-25 nucleotides in length, that regulate gene expression by targeting specific messenger RNAs (mRNAs) for translational repression or degradation. Expression patterns of miRNAs differ between normal and tumor tissues [16, 17]. Depending on their target, miRNAs can act either as tumor suppressors or oncogenes; downregulation of an miRNA that targets an oncogene, or an overexpression of an miRNA that targets a tumor suppressor gene, can promote carcinogenesis [16, 17].

EPIGENETIC DRUGS

Two strategies for epigenetic therapy are currently in use: small molecules that inhibit epigenetic-modifying enzymes and manipulation of miRNA expression.

Amongst the small molecule inhibitors are HDAC inhibitors and DNMT inhibitors. HDAC inhibitors (HDACi) are classified into 4 groups according to their chemical structures: hydroxamates (SB393, Vorinostat, Panobinostat), cyclic peptides (Romidepsin), benzamides (Entinostat and Mocetinostat) and aliphatic fatty acids (Valproic Acid) [18].

The majority of HDACi inhibit zinc-dependent HDACs by interacting with the zinc ion. In cancer cells, the inhibition of histone deacetylation restores expression of tumor suppressor genes that were previously silenced by epigenetic mechanisms [18, 19].

DNMT inhibitors are divided into nucleoside analogues and non-nucleoside analogs [4]. Nucleoside analogues, such as Azacitidine, Decitabine and FdCyd, are cytosine analogs modified at the C5 position. Inside the cell they are metabolized and incorporated into DNA molecules [4]. DNA methyltransferases can bind to these modified nucleotides but their modification at C5 prevents their methylation. It also prevents the dissociation of the enzyme thereby reducing DNMT activity at other sites [4]. Non-nucleoside analogues, such as Hydralazine, Procainamide and MG98, inhibit methylation by binding to the catalytic region of the enzyme [4].

Another focus of epigenetic therapy is the manipulation of miRNA expression and activity. Several

strategies have been employed to silence miRNAs that are overexpressed in cancer. These include anti-miRNA oligonucleotides (AMOs), peptide nucleic acids (PNAS), miRNA-masking antisense oligonucleotides (miR-mask) and miRNA sponges [16]. Restoration of miRNA expression that has been downregulated in cancer is

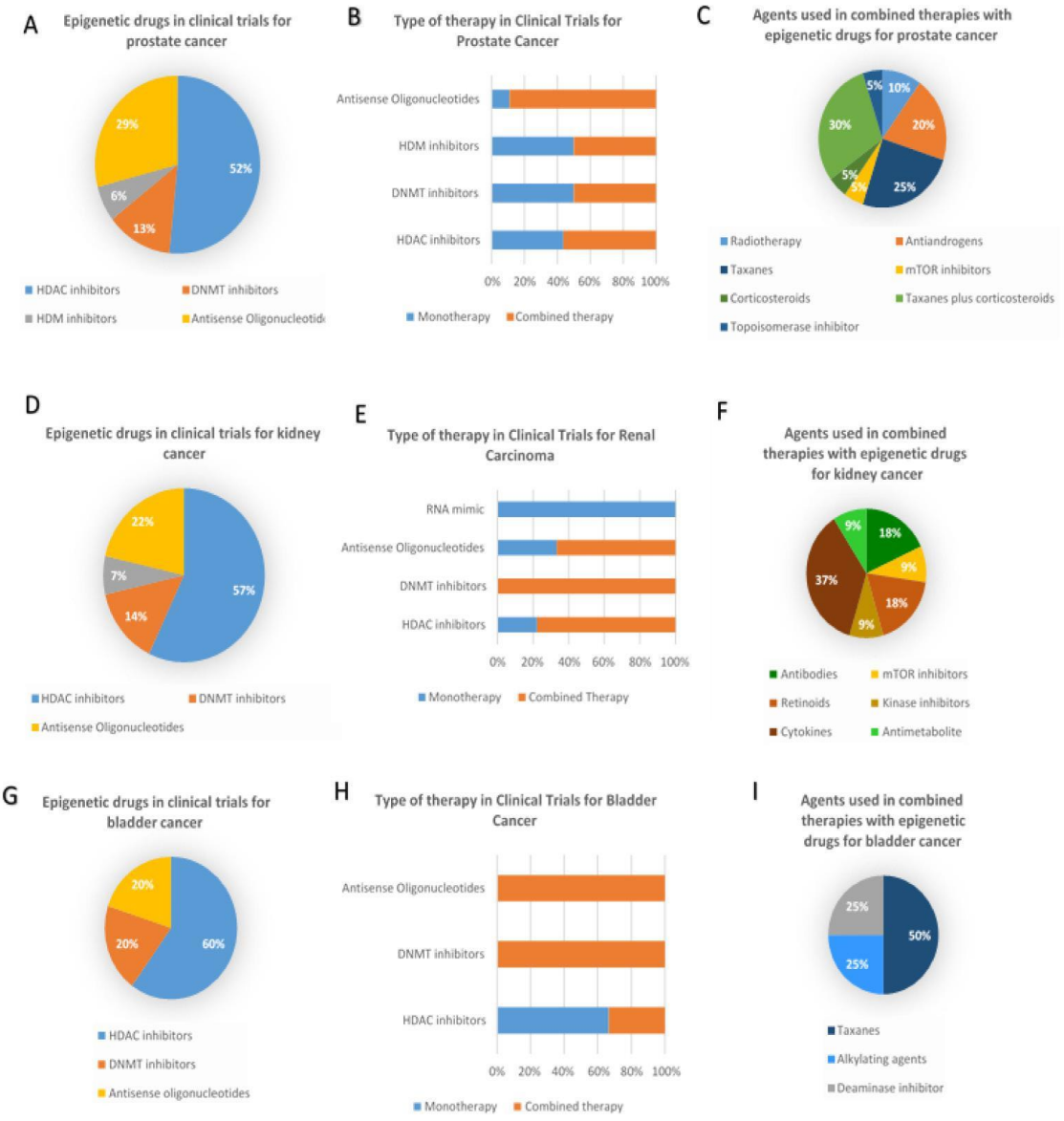

Figure 1: Epigenetic therapies in clinical trials for prostate, bladder and kidney cancers. A. Percentage of clinical trials employing each types of epigenetic therapeutic agents in prostate cancer; **B.** Percentage of clinical trials using mono or combined therapy as therapeutic strategy with the different classes of epigenetic drugs in prostate cancer; **C.** Percentage of clinical trials where different agents are used in combined therapies for prostate cancer; **D.** Percentage of clinical trials employing each types of epigenetic therapeutic agents in kidney cancer; **E.** Percentage of clinical trials using mono or combined therapy as therapeutic strategy with the different classes of epigenetic drugs in kidney cancer; **F.** Percentage of clinical trials where different agents are used in combined therapies for kidney cancer **G.** Percentage of clinical trials employing each types of epigenetic therapeutic agents in bladder cancer; **H.** Percentage of clinical trials using mono or combined therapy as therapeutic strategy with the different classes of epigenetic drugs in bladder cancer; **I.** Percentage of clinical trials where different agents are used in combined therapies for bladder cancer

achieved by administration of synthetic miRNAs or by induced expression of miRNA coding genes using viral constructs, such as adenovirus-associated vectors [16].

Dysregulation of epigenetic marks leads to changes in gene expression that, in cancer cells, can result in activation of oncogenes or inactivation of tumor suppressor genes, both of which can contribute to cancer. Unlike genetic mutations, however, epigenetic changes are reversible. Therefore, the development of drugs capable of restoring the normal epigenetic patterns of cells has great therapeutic potential. In this review we discuss the efficacy of this novel therapeutic approach through the analysis of clinical trials of epigenetic therapies conducted in prostate, kidney and bladder cancers.

METHODS

We performed a comprehensive literature review and searched for clinical trials from the United States (https://clinicaltrials.gov/) and European (https://www.clinicaltrialsregister.eu/) databases. Relevant articles on the subject were also retrieved from PubMed database using keywords encapsulating all types of epigenetic therapies and urologic cancers (examples: "epigenetic therapy" AND "urologic cancer", "prostate cancer" AND "HDACi", "kidney cancer" AND "DNMTi"). To guarantee that most of the data on the subject was included, the reference sections of the captured articles were also filtered for relevant articles.

Prostate cancer - epigenetics

Dysregulation of epigenetic-modifying enzymes disturbs normal epigenetic patterns and is associated with cancer development and progression. In prostate cancer, DNA methyltransferases are upregulated [20, 21]. Histone-modifying enzymes, such as HDACs are upregulated in prostate cancer [22]. HMTs and HDMs show variable changes in expression with a tendency for upregulation of HMTs and lower expression of HDMs [23, 24]. Prognostically, overexpression of HDAC2 is associated with a shortened time before prostate cancer recurrence as shown in a subgroup of patients with Gleason Score 7 carcinomas, [6].

Specific histone modifications have also been associated with prostate cancer [25, 26]. The levels of histone marks H3Ac and H3K9me2 are significantly lower in tumor tissue when compared to normal tissue [26]. Conversely, an increase in H3K27me3 is found in metastatic tissue relative to localized tumors and normal prostatic tissue [25]. Finally, higher levels of H3K4me1 are associated with a higher probability of recurrence [26].

Changes in DNA methylation are also evident in prostate cancer and are targets for epigenetic therapy. The *CCDN2*, *GSTP1* and *RARβ2* genes, involved in cell cycle control, DNA repair mechanisms and hormonal responses respectively, are hypermethylated in prostate cancer. Alteration of their normal methylation status is correlated with poor clinical prognosis [26]. As with many epigenetic alterations, these biomarkers are useful in diagnosis and prognosis of disease [25, 26].

Finally, miRNA levels are also altered in prostate cancer, affecting the expression of genes involved in cell cycle control, apoptosis, migration, and invasion [27]. Levels of miRNAs also have the potential to be used as biomarkers for diagnosis and prognosis [25]. As an example, miR-141 is upregulated in prostate cancer [25, 28]. Serum levels of this miRNA can distinguish between tumor and healthy tissue and higher levels of miR-141 are associated with worse prognosis [28]. miR-449a is another miRNA that is downregulated in prostate cancer. It targets HDAC1, so its downregulation contributes to overexpression of this enzyme, showing that epigenetic-modifying enzymes are often regulated epigenetically [27, 29].

Prostate cancer - current treatment

Prostate cancer treatment is disease stage-specific. Epigenetic therapies have thus far been limited to the advanced form of castrate resistant prostate cancer (CRPC). Currently there are several chemotherapeutic agents approved for the treatment of advanced CRPC: Sipuleucel T, Docetaxel, Cabazitaxel, Abiraterone, Alpharadin and Enzalutamide [30-35]. Although all of these agents have shown efficacy, strategies for the sequence of administration and their combination are still being optimized [36]. Treatment resistance is a major concern with some of these agents, including Abiraterone and Enzalutamide, reflecting the need for ongoing development of novel therapeutic strategies [36]. Since epigenetic dysregulation contributes to the development of treatment resistance, epigenetic therapy is an intriguing addition to the CRPC therapy arsenal [37].

Prostate cancer - pre-clinical data

Pre-clinical studies in prostate cancer cell lines demonstrate that treatment with HDACi can restore susceptibility to chemotherapeutic agents such as taxanes, antiandrogens, and mTOR inhibitors [38-40]. Combined therapy using HDACi and taxanes prevents tumor growth and increases cell death rate when compared to a monotherapeutic approach [38]. Liu *et al* showed that low doses of the HDACi Panobinostat can restore the susceptibility of prostate cancer cells to hormonal therapy with the nonsteroidal antiandrogen Bicalutamide [39]. The HDACi Belinostat (PXD101) can also downregulate

Table 1: Clinical trials of epigenetic drugs in prostate cancer

Drug	Combined Therapy	Enzimatic Class	Approval Stage	Status	Indication	Results	Reference/Clinical trial identification
SB939	-	HDAC inhibitor	Phase 2	Completed	Castration Resistance Prostate Cancer (CRPC)	6% of the patients had a PSA response 64% of the patients had a conversion from an unfavorable CTC profile to a favorable one	Eigl *et al.* 2015 (NCT01075308)
Panobinostat	-	HDAC inhibitor	Phase 2	Completed	CRPC	14,3% of the patients had a PSA decrease <50% but no objective responses were seen 11,4% of the patients had stable disease for at least 24 weeks	Rathkopf *et al.* 2013 (NCT00667862)
Panobinostat	Docetaxel	HDAC inhibitor	Phase 1	Completed	CRPC	63% had a PSA decrease >= 50%	Rathkopf *et al.* 2010
Panobinostat	Radiotherapy	HDAC inhibitor	Phase 1	Completed	Prostate Cancer, esophageal cancer and neck cancer	No study results or publications provided	NCT00670553
Panobinostat	Docetaxel/prednisone	HDAC inhibitor	Phase 2	Completed	CRPC	No study results or publications provided	NCT00878436
Panobinostat	Bicalutamide	HDAC inhibitor	Phase 1	Completed	CRPC	No study results or publications provided	NCT00663832
Vorinostat	-	HDAC inhibitor	Phase 2	Completed	Progressive metastatic prostate cancer	No PSA declines >=50% were observed Median of progression free survival=2,8 months with a median overall survival of 11,7 months	Bradley *et al.* 2010 (NCT00330161)
Vorinostat	Docetaxel	HDAC inhibitor	Phase 1	Terminated	Advanced solid tumor including prostate cancer, urothelial carcinoma and kidney cancer	This study was terminated due to excessive toxicity as five patients experienced dose-limiting toxicities (DLT) No responses were observed	Schneider *et al.* 2012 (NCT00565227)
Vorinostat	Temsirolimus	HDAC inhibitor	Phase 1	Terminated	Metastatic prostate cancer	This study was terminated due to lack of efficacy	NCT01174199
Vorinostat	Androgen deprivation therapy	HDAC inhibitor	Phase 2	Completed	Localized prostate cancer	No study results or publications provided	NCT00589472
Vorinostat	-	HDAC inhibitor	Phase 1	Completed	Advanced solid tumors including prostate cancer	No study results or publications provided	NCT00005634
Romidepsin	-	HDAC inhibitor	Phase 2	Completed	Metastatic prostate cancer	No study results or publications provided	NCT00106418
Romidepsin	-	HDAC inhibitor	Phase 2	Completed	metastatic castration-resistant prostate cancer (MCRPC)	63% of the patients had progressive disease with a median time to progression of 49,5 days PSA decline >=50% was observed in 5,7% of the patients	Molife *et al.* 2009
Curcumin	Docetaxel	HDAC inhibitor	Phase 2	Ongoing	MCRPC	Final data collection date for primary outcome measure: January 2017	NCT02095717
Curcumin	-	HDAC inhibitor	Phase 2	Ongoing	Prostate cancer	Estimated primary completion date: June 2020	NCT02064673
Curcumin	Radiotherapy	HDAC inhibitor	-	Completed	Prostate cancer	No PSA response was observed but the severity of radiotherapy related urinary symptoms was reduced,	Hejazi J. *et al.* 2013
Dissulfiram	-	DNMT inhibitor	Phase 1	Completed	Non-metastatic recurrent prostate cancer	Five patients achieve a transient demethylation response Six patients discontinue therapy due to adverse effects	Schweizer *et al.* 2013
Azacitidine	Combined Androgen Blockade (CAB)	DNMT inhibitor	Phase 2	Completed	CRPC	Overall median PSA doubling time increased significantly (2.8 vs 1.5 months of the baseline). Median of progression free survival=12,4 weeks Fourteen patients had some PSA decline and 1 patient had a PSA decline >=30%	Sonpavde *et al.* 2011
Azacitidine	-	DNMT inhibitor	Phase 2	Completed	Prostate cancer	No study results or publications provided	NCT00384839
Azacitidine	Docetaxel/prednisone	DNMT inhibitor	Phase1/2	Terminated	CRPC	This study was terminated due to withdrawal of funding Complete and partial response were achieved by one and two patients, respectively	NCT00503984
Phenelzine sulfate	-	HDM inhibitor/monoamine oxidase A inhibitor	Phase 2	Ongoing	Non-metastatic recurrent prostate cancer	Study completion date: August 2018	NCT02217709

Phenelzine sulfate	Docetaxel	HDM inhibitor/monoamine oxidase A inhibitor	Phase 2	Ongoing	Progressive prostate cancer	Final data collection date for primary outcome measure: January 2016	NCT01253642
OGX-011	Docetaxel/ prednisone	Antisense oligonucleotide that targets clusterin	Phase 1	Completed	advanced cancer including prostate, bladder and kidney cancer	Six patients with hormone-refractory prostate cancer had a PSA decline >=50%	Saad et al. 2011 (NCT00471432)
OGX-011	Docetaxel/ prednisone and doxorubicin/ mitoxantrone	Antisense oligonucleotide that targets clusterin	Phase 3	Completed	MCRPC	No objective responses were seen 41% of the patients discontinued treatment due to serious adverse events	Chi et al. 2008 (NCT01188187)
OGX-011	Cabazitaxel/ prednisone	Antisense oligonucleotide that targets clusterin	Phase 3	Ongoing	CRPC	Study completion date: December 2016	NCT01578655
Oblimersen	Docetaxel	Antisense oligonucleotide that targets Bcl-2	Phase 2	Completed	CRPC	PSA response was observed in 46% and 37% of the patients treated with docetaxel alone and docetaxel+oblimersen, respectively Partial response was observed in 18% and 24% of the patients in the referred groups and major toxic events were reported in 22,8% and 40,7% respectively	Sternberq *et al*. 2009 (NCT00085228)
Oblimersen sodium (Genasense)	Mitoxantrone	Antisense oligonucleotide that targets Bcl-2	Phase 1	Completed	CRPC	Two patients had a PSA reduction >=50%, 1 patient had a PSA resuction <50%, and 5 patients had stable disease	Chi *et al*. 2001
OGX-427	Prednisone	Antisense oligonucleotide that targets heat shock protein27	Phase 2	Completed	CRPC	No study results or publications provided	NCT01120470
OGX-427	Abiraterone	Antisense oligonucleotide that targets heat shock protein27	Phase 2	Ongoing	MCRPC	Study completion date: December 2017	NCT01681433
ISIS 1837	Docetaxel/ prednisone	Antisense oligonucleotide that targets eIF4E	Phase 2	Completed	Metastatic resistant castrate prostate cancer	No study results or publications provided	EudraCT Number: 2010-022239-12
ISIS 3521/ISIS 5132	-	Antisense oligonucleotides that targets PKC-α and Raf-1, respectively	Phase 2	Completed	CRPC	No objective responses were observed but three patients had stable disease for 5 or more months PSA values of five patients did not rise more than 25% for >=120 days	Tolcher *et al*. 2002
LY2181308	Docetaxel/ prednisone	Antisense oligonucleotide that targets survivin	Phase 2	Completed	CRPC	No differences in efficacy were observed between the control and the experimental group. Higher incidence of adverse effects in the LY2181308 treated group.	Wiechno *et al*. 2014

the androgen receptor, preventing the onset of castration resistant prostate cancer *in vivo* in the context of hormonal therapy [41].

An *in vitro* study of the HDACi Panobinostat in combination with the mTOR inhibitor Rapamycin in prostate cancer cell lines resulted in a decrease in *HIF1-α* expression leading to inhibition of angiogenesis [40]. Combined therapy with these agents was more efficient than either one administrated alone [40].

Cancer stem cells are thought to be responsible for treatment resistance and tumor recurrence [42] and present epigenetic alterations that contribute to their ability to resist therapy [42]. Epigenetic therapeutics may therefore have the potential to target not only the bulk tumor but also this key subset of cells [42]. A study carried out by Frame *et al* revealed that prostate stem-like cells are more resistant to radiotherapy [43]. However, combined therapy with HDACi restored sensibility to radiotherapy [43]. Additionally, prostate stem-like cells treated jointly with the HDACi Trichostatin A and radiotherapy showed a significant reduction in the number of cell colonies formed when compared to treatment with radiation alone [43].

Cancer is a heterogeneous disease and the identification of biomarkers that predict whether a specific therapy (including epigenetic therapies) will be beneficial, is essential to improving cancer treatment. Recently, it has been reported that prostate cells positive for the presence of androgen receptor and cellular prostatic acid phosphatase show greater response to treatment with HDACi than cells without this pattern of expression [44].

At the level of DNA methylation, reversion of methylation can restore expression of genes silenced by this epigenetic mechanism. Treatment of human prostate cancer cells with Procainamide, a non-nucleoside DNMT inhibitor, results in a decrease in *GSTP1* methylation levels and a consequent increase in gene expression [45]. *In vivo*, treatment of immunodeficient mice carrying xenograft tumors with Procainamide resulted in a significant reduction in tumor size, suggesting clinical efficacy [45].

Resistance to hormonal therapy in prostate cancer is mediated by several mechanisms. Alterations at the DNA level include androgen receptor gene amplifications and point mutations [46]. However, these modifications account for only a minority of cases.

Downstream activation of the androgen receptor pathway and activation of an alternative signaling pathway can also contribute to hormonal therapy resistance [46]. Hypermethylation of the androgen receptor promoter region correlates with decreased androgen receptor expression and is also associated with the development of hormonal therapy resistance [47]. *In vivo* studies reveal that long-term treatment of prostate cancer cells with the DNMTi Azacitidine led to a significant reduction in cell proliferation due to increased androgen receptor expression. Moreover, androgen receptor induction restored sensitivity to the antiandrogen agent Bicalutamide [47].

The DNA demethylating agent, Disulfiram, has also been tested in prostate cancer cells. Treatment with Disulfiram resulted in the reestablishment of *APC* and *RARβ* gene expression, both of which are known to be hypermethylated and inactive in prostate cancer [48]. Cell growth inhibition was observed *in vitro*, and *in vivo* using prostate cancer xenograft models [48].

Finally, microRNAs modulators have been tested in preclinical studies as potential therapeutic options for prostate cancer. miR-16 regulates the expression of genes involved in cell-cycle control and apoptosis such as *CDK1*, *CDK2* and *BCL2* [49, 50]. Transfection of a synthetic miR-16 reduced the proliferative capacity of several prostate cancer cell lines [49]. *In vivo*, Takeshita *et al* used the atelocollagen method to deliver miR-16 to bone metastases via the mouse tail vein. They subsequently observed a suppression in metastasis growth, indicating not only efficacy of the treatment but also of the delivery method [49].

Like HDACi and DNMTi, antisense oligonucleotides can restore the sensitivity of cancer cells to chemotherapeutic agents. Upregulation of the *Bcl2* and *CLU* genes in prostate cancer is linked to chemoresistance and cancer progression [51, 52]. Knockdown of these genes by antisense oligonucleotides decreases gene expression and reestablishes tumor sensitivity to taxane-based chemotherapy [51, 52]. Furthermore, transfection of miR-449a into prostate cancer cells lines caused cell cycle arrest and a decrease in HDAC1 levels, an effect also observed after knockdown of *HDAC1* using an siRNA
[29]. The inhibitory effect of miR-449a on cell cycle progression was associated with increased expression of the protein p27 [29].

These results demonstrate the potential for epigenetic therapies to advance prostate cancer treatment.

Prostate cancer - clinical data

Clinical trials using HDAC inhibitors for the treatment of prostate cancer showed a PSA response in five studies, three of which resulted in a decrease in PSA levels of ≥50% (Table 1: NCT01075308, NCT00667862, NCT00330161, NCT00106418, [53-57]). The best results were obtain with the administration of the HDACi Panobinostat (Table1: NCT00667862, [54, 55]. Stable disease was reported in two clinical trials, but in one of them conversion from an unfavorable circulating tumor cell profile to a favorable one was observed in 64% of the patients (Table 1: NCT01075308, NCT00667862, NCT00330161, [53, 54, 56]).

Most common side effects were grade 2 fatigue and nausea. In addition, HDACi SB939 caused five patients to experience one or more grade 3 complications (Table 1: NCT01075308, [53]). More severe side effects were noted with HDACi Panobinostat, resulting in 71, 4% of the patients experiencing one or more grade 3 adverse effects and four subjects reporting grade 4 adverse effects (Table1: NCT00667862, [54]). Another trial using Panobinostat reported no grade 4 toxicities when administered as monotherapy [55], however, when administered in combination with Docetaxel, seven patients experienced grade 4 toxicities [55] (Table 1). HDACi Vorinostat also showed a complex side effect profile. When administered alone in patients pre-treated with chemotherapeutic agents it led to the development of grade 3/4 toxicities in 48% of the patients, with 41% of the patients forced to discontinue therapy due to their severity (Table 1: NCT00330161, [56]). A second trial of Vorinostat in combination with docetaxel was terminated early due to excessive toxicity as five patients experienced dose-limiting toxicities, including two patients experiencing neutropenic fever and sepsis. The other three patients reported an anaphylactic reaction, a myocardial infarction and a gastrointestinal bleed, respectively (Table 1: NCT00565227, [58]). Finally, a trial of the HDACi Romidepsin in metastatic prostate cancer resulted in no grade 4 toxicities, and grade 3 events represented only 4.7% of all reported adverse effects (Table 1: NCT00106418, [57]).

Curcumin, a compound found in the spice turmeric, is another HDACi [59]. A trial of Curcumin in prostate cancer showed no PSA response when used in combination with radiotherapy (Table 1: NCT01917890, [60]). However, there was a significant reduction in urinary symptoms, one of the most common side effects of radiotherapy (Table 1: NCT01917890, [60]). Two additional trials testing Curcumin in the treatment of prostate cancer are ongoing (Table 1: NCT02095717, NCT02064673).

DNMT inhibitors have also showed promising results in clinical trials of prostate cancer. When treated with the DNMTi disulfiram, five patients achieved a transient demethylation response. No grade 4 adverse effects were observed in this trial but 6 patients were forced to quit due to treatment toxicity (Table 1: NCT01118741, [61]). The DNMTi Azacitidine was trialed in chemonaive patients with CRPC in combination with combined androgen blockade (CAB). PSA doubling time increased relative to patients receiving only CAB and no grade 4 toxicities were reported, although 4 patients had to

stop treatment due to grade 3 toxicities [62].

Azacitidine has also been tested in combination with Docetaxel and Prednisone in CRPC (Table 1: NCT00503984). Therapeutic response was assessed by magnetic resonance imaging with a complete response considered the disappearance of target lesions, and a partial response considered a ≥30% decrease in the sum of the longest diameter of targeted lesions. Complete and partial responses were achieved by only one and two patients, respectively. A PSA response was observed in 10 patients. Despite some positive results, the study was terminated due to withdrawal of funding (Table 1: NCT00503984). More studies are needed to assess the clinical potential of this agent.

Another category of epigenetic drugs with clinical potential in cancer treatment are HDM inhibitors. Phenelzine is a monoamine oxidase A (MAOA) inhibitor used in the treatment of psychiatric disease. MAOA is an enzyme responsible for the deamination of neurotransmitters, such as dopamine, serotonin and norepinephrine, that are important in a variety of neurological and psychiatric illnesses [63]. MAOA has close homology to LSD1, a histone demethylase, which catalyzes removal of the methyl group from H3K4me1 and H3K4me2. As a result of this homology, Phenelzine is able to bind and inhibit LSD1. [63]. Lower levels of H3K4me2 are correlated with higher risk of recurrence in prostate cancer [64]. Phenelzine is currently being trialed as a monotherapy for the treatment of recurrent prostate cancer and in combination with docetaxel for the treatment of progressive prostate cancer. Given that Phenelzine is already an approved medication, positive responses in these clinical trials will open the door to using this class of epigenetic drugs in clinical practice in the near future.

In the area of miRNA modulation, four clinical trials of antisense oligonucleotides have reported a positive PSA response, with three trials describing a PSA response >=50% (Table 1: NCT01188187, NCT00471432, NCT00085228, [65-68]). One of these trials, evaluating the efficacy of the antisense oligonucleotide OGX-011 in combination with docetaxel or mitoxantrone, reported a PSA decrease ≥50% in 6 of 14 patients with CRPC (Table 1: NCT00471432, [66]). Most of the adverse effects were grade 1 and 2 only, however adverse effects of grade 3 or higher affected 60% of the patients receiving the antisense oligonucleotide in combination with docetaxel/ prednisone and 73% of the patients receiving the OGX-011 in combination with mitoxantrone/prednisone (Table 1: NCT00471432, [66]). The most common grade 3 or higher adverse effects in both groups were fatigue and lymphopenia (Table 1: NCT00471432, [66]). OGX-011 was also tested in combination with docetaxel and prednisone in a phase III clinical trial but, despite some positive results observed in phase II of the study where 58% of the patients had a PSA response >=50%, no significant results were observed in phase III (Table 1: NCT00471432, NCT01188187, [66, 69]). In the group receiving the combined therapy OGX-011/docetaxel/ prednisone, 41% of the patients had to discontinue the treatment program due to adverse effects of the therapy (≥3 grade) (Table 1: NCT01188187, [69]).

Administration of the antisense oligonucleotide LY2181308 to decrease the expression of survivin, an anti-apoptotic gene involved in therapy resistance was tested in a randomized phase 2 clinical trial performed by Wiecho *et al* for the treatment of CRPC [70]. The patients allocated in the group treated with LY2181308 reported higher incidence of grade 3 and 4 adverse effects without any improvement in progression free survival or overall survival of the patients [70].

Disappointing results were obtained with another antisense oligonucleotide, Oblimersen, that was trailed by Sternberg *et al* both alone and in combination with docetaxel (Table 1: NCT00085228, [67]). The authors observed a PSA response in 46% of the patients treated with docetaxel alone, versus 37% of the patients treated with the antisense oligonucleotide and docetaxel (Table 1: NCT00085228, [67]). In the group of patients receiving the combined therapy, major toxic events were observed in 40.7% of the patients, compared to 22.8% of the patients receiving the antisense oligonucleotide alone, indicating that docetaxel increased oblimersen-related toxicity. This suggests that combined therapy with antisense oligonucleotides and taxanes might not the best therapeutic approach (Table 1: NCT00085228, [67]). In another clinical trial Oblimersen was administrated in combination with mitoxantrone, with 2 patients out of 25 showing a PSA decrease equal or superior to 50%. One patient had a PSA response inferior to 50% while stable disease was observed in a further five patients [68].

Although no clinical benefits were observed in a study testing the antisense oligonucleotides ISIS 3512 and ISIS 5132, two patients who received the oligonucleotide ISIS 3512 and one patient who received ISIS 5132 did not show disease progression for at least five months [71]. Finally, results are currently unavailable from eight completed clinical trials and two other trials are ongoing (Table 1).

Kidney cancer - epigenetics

Genetic and epigenetic dysregulation of genes involved in pathways such as the hypoxia-inducible pathway, the mTOR pathway, and the *cMET-RAF-MEK-ERK* pathway contribute to the progression of kidney cancer [72]. Changes in the levels of epigenetic-modifying enzymes are an important factor in altering expression of genes involved in cancer-related pathways. In renal cell carcinoma (RCC), an increase in the levels of histone demethylases such as *UTX* , *JMJD2* and *EZH2*, results in a reduction in H3K27me and promotes progression of the disease [73, 74]. Also in RCC, almost 60% of patients

Table 2: Clinical trials of epigenetic drugs in kidney cancer

Drug	Combined Therapy	Enzimatic Class	Approval Stage	Status	Indication	Results	Reference/Clinical trial identification
Vorinostat	Bevacizumab	HDAC inhibitor	Phase 1/2	completed	Unresectable or metastatic kidney cancer	48,6% of the patients had absence of disease progression at 6 months 8,11% of the patients experienced serious adverse events	NCT00324870
Vorinostat	Isotretinoin	HDAC inhibitor	Phase 1/2	Completed	Advanced renal cell carcinoma	MTD of Vorinostat in combination with isotretinoin=0,5 mg/kg	NCT00324740
Vorinostat	-	HDAC inhibitor	Phase 2	Completed	Advanced renal cell carcinoma	An objective response was observed in 36% of the patients 63% of the patients demonstrate progressive disease and one patient had serious adverse events	NCT00278395
Vorinostat	Pembrodizumab	HDAC inhibitor	Phase 1	Ongoing	Advanced renal or urothelial cell carcinoma	Final data collection date for primary outcome measure: May 2018	NCT02619253
Panobinostat	-	HDAC inhibitor	Phase 2	Completed	Refractory clear cell renal carcinoma	Median of progression free survival=1,7 months 30% of the patients experienced serious adverse events	Hainsworth *et al*. 2011 (NCT00550277)
Panobinostat	Everolimus	HDAC inhibitor	Phase ½	Terminated	Metastatic or unresectable renal cell cancer	The study has been terminated (patients off study, principal investigator left institute)	NCT01582009
Panobinostat	Sorafenib	HDAC inhibitor	Phase 1	Ongoing	Advanced renal cell carcinoma	Study completion date: November 2016	NCT01005797
Entinostat	Isotreitinoin	HDAC inhibitor	Phase 1	Completed	solid tumor including kidney cancer, urothelial carcinoma and prostate cancer	No objective responses were observed but stable disease was noticed in patients with kidney, prostate and pancreatic cancer Recommended doses for phase 2: 4 mg/m of entinostat once weekly and 1mg/kg of isotretinoin per day	Pili et al. 2012
Entinostat	IL-2	HDAC inhibitor	Phase 1/2	Ongoing	Metastatic kidney cancer	No date given for study completion	NCT01038778
Decitabine	Interferon alpha2B	DNMT inhibitor	Phase 2	Terminated	Advanced renal cell carcinoma	The study was terminated due to low accrual and unavailable treatment agent.	NCT00561912
Decitabine	IL-2	DNMT inhibitor	Phase 1	Completed	Melanoma or renal cell cancer	Three patients with renal cell cancer had stable disease	Gollob *et al*. 2006
GTI-2040	Capecitabine	Antisense oligonucleotide that targets R2 subunit of ribonucleotide reductase	Phase 2	Completed	Advanced/metatastic renal cell carcinoma	52% of the patients had stable disease with median duration of 4 months One partial response was observed	Desai *et al*. 2004
MG98	-	Antisense oligonucleotide that targets DNMT1	Phase 2	Completed	Metastatic renal carcinoma	Six patients had stable disease but no objective responses were seen	Whinquist E. *et al* 2006
Oblimersen	Interferon alpha	Antisense oligonucleotide that targets bcl2	Phase 2	Completed	Metastatic renal cell cancer	No study results or publications provided	NCT00059813
MRX34		RNA mimic	Phase 1	Terminated	Renal cell carcinoma	This study was terminated due to serious adverse events	NCT01829971

overexpress *HDAC1* and *HDAC2* [75]. No prognostic value has been associated with these alterations [75]. *HDAC3* is also highly expressed, but only in the papillary carcinoma subset [75].

At the level of individual epigenetic changes, low levels of H3K4me2, H3K18Ac, and H3K9me2 are associated with poor prognosis and lower survival probability in RCC. , Mechanistically, H3K4me2 and H3K18Ac are associated with active transcription while H3K9me2 is associated with transcriptional repression [73, 74]. H3K27me is another histone modification that correlates with poor clinical outcome, result of overexpression of histone demethylases in RCC [73, 74].

Clear cell RCC is the most common form of renal cell carcinoma, and is associated with inactivation of the tumor suppressor gene *von-Hippel Lindau* (*VHL*) by either genetic and epigenetic factors [76, 77]. *VHL* inactivation in both sporadic and familial forms can occur due to point mutations or deletions at the genetic level, or due to DNA hypermethylation at the gene promoter [76, 77].

DNA methylation also affects the regulation of several genes in RCC and has the potential to be used as biomarker and as a therapeutic target [78]. For example, hypermethylation of *RASSF1A* and *HIC* in patients with RCC is associated with a poor prognosis [79, 80].

Several miRNAs showed altered expression in RCC, resulting in changes to important cellular functions such as apoptosis, angiogenesis and the epithelial mesenchymal transition [27, 81]. Examples of miRNAs with altered expression in RCC include miR-210, miR-34a, miR-30c, miR-29b and miR-23b [27, 81].

Kidney cancer - current treatment

Radical nephrectomy is the standard of care for localized renal cell carcinoma. However, high rates of recurrence after surgery demand the development of new adjuvant therapies. Both radiotherapy and hormone therapy have proven ineffective in advanced stages of disease and chemotherapy has a response rate inferior to 10% [82, 83].

Kidney cancer - pre-clinical data

Preclinical studies using epigenetic drugs for kidney cancer treatment show some promise. In renal cancer cell lines, the HDACi Panobinostat induced cell cycle arrest and apoptosis and also resulted in a reduction in tumor size in xenograft mice models [84]. Pre-clinical studies of DNMT inhibitors in kidney cancer have also shown promise with evidence of reactivation of silenced genes and growth inhibition of cancer cells [85, 86].

One ongoing issue in RCC treatment is resistance to immunomodulatory therapy with interferons. This can occur via promoter hypermethylation and silencing of interferon response genes [87]. Treatment of renal cancer cell lines with 5-Aza-2'-deoxycitidine (5-Aza-dC), increased expression of interferon response genes and restored interferon induced apoptosis [87]. In addition, treatment of RCC cells with the antisense DNMT1 oligonucleotide MG98 also restored susceptibility to interferon therapy [88].

Downregulation of miR-30c in RCC is associated with promotion of the epithelial mesenchymal transition [89]. Reduced expression of this miRNA is associated with hypoxia and *VHL* cell status with lower levels of miR-30c being observed in *VHL*-deficient RCC cell lines [89]. Transfection of a RNA mimic to restore miR-30c levels caused an increase in E-cadherin expression and reduced cell migration capacity [89].

Kidney cancer - clinical trials

Trials of HDACi in RCC have shown mixed responses. HDACi Vorinostat used as a monotherapy showed an objective response in 36% of patients, however 63% of the patients presented disease progression at 6 months (Table 2: s). When used in combination with Bevacizumab, 48, 6 % of the patients showed stable disease at 6 months (Table 2: NCT00324870). By contrast, treatment with Panobinostat alone resulted in no objective responses, and a median of progression-free survival of 1.7 months (Table 2: NCT00550277, [90]). Treatment was generally well tolerated, but 7 patients reported thrombocytopenia grade 3 or higher (Table 2: NCT00550277, [90]). In a separate small phase I trial, the HDACi Entinostat was administered in combination with Isotretinoin. One patient, who had presented with disease progression after treatment with cytokines and anti-angiogenic therapy, subsequently showed stable disease
[91]. The patient had a reduction in tumor size after 4 months of therapy and did not show signs of disease progression at 12 months [91]. However, the number of patients enrolled in the study (2) was insufficient to draw any conclusions [91].

The only DNMT inhibitor tested in RCC is Decitabine. When used in combination with the cytokine IL-2 in a phase II study of advanced RCC, three out of five patients showed stable disease [92]. Another study combining Decitabine with interferon-α was terminated early due to low accrual (Table 1, NCT00561912).

Treatment with antisense oligonucleotides has also resulted in stabilization of disease in some RCC patients. The antisense oligonucleotide GTI-2040, targeting the R2 subunit of ribonucleotide reductase, was tested in patients with metastatic disease in a phase II trial and generated a partial response for approximately eight months [93]. One patient experienced a dose limiting toxicity (grade 3 diarrhea) and adverse effects of all grades were reported in this trial including grade 4 pancytopenia, pulmonary embolism and bone pain [93]. In a trial reported by Winquist *et al*, in which the antisense oligonucleotide MG98 was administered to 15 patients, no objective responses were observed with nine patients presenting progression of the disease. MG98 targets DNMT1 but no decrease in enzyme activity was observed [94]. Also, grade 3 and 4 adverse effects forced 8 patients to discontinue treatment, primarily due to elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels [94].

miRNA mimics have also been trialed in RCC. MRX34, an miRNA mimic of the tumor suppressor miRNA34, was tested in a phase I clinical trial for advanced or metastatic cancers, including RCC, but the trial was terminated early due to serious immunologic adverse events (Table 2: NCT01829971).

Bladder cancer - epigenetics

Epigenetic modifications are important in bladder cancer development [95]. An increase in global histone methylation was reported in bladder cancer samples, particularly in the subset of patients with non-muscular invasive bladder cancer. In these patients, a global increase in H3K9 and H3K27 methylation was associated with high-grade tumors. However, the authors did not find any correlation between histone methylation and tumor recurrence or survival [96]. Interestingly, another study by the same group revealed that a decrease in methylation levels of other histone proteins, namely H3K4 and H3K20, could be a prognostic biomarker for muscle invasive bladder cancer [97]. The presence of different histone methylation patterns in muscle invasive and non-invasive bladder cancer, suggests that patients of these subgroups will respond differentially to epigenetic therapies affecting histone methylation. These data reinforce the need for biomarker discovery to predict responses to epigenetic therapy.

With respect to DNA methylation in bladder cancer, Friedrich *et al* reported hypermethylation of the genes *DAPK*, *BCL2* and *TERT* in urine samples from patients

Table 3: Clinical trials of epigenetic drugs in bladder cancer

Drug	Combined Therapy	Enzimatic Class	Approval Stage	Status	Indication	Results	Reference/Clinical trial identification
Belinostat	Carboplatin or paclitaxel	HDAC inhibitor	Phase 1/2	Phase 1 concluded, Phase 2 ongoing	Bladder cancer	Four out of fifteen patients had complete or partial response patients had progressive disease with median time to progression of 136 days	NCT00421889
Vorinostat	-	HDAC inhibitor	Phase 2	Terminated for futility	Locally Recurrent or Metastatic Cancer of the Urothelium	No objective response was observed Median overall survival: 4,3 months Median progression free survival: 1,1 months	NCT00363883
Mocetinostat	-	HDAC inhibitor	Phase 2	Ongoing	Patients with advanced urothelial Carcinoma and inactivating alterations of acetyltransferase genes	Study completion date: December 2017	NCT02236195
FdCyd	Tetrahydrouridine	DNMT inhibitor	Phase 2	Ongoing	Advanced cancer including bladder Cancer	Study completion date: May 2017	NCT00978250
OGX-427	Docetaxel	Antisense oligonucleotide that targets heat shock protein 27	Phase 2	Ongoing	Advanced urothelial Carcinoma	Study completion date: February 2017	NCT01780545

with bladder cancer [98]. Detection of methylation patterns in urine samples has proven to be a good diagnostic strategy in bladder cancer. [99, 100]. Methylation of some gene promoters can also be indicative of prognosis, for example, methylation of the *RUNX3* promoter is associated with a higher risk of progression and lower survival [101].

Differential miRNA expression is another epigenetic feature of bladder cancer and can distinguish between cancer patients and healthy subjects [27, 102]. The miRNAs implicated in bladder cancer target genes involved in cell cycle control, cell proliferation, cell differentiation and signal transduction pathways [27, 102]. Both upregulation and downregulation of miRNA expression can potentiate cancer development. In bladder cancer, loss of miR-200 is associated with epithelial mesenchymal transition while upregulation of miR-21 and miR-129 is associated with high grade tumors and poor prognosis, respectively [27, 102, 103].

Bladder cancer - current treatment

Muscle invasion is a critical factor in the selection of the right therapeutic option for bladder cancer. In the case of non-muscle invasive bladder cancer, the standard clinical approach is transurethelial resection followed by administration of chemotherapeutic or immunotherapeutic agents [104]. For muscle-invasive bladder cancer, a more aggressive form, the standard of care is radical cystectomy [105]. In cases of disease relapse, occurring in approximately 30% of patients, combinatory chemotherapy regimens containing cisplatin are used. These include MVAC (Methrotrexate, Vinblastine, Achiamycin and Cisplatin) and GC (Gemcitabine and Cisplatin). Despite positive early responses to these therapeutic modalities, the median survival rate after treatment is only 12 months [104-106]. For advanced and metastatic bladder cancer, where surgery is not a valid approach, the only treatment option is palliative chemotherapy. This reflects the need for the development and implementation of new therapeutic agents [104].

Bladder cancer - pre-clinical data

Proteomic studies after exposure of bladder cancer cells to HDAC inhibitors reveals that HDAC activity influences many cellular pathways involved in carcinogenesis [107]. The treatment of bladder cancer cell lines with these agents resulted in cell growth suppression and induction of cell death [107]. Wang *et al* showed that the HDACi Vorinostat was able to induced cell growth inhibition in bladder cancer cells in part due to downregulation of survivin, an apoptosis inhibitory protein [108]. Importantly, Vorinostat had a synergistic effect with chemotherapeutic agents including Cisplatin, Mitomycin c, and Adriamycin. Combined therapy of using Vorinostat and Cisplatin prevented cancer progression in an animal model [108].

Positive responses were also obtained in bladder cancer cells after administration of the DNMTi Belinostat. A significant decrease in cell proliferation was observed *in* vitro, and *in vivo* with the use of a transgenic mouse model [109].

Epigenetic drugs can be used in combination with other agents to enhance their efficacy. Shang *et al* evaluated the DNMT inhibitor 5-Aza-2-deoxycytidine in combination with chemotherapeutic agents in bladder transitional cell carcinoma cell lines. The authors demonstrated that DAC enhances susceptibility to Cisplatin, a common agent used as neoadjuvant therapy for bladder cancer, in a synergistic way. Both agents induced cell cycle arrest at G2/M phase, with Cisplatin also inducing tumor cell apoptosis [110].

Rieger *et al* compared the efficacy and efficiency of

siRNAS and antisense oligonucleotides against *Bcl-xL* in bladder cancer cell lines. Their effect in combined therapy with chemotherapeutic agents was also tested. Both agents enhanced tumor cell apoptosis when administrated with Cisplatin, however *Bcl-xL* knockout was more efficient with siRNAs than with antisense oligonucleotides [111]. Another study showed that simultaneous knockout of *Bcl-xL* and *survivin* with siRNAs in bladder cancer cells led to greater sensitization of the cells to chemotherapeutic agents [112].

Bladder cancer - clinical data

Relative few studies of epigenetic therapies have been undertaken in bladder cancer. Only one of three clinical trials employing HDAC inhibitors showed a positive response; in this trial, four of fifteen patients treated with the HDACi Belinostat in combination with Carboplatin or Paclitaxel showed complete or partial response to treatment, while five patients presented disease progression with a median time to progression of 136 days (Table 3, NCT00421889). Further studies employing this HDACi in combination with other agents are essential to confirm its therapeutic potential [109]. The only study using the HDACi Vorinostat terminated due to lack of efficacy (Table 3, NCT00363883). Three other phase 2 clinical trials employing epigenetic drugs for bladder cancer treatment are currently ongoing (Table 3: NCT02236195, NCT00978250, NCT01780545).

CONCLUSIONS

Despite the obvious importance of epigenetics in the development of cancer, few epigenetic therapies have thus far reached advanced clinical testing. As the data above demonstrates, pre-clinical data has not translated into the hoped-for clinical responses. This is likely secondary to the nonspecific actions of epigenetic drugs and the consequent toxicities associated with their administration.

Many of the epigenetic therapies being tested have global epigenetic effects on both cancerous and non-cancerous tissues. Moreover, some of them have additional non-epigenetic effects that limit their efficacy. HDAC enzymes, for instance, target non-histone proteins involved in oncologic pathways unrelated to epigenetic regulation [113]. It is important to consider that the observed therapeutic responses to HDACi treatment may thus be the result of the altered activity of these proteins and not to the reversal of specific epigenetic marks [113]. Similarly, demethylating agents are not specific to genes involved in carcinogenesis but result in global demethylation of the genome, an epigenetic signature associated with genomic instability that can lead to severe side effects [113].

Of all the clinical trials analyzed in this study, 16 included evaluation of gene expression and/or DNA methylation as a secondary objective of the trial. We feel that analysis of gene expression and epigenetic patterns should be included in all clinical trials using epigenetic agents in order to assess the causal link between drug induced alterations and therapeutic responses. A better knowledge of the specific mechanism of action of these agents is essential to overcoming their clinical limitations and improving therapeutic success.

Antisense oligonucleotides (ASOs) avoid some of the issues described above as as they are designed to be more target specific. Thus far, two separate studies of ASOs as monotherapy have shown no objective responses [71, 94], however when used in combination with other agents the results have been more promising [66, 93]. However, ASOs also have their limitations secondary to toxicity and delivery efficiency [67, 69].

Currently, cancer treatment is determined largely according to cancer stage, even though patients with similar stage cancers may respond differently to the same type of therapy. The promise of "personalized medicine" is the idea that tailoring treatment to an individual patient will optimize efficacy while minimizing toxicity. Personalization should be an ongoing goal for all cancer therapy development, including epigenetic therapies. Biomarker development should thus be a central goal in the development of epigenetic therapies, both so that the correct patient receives the correct therapy, and to ensure that therapies that have value in a subset of patients are not passed over because of lack of efficacy in other patients. Epigenetic therapies are still in their infancy as a therapeutic class and pre-clinical promise has not yet translated into clinical efficacy. However, the development of target-specific agents, and the careful combination of epigenetic therapies with traditional modalities should enable them to achieve clinical success in the near future.

CONFLICTS OF INTEREST

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