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MicroRNAs as bile-based biomarkers for pancreatic and biliary tract cancers

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) and biliary tract cancers (BTC) are lethal diseases and have poor survival rates. MicroRNAs (miRNAs) are small non-coding RNAs that regulate gene expression at the post-transcriptional level. They are commonly deregulated in cancer and are incredibly stable molecules in tissues and biofluids. Therefore, miRNAs have potential as clinical useful biomarkers for the detection of cancer. PDAC and BTC often present with obstruction of the common bile duct leading to painless jaundice. Therefore, finding novel miRNAs in the bile-fluid associated with PDAC and/or BTC would be a considerable discovery, due to its close proximity to the malignant lesion. Exosomes are small extracellular vesicles of endocytic origin, which contain miRNAs. They are secreted into the extracellular space by a variety of cells, including cancer cells. They play a crucial role in cell-cell communication in the tumour microenvironment, transferring information through their cargo, and are implicated in cancer development and metastasis. Here, I develop upon these initial observations and demonstrate that bile miRNAs can discriminate PDAC and BTC from benign disease. Profiling revealed miRNAs are able to differentiate between benign and malignant lesions. These candidates were further validated in an independent cohort of bile samples and three miRNAs (miR-148a, miR-194 and miR-125b) were able to not only differentiate benign from malignant lesions, but also discriminate between PDAC and cholangiocarcinoma (CCA). Furthermore, the expression of these three miRNAs was measured in exosomes from six pancreatic cell lines showing a higher expression of these miRNAs in exosomes compared to the cell pellet. A significantly higher expression of miR-148a was observed in exosomes compared to the cell pellet. Therefore, there is potential to use these bile-based miRNAs to improve diagnosis and stratify patients, thereby informing treatment decisions and improving survival by “personalising” their subsequent therapies.

Declaration of originality

I herewith certified that all material in this dissertation which is not my own work has been properly acknowledged.

Methods Collection of bile specimens from patients with PDAC, CCA, ampullary cancer, intraductal papillary mucinous neoplasms and benign disease was performed by Prof. Long Jiao, Prof. Nagy Habib, and Dr Artur Zanellato at Hammersmith Hospital (Imperial College London NHS Trust, London, UK), Dr Elisa Giovannetti at VUMC Amsterdam (The Netherlands) and Dr Nicola Funnel in University Hospital of Pisa (Italy).

Methods MicroRNA microarray expression profile using nCounter miRNA Expression was performed by Nanostring Technologies, Seattle (USA).

Methods Fixation of exosomes from pancreatic cancer cell medium for transmission electron microscopy imaging was performed by Dr Padmini Sarathchandra at Harefield Hospital (London, UK)

Results Bioinformatic analyses were performed by Dr Leandro Castellano at Imperial College London (United Kingdom).

Results Transmission electron microscopy images of exosomes were obtained by Dr Padmini Sarathchandra at Harefield Hospital (London, UK)

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Full manuscripts arising

Below are the complete papers that have been published during this PhD thesis.

*indicates joint first authorship.

1. Frampton AE*, **Prado MM***, Lopez-Jimenez E, Fajardo-Puerta AB, Jawad ZAR, Lawton P, Giovannetti E, Habib NA, Castellano L, Stebbing J, Krell J, Jiao LR. Glypican-1 is enriched in circulating exosomes in pancreatic cancer and correlates with tumour burden. *Oncotarget*, 04/2018; 10;9(27):19006-19013. doi: 10.18632/oncotarget.24873. eCollection 2018 Apr 10.
2. **Mato Prado M**, Frampton AE, Giovannetti E, Stebbing J, Castellano L, Krell J. Investigating miRNA-mRNA regulatory networks using crosslinking immunoprecipitation methods for biomarker and target discovery in cancer, *Expert Rev Mol Diagn*, 11/2016; 16(11):1155-1162
3. Le Large TY, **Mato Prado M**, Krell J, Bijlsma MF, Meijer LL, Kazemier G, Frampton AE, Giovannetti E. Bioinformatic analyses reveals pancreatic cancer molecular subtypes specific to the tumor and the microenvironment, *Expert Rev Mol Diagn*, 07/2016; 16(7):733-6
4. Fajardo-Puerta AB, **Mato Prado M**, Frampton AE, Jiao LR. Gene of the month: HGF, *J Clin Pathol.*, 07/2016; 69(7):575-9
5. Adam E. Frampton*, Jonathan Krell*, **Mireia Mato Prado**, Tamara M.H. Gall, Nima Abbassi-Ghadi, Elisa Giovannetti, Niccola Funel, Leandro Castellano, Nagy A. Habib, Panagiotis Vlavianos, Justin Stebbing and Long R. Jiao. Prospective validation of microRNA signatures for detecting pancreatic malignant transformation in endoscopic-ultrasound guided fine-needle aspiration biopsies, *Oncotarget*, 05/2016; 7(19):28556-69

6. Le Large TY, **Prado MM**, Kazemier G, Frampton AE, Giovannetti E. Circulating microRNAs as diagnostic biomarkers for pancreatic cancer, *Expert Rev Mol Diagn*, 11/2015; 15(12):1525-9
7. **Mato Prado M**, Frampton AE, Stebbing J, Krell J. Single-cell sequencing in cancer research, *Expert Rev Mol Diagn*, 11/2015; 1-5
8. **Mato Prado M**, Frampton AE, Stebbing J, Krell J. Gene of the month: NANOG, *J Clin Pathol*. 10/2015 Oct; 68(10):763-5.
9. Frampton AE*, Krell J*, Jamieson NB, Gall TM, Giovannetti E, Funel N, **Mato Prado M**, Krell D, Habib NA, Castellano L, Jiao LR, Stebbing J. MicroRNAs with prognostic significance in pancreatic ductal adenocarcinoma: A meta-analysis, 07/2015; 51(11):1389-404.

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Abbreviations

AC: ampullary carcinoma

ADEX: aberrantly differentiated endocrine exocrine

adj: adjusted

AFM: atomic force microscopy

Ago: Argonaute

AGO-CLIP: Argonaute crosslinking immunoprecipitation

AGO-IP: Argonaute immunoprecipitation

AJCC: American Joint Committee on Cancer

ATP: adenosine tri-phosphate

AUC: area under curve

BCT: benign cystic tumours

BSA: bovine serum albumin

BTC: biliary-tract cancer

°C: centigrade

CA 19-9: carbohydrate antigen 19-9

CAF: cancer-associated fibroblasts

CCA: cholangiocarcinoma

CDCA: chenodeoxycholic acid

cDNA: complementary DNA

C. elegans: *Caenorhabditis elegans*

CLASH: crosslinking immunoprecipitation and sequencing of hybrids

CLIP: crosslinking immunoprecipitation

CO₂: carbon dioxide

CP: chronic pancreatitis

CT: computed tomography

C_T: comparative threshold cycle

CSF: cerebrospinal fluid

DC: dendritic cell

dCCA: distal cholangiocarcinoma

ddH₂O: distilled water

DGCR8: Di George critical region gene 8

DLS: dynamic light scattering

DMBT1: deleted in malignant brain tumours 1

DMEM: Dulbelco's Modified Eagle Medium

DNA: deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

dsRBD: double-stranded RNA binding domain

dTTP: deoxythymidine triphosphate

EDTA: ethylenediaminetetraacetic acid

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

EM: electron microscope or electron microscopy

EMT: epithelial-mesenchymal transition

ERCP: endoscopic-retrograde cholangiopancreatography

ESCRT: endosomal sorting complex required for transport

ETS: environmental tobacco smoke

EUS: endoscopic ultrasonography

EUS-FNA: endoscopic ultrasonography fine-needle aspiration

EV: extracellular vesicle

FBS: foetal bovine serum

FC: fold change

FFPE: formalin-fixed paraffin-embedded

FGF-19: fibroblast growth factor 19

Fig: figure

FNA: fine-needle aspiration

FU: fluorouracil

g: G-force

GEM: gemcitabine

GTP: guanosine tri-phosphate

HCC: hepatocellular carcinoma

HCl: hydrochloric acid

hEGF: human epidermal growth factor

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER: human epidermal receptor

HIF-1: hypoxia inhibitor factor 1

HITS-CLIP: high-throughput sequencing crosslinking immunoprecipitation

H₂O: water

HR-MM: hypoxia-resistant multiple myeloma

HRP: horseradish peroxidase

iCCA: intra-hepatic cholangiocarcinoma

iCLIP: individual-nucleotide resolution crosslinking immunoprecipitation

IgA: immunoglobulin A

IgG: immunoglobulin G

ILV: intraluminal vesicle

IP: immunoprecipitation

IPMN: intraductal papillary mucinous neoplasm

KCl: potassium chloride

kDa: kilo Dalton

KH₂PO₄: monopotassium phosphate or potassium dihydrogen phosphate

LCSGJ: Liver Cancer Study Group of Japan

MCN: mucinous cystic neoplasms

MEM: minimum essential medium

mg: milligram

MIF: macrophage migration inhibitory factor

min: minute

miRNAs (miR): microRNAs

ml: millilitre

mM: millimolar

MRCP: magnetic resonance cholangiopancreatography

MRE: microRNA recognition element

MRI: magnetic resonance imaging

mRNA: messenger RNA

MSKCC: Memorial Sloan-Kettering Cancer Center

MVB: multivesicular body

MVE: multivesicular endosome

NaCl: sodium chloride

Na₂HPO₄: sodium phosphate dibasic or disodium phosphate

NCCJ: National Cancer Center of Japan

ncRNA: non-coding RNA

ng: nanogram

nm: nanometer

nt: nucleotide

NTA: nanoparticle tracking analysis

ORF: open reading frame

OS: overall survival

PACT: protein activator of PKR

PanIN: pancreatic intraepithelial neoplasm

PAR-CLIP: photoactivatable ribonucleoside-enhanced crosslinking and immunoprecipitation

PBS: phosphate buffered saline

PC: pancreatic cancer

PCR: polymerase chain reaction

pCCA: perihilar cholangiocarcinoma

PDAC: pancreatic ductal adenocarcinoma

PET: positron emission tomography

PFA: paraformaldehyde

PNET: pancreatic neuroendocrine tumours

Pre-miRNA: precursor microRNA

Pri-miRNA: primary microRNA

PSC: primary sclerosing cholangitis

R0: no tumour involvement of resection margin

RISC: RNA-induced silencing complex

RLC: RISC loading complex

RNA: ribonucleic acid

RNase: ribonuclease

RNAi: RNA interference

RNA-seq: RNA sequencing

RNA pol: RNA polymerase

ROC: receiver operating characteristic

RPMI: Roswell Park Memorial Institute

rRNA: ribosomal RNA

RT-PCR: reverse transcription polymerase chain reaction

RT-qPCR: reverse transcription quantitative polymerase chain reaction

SD: standard deviation

SILAC: stable isotope labelling amino acid in cell culture

siRNA: small interfering RNA

SNAP: Soluble N-ethylmaleimide-sensitive fusion Attachment Protein

SNARE: Soluble N-ethylmaleimide sensitive fusion Attachment Receptor

snRNA: small nuclear RNA

snoRNA: small nucleolar RNA

Taq: *thermus aquaticus*

TEM: transmission electron microscope/microscopy

TGF- β : transforming growth factor type β

TLR: toll-like receptor

TNM: tumour, nodes, metastasis

TRBP: TAR RNA-binding protein

tRNA: transfer RNA

UICC: Union for International Cancer Control

μ l: microliter

μ m: micrometre

US: ultrasound or ultrasonography

UTR: untranslated region

CHAPTER 1. INTRODUCTION

1.1 Pancreatic cancer

1.1.1 The pancreas

The pancreas is a gland located in the abdominal cavity between the stomach and the spine. It can be divided into three different parts (head, body and tail) and it is composed of two different types of cells that also have different functions. Exocrine cells are formed by ducts, which contain acini (small sacs), and produce pancreatic enzymes that are released directly into the pancreatic duct and then into the duodenum to help digestion and break down metabolites such as fatty acids into small molecules; whereas endocrine cells, also called islets of Langerhans, generate different hormones, which play vital roles in metabolism (particularly insulin) and are released directly into the bloodstream.

1.1.2 Types of pancreatic cancer

The vast majority of pancreatic tumours belong to the exocrine type (95%) and within this category the most common type of tumours are adenocarcinomas (90%). Pancreatic ductal adenocarcinoma (PDAC) can grow anywhere in the pancreas, usually starts in the cells lining the pancreatic duct, and it is often found in the head of the pancreas. There are other rare types of exocrine tumours including acinar cell carcinoma, adenosquamous carcinoma, mucinous cyst neoplasms, pancreatoblastoma, colloid carcinoma, hepatoid carcinoma and giant cell tumour. The endocrine tumours are much less common (5%) and are also called pancreatic neuroendocrine tumours (PNETs) or islet cell tumours because they arise from endocrine cells in the islets of Langerhans that develop an abnormal growth. This type of tumours are either functional or non-functional meaning that functional

neuroendocrine tumours overproduce hormones and as a consequence patients present hormone-related symptoms, whereas the non-functional tumours do not produce hormones and hence, do not cause any hormone-related symptoms. A greater number of PNETs are non-functional tumours. These tumours are named based on the hormone cells that are affected (i.e. insulinoma, glucagonoma, gastrinoma). The tumour growth is slower in endocrine tumours compared to exocrine tumours and the therapeutic treatment differs between these two categories, generally presenting a better prognosis PNETs than PDAC.

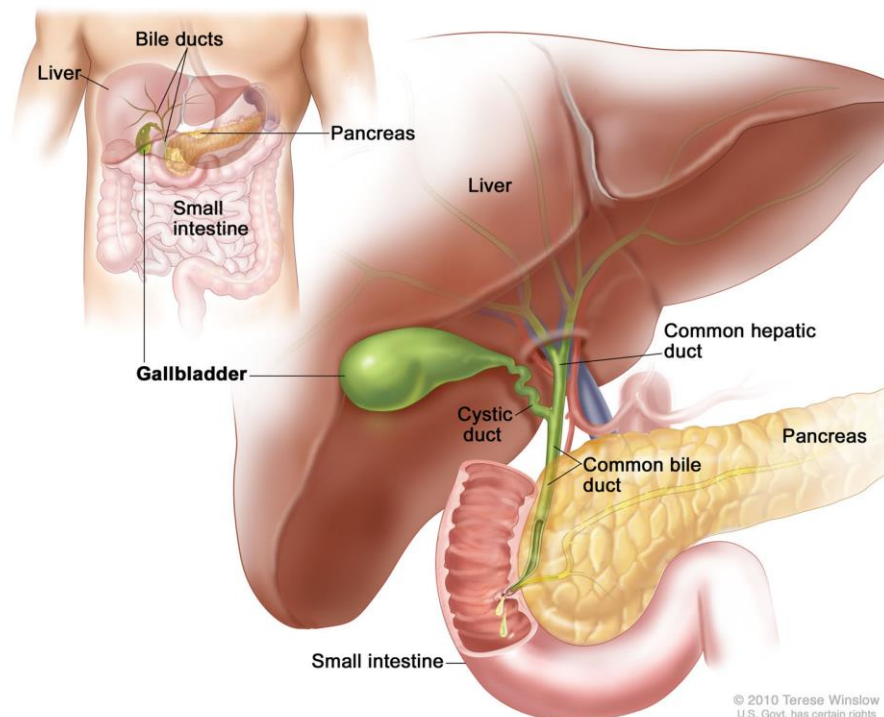


Figure 1.1. Anatomy of the biliary tract system.

The gallbladder lies beneath the lower right lobe of the liver and stores bile before releasing it through the common bile duct into the small intestine. The pancreas is located in the abdominal cavity between the stomach and the spine. It can be divided in three parts: head, body and tail. *For the National Cancer Institute © (2018) Terese Winslow LLC, U.S. Govt. has certain rights.*

Pancreatic adenocarcinoma can also originate from pancreatic benign cystic tumours (BCTs) that can be either inflammatory or proliferative (1). Mucinous cystic neoplasms (MCNs) and intraductal papillary mucinous neoplasms (IPMNs) are two types of BCTs with the potential to progress to *in situ* or invasive carcinoma (2). IPMNs are intraductal neoplasms with tall, columnar, mucin-containing epithelium with or without papillary projections. They are connected to the pancreatic duct (main or side-branch), which is generally enlarged (3), whilst MCNs are separated from the ductal system (4). The highest percentage of malignancy is comprised in the main branch of the pancreatic duct (5), so IPMN lesions exhibit a higher malignant potential in comparison to MCNs.

1.1.3 Epidemiology and statistics

Pancreatic cancer was first described by Mondière in 1836 (6) and since then an exponential increase in its incidence has been reported worldwide. It is the fifth most common cause of cancer death in Europe and in the United Kingdom (UK), and the seventh most common cause of death worldwide (7, 8). It has been predicted that PDAC will become the second leading cause of cancer-related death in the USA in 2020 (9). In 2012, about 338,000 people had PDAC worldwide but only 3% survived; whilst in Europe 409,911 new cases were diagnosed and 353,580 cases (86%) died from PDAC (7). In the UK (2015), a total number of 9,921 new cases were diagnosed but only 11% survived (8). The 5-year relative survival for PDAC is currently 3% in the UK and less than 5% worldwide (10). If the tumour is resectable, the 5-year survival rate increases between 7-25% in the UK. However, most patients (80-85%) present advanced unresectable carcinoma and only 20% of patients with malignant disease localised to the pancreas will benefit from surgery. In the UK, only a small percentage of patients (8%) are suitable to undergo surgery (8).

1.1.4 Aetiology and clinical characteristics of pancreatic cancer

Pancreatic cancer, and particularly pancreatic ductal adenocarcinoma (PDAC), has a very poor prognosis due to its invasive nature to spread to surrounding organs. Generally, PDAC is diagnosed in elderly people (> 70 years) and almost all cases are diagnosed after the age of 55 years. However, men present a higher incidence rate of PDAC than women (11).

Early stages of PDAC are usually clinically silent given that the early symptoms are quite vague and unspecific, which are often ignored by the patient and the doctor. These early symptoms include epigastric bloating, vomiting, diarrhoea and constipation followed by painless jaundice and weight loss as the disease progresses. Abdominal and mid-back pain are usually late symptoms (12). The obstruction of the pancreatic duct could occasionally result in episodes of pancreatitis (13). Additionally, important physical signs such as palpable liver and gallbladder, thrombophlebitis and abdominal tenderness should be noted given that usually indicates an advanced or unresectable disease. Nevertheless, other conditions including chronic pancreatitis and choledocholithiasis can mimic very similar signs and symptoms (12).

The range of risk factors associated to PDAC is reasonably wide. The two most dominant risk factors are cigarette smoking and a family history of chronic pancreatitis, followed by diabetes mellitus (14) – the latter may be a sequela of the disease rather than a risk factor. It has been suggested that cigarette smoking as well as daily exposure to environmental tobacco smoke (ETS) contribute to an augmented risk of developing PDAC by more than 70% (15). Moreover, pancreatitis (acute or chronic) is an inflammatory disorder associated with the progression of PDAC. Despite patients with chronic pancreatitis (CP) are at a relatively high risk of developing PDAC, only around 5% of patients diagnosed with CP will develop a pancreatic carcinoma over a period of 20 years (16). It seems that progressive inflammatory episodes in chronic

pancreatitis are responsible of the neoplastic transformation of pancreatic cells that triggers towards pancreatic cancer progression (17).

A family history of pancreatic cancer, defined as a family where a pair of first-degree relatives has been diagnosed with malignant pancreatic tumours, is an extremely relevant risk factor, which accounts for 7-10% of individuals affected with this disease (18). These patients tend to present more precancerous lesions and have an increased risk of developing PDAC compared to the general population (19). Despite the fact that testing for germline genetic changes is not a common practice in patients with a family history of PDAC, mainly due to failing in recognising a familial cancer syndrome from the family history, genetic tests should be considered for those individuals with many 1st-degree relatives with pancreatic abnormalities which include germline mutations associated with inherited susceptibility for PDAC such as BRCA2, PALB2, CDKN2A, STK11 and PRSS1 genes, and Lynch syndrome (13).

Furthermore, type II diabetes mellitus has been associated with an increased risk of suffering pancreatic cancer. The relationship between PDAC and diabetes is complex as it can be either a predisposing factor of PC or an early manifestation of the disease (20). Other risk factors include obesity, ethnic origin (African-American are at high risk), a high-fat diet, male gender, advanced age, alcohol consumption, occupational exposures (e.g. chlorinated hydrocarbon solvents and nickel) and possibly infection with *Helicobacter pylori* and periodontal disease (13). Hence, pancreatic cancer is a very complex and multifactorial disease.

1.1.5 Molecular characteristics of pancreatic cancer

Pancreatic cancer is a heterogeneous disease; therefore, it is essential to effectively classify precancerous lesions to investigate pancreatic carcinogenesis and develop new screening test to detect pancreatic neoplasms at an early stage.

It is believed that adenocarcinomas from the pancreas arise from non-invasive pancreatic duct lesions to infiltrating carcinomas by accumulating multiple genetic alterations. In 1998, Brat *et al.* established that duct lesions could later develop in infiltrating adenocarcinomas. Observations in 3 patients where a portion of their pancreas was resected strongly support the role of duct lesions in the development of PDAC (21). Currently, these pancreatic duct lesions are called PanINs (pancreatic intraepithelial neoplasm) and are grouped into three categories: PanIN-1, PanIN-2 and PanIN-3 according to the histological analysis (22). They are the best characterised precursors of PDAC, microscopic, non-invasive epithelial neoplasms formed in ducts from columnar-to-cuboidal cells with < 5 mm of diameter (23). Furthermore, PanIN-1 lesions can be divided into PanIN-1A (flat epithelial lesions) and PanIN-1B (papillary epithelial lesions) and can be detected in up to 40% of adult pancreata not involved in an invasive carcinoma. PanIN-2 lesions are mostly papillary and present some nuclear abnormalities such as loss of polarity, nuclear crowding, enlarged nuclei and hyperchromatism. PanIN-3 lesions are usually papillary or micropapillary but are rarely flat. They are characterised by loss of nuclear polarity and are present in 30-50% of pancreata with invasive ductal carcinoma phenotype (23).

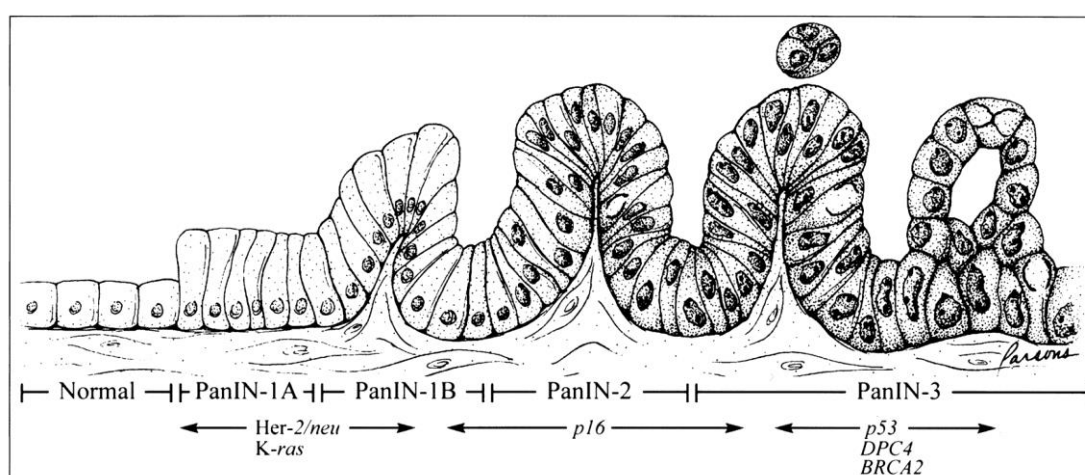


Figure 1.2. Progression model for pancreatic cancer.

Normal duct epithelium progresses from pancreatic duct precursor lesions (PanINs) to infiltrating carcinoma by accumulating multiple genetic alterations. *Reproduced from Hruban et al., Clinical Cancer Research 6, 2969-2972 (2000).*

Moreover, the evaluation of these lesions aids to define the molecular and genetic alterations present in each group. There is an accumulation of genetic alterations that are associated with histological progression from low-grade PanINs (PanIN-1) to intermediate grade (PanIN-2), to high-grade PanIN (PanIN-3), to invasive cancer. Hence, a progression model for pancreatic cancer has been developed. As it can be observed in **Figure 1.2**, early genetic events of PDAC tumourigenesis include activation of *K-ras* oncogene and overexpression of *HER-2/neu*; whereas inactivation of tumour-suppressor gene *p16* (*p16INK4A/CDKN2A*) occurs slightly later (PanIN-2) and loss of *p53*, *DPC4* and *BRCA2* tumour-suppressor genes occur relatively late in the development of pancreatic neoplasia (PanIN-3) (24). It has been shown that PanIN-2 lesions often contain as many mutations as PanIN-3 and invasive tumour samples, indicating that pancreatic tumours could progressed from PanIN-2 lesions without forming PanIN-3 lesions (25). Nevertheless, it is important to bear in mind that not all PanIN lesions will progress to infiltrating carcinomas (24).

Four genes were identified in pancreatic cancer to be commonly mutated, and these include the *KRAS* oncogene and three tumour suppressor genes: *p16/CDKN2A*, *TP53* and *SMAD4*. Furthermore, patients with a strong family history of pancreatic cancer are more predispose to develop this type of cancer due to inheritance of genetic abnormalities. In particular, four genes (*BRCA2*, *p16/CDKN2A*, *STK11* and *PRSS1*) are known to cause familial aggregation of PC when they are mutated in the germline (19, 26-28). However, they only account for less than 20% of the familial aggregation of PDAC. It is suggested that genetic alterations in the pancreas could start in the germline with inherited mutations in genes including *BRCA2*, *p16/CDKN2A*, *STK11*, *PALB2*, *ATM* and *PRSS1* and then progress to non-invasive precursor lesions that accumulate mutations in *KRAS*, *p16/CDKN2A*, *TP53* and *SMAD4* and then on to invasive PDAC (26).

The *KRAS* gene is located on chromosome 12 (12p) and is activated in almost all pancreatic cancers (90%). It is one of the most common genetic changes observed in early intraductal lesions and infiltrating PDAC (21, 29). Generally, normal duct epithelial cells do not present *KRAS* mutations (30). The majority of activating point mutations in the *KRAS* gene occur at codon 12 in PC and a few target codons 13 or 61 (31). These mutations occur early in pancreatic development, before an invasive carcinoma develops (31).

The tumour suppressor gene *p16/CDKN2A* encodes for the p16^{INK4A} protein and it regulates the cell cycle via the p16/Rb pathway (31). Its inactivation in pancreatic cancer accounts for more than 95% (32).

TP53 is another tumour suppressor gene located on chromosome 17 (17p), which codes for the p53 protein (31). This protein is important in many biological cell events such as regulation of the cell cycle checkpoint, maintenance of G₂/M arrest and induction of apoptosis. In approximately 55-75% of pancreatic cancers this gene is inactivated (31).

The *SMAD4*, also known as *DPC4*, is a tumour suppressor gene located on the chromosome 18 (18q) and is genetically inactivated in 55% of pancreatic cancers (31). This gene codes for the protein Smad4, or *dpc4*, that is involved in signalling via the transforming growth factor type β (TGF- β). The activated form of TGF- β promotes an intracellular cascade resulting in Smad4 being localised into the nucleus where it regulates the expression of specific target genes (31). According to Iacobuzio-Donahue, the loss of *SMAD4* was associated with metastases (26).

Other tumour suppressor genes are involved in pancreatic cancer but in a rather low frequency, less than 10%, and are the following: *MKK4*, *TGFRI* (ALK 5), *STK11/LKB1*, *ACVR2*, *FBXW7* (*CDC4*) and *EP300* (31).

Yachida and colleagues suggested that only 37–39% of carcinomas contain genetic alterations in all genes. In many cases pancreatic cancer presents mutations in two driver genes (*KRAS* and *CDKN2A*); however, other carcinomas have alternative combinations such as *KRAS* and *TP53* but *SMAD4* was never included (33). Individuals with only two driver genes mutated in PC had a significantly longer disease free and median overall survival (33). In contrast, Rachakonda and colleagues showed that *KRAS* mutations were associated with a reduced survival time in PC. Moreover, patients with concomitant mutations in *KRAS* and in *CDKN2A* have the lowest survival rate (29). In addition, Yachida *et al.* found that most of the genetic alterations in pancreatic metastases were also identified in paired primary cancers from the same subjects (33).

In 2012, Biankin exposed a comprehensive evaluation of PDAC using whole exome sequencing and copy number analysis of 99 tumours (34). The study showed a large number of novel mutated genes including those involved in DNA damage repair (*ATM*), chromatin remodelling (*EPC1* and *ARID2*) and others (*ZIM2*, *NALCN*, *SLC16A4*, *MAP2K4* and *MAGEA6*). Recently, Bailey revealed an integrated genomic analysis combining whole-genome and deep-exome sequencing using 456 pancreatic ductal adenocarcinoma samples to identify mutational mechanisms and crucial genomic events in pancreatic carcinogenesis (35). The study defined 4 subtypes of PDAC: squamous, pancreatic progenitor, immunogenic and aberrantly differentiated endocrine exocrine (ADEX) associated with different survival and distinct histopathological characteristics. This classification overlaps with the Collisson classification (36) in three of the subtypes, except for the immunogenic subtype.

1.1.6 Clinical diagnosis of pancreatic cancer

Pancreatic cancer patients can be classified following the TNM staging (Tumour, Node, Metastasis) shown in **Table 1**, which is helpful in determining prognosis; although a simplified classification - commonly used for treatment purposes – divides patients into four different categories according to the clinical stage of the tumour including resectable, borderline resectable, locally advanced and metastatic (13, 37). Depending on the stage of the disease a different therapeutic treatment will be given.

Table 1.1. Staging of PDAC (AJCC).

Stage	Pre-operative status	TNM level	Description
I	Resectable	T1 or T2, N0, M0	No extrapancreatic disease, no encasement of the coeliac axis or SMA
II	Potentially resectable	T1 or T2, N1, M0 T3, N0 or N1, M0	Regional lymph nodes may be involved, no encasement of coeliac axis or SMA, possible extrapancreatic involvement
III	Unresectable	T4, N0 or N1, M0	Regional lymph nodes may be involved, encasement of coeliac axis or SMA
IV	Unresectable	T (any), N (any), M1	Liver peritoneal, lung metastases

Early detection of pancreatic cancer has improved over the last decades. At first, early diagnosis of this malignant disease was limited to patients with obstructive painful jaundice and laboratory test included measurements of amylase in serum and urine, serum carbohydrate antigens such as CA19-9, abnormal glucose tolerance and glycosuria. Moreover, imaging techniques were also used which allowed clinicians to recognise the clinical characteristics of pancreatic cancer at an early stage (38). However, asymptomatic patients presented with locally advanced or metastatic disease due to the lack of an effective screening test to detect PDAC at an earlier stage (37). Similarly, low- and high-risk pre-malignant lesions can be difficult to differentiate, therefore, an accurate clinical evaluation and a correct pre-operative

diagnosis is essential. For instance, sensitivity and specificity of endoscopic ultrasound fine-needle aspiration (EUS-FNA) to predict malignancy in pancreatic cyst lesions is 24% and 97%, respectively (39), and current cross-sectional imaging modalities are not sufficiently reliable to differentiate between benign and malignant lesions (40, 41). Hence, development of better techniques to distinguish benign from pre-malignant and malignant pancreatic cyst tumours is urgently necessary.

Currently, substantial improvements in non-invasive imaging techniques including ultrasonography (US), computed tomography (CT), magnetic resonance imaging (MRI), endoscopic ultrasonography (EUS), endoscopic retrograde cholangiopancreatography (ERCP) and positron emission tomography (PET) have enhanced the ability to diagnose PDAC and plan appropriate therapies for patients. Adequate combination of these different imaging modalities aid for diagnosis and staging of PDAC and they can also help in reducing the number of unnecessary surgical explorations; however, all of them have their limitations (12). For instance, CT scans are the best initial test for pancreatic cancer and staging of the disease, providing 80% accuracy for prediction of resectability. However, in cases where patients cannot tolerate intravenous contrast for CT scans the tumour staging is carried out with MRI, instead. Another frequently used imaging technique is EUS, which is used as a screening test due to its ability to detect small pre-invasive lesions. Additionally, EUS or CT-guided fine-needle aspirations are used for cytological diagnosis providing a sensitivity of 80%. In cases where a metastasis is suspected, a PET-CT scan is carried out although is currently not part of routine staging (13). Furthermore, an accurate screening blood test for early detection of PDAC is still to be found. Detection of serum marker CA19-9 in blood higher than 100-200 U/mL helps to predict unresectability and survival after surgery (13). It is also the most commonly used biomarker to monitor the disease providing information on prognosis and therapeutic response after surgical resection and chemotherapy (42). Unfortunately,

there are not specific tumour biomarkers that have proven sufficient specificity and/or sensitivity for diagnosis.

1.1.7 Treatment of pancreatic cancer

1.1.7.1 Surgical treatment

In the past, surgical resection was the treatment of choice to cure pancreatic cancer; although the potential to cure this tumour by only surgical treatment alone was low. However, surgical resection is the only currently effective therapeutic treatment to cure pancreatic cancer. There are different surgical procedures that can be performed but nowadays the most common procedure that provides a curative resection of the pancreas is a pancreaticoduodenectomy or Whipple procedure (43). Patients who successfully underwent a surgical resection of the pancreatic tumour (R0, margin negative) and follow an adjuvant therapy have a median survival rate of 20 months or more (44).

1.1.7.2 Therapeutic treatment

Radiotherapy at conventional doses (3000-4000 rad) had very little or no effect on pancreatic adenocarcinoma patients, indicating that these tumours are resistant to radiation and this type of therapy alone will not eradicate the malignant tumour. Likewise, chemotherapy was used as a palliative treatment because only a few drugs were well studied and the available data of standard anticancer agents was not sufficient.

Currently, all these three modalities of treatment have improved considerably, especially chemo- and radiotherapy, which nowadays have a significant effect on pancreatic cancer patients' survival. The first chemotherapeutic agent approved by the Food and Drug Administration (FDA) for the treatment of pancreatic cancer was

gemcitabine. In 1996, it was given to patients with locally advanced or metastatic PDAC who were previously treated with fluorouracil (5-FU). Gemcitabine in combination with 5-FU, capecitabine, oxaliplatin and cisplatin failed to improve survival compared with gemcitabine alone (45-51). On the other hand, a widely used treatment is the combination of *nab*-paclitaxel with gemcitabine – which resulted in a near 2-month survival benefit; however, it is quite toxic compare to gemcitabine alone and might not be suitable for elderly patients and/or patients with a poor performance status (52). An alternative first-line therapy for healthy individuals is FOLFIRINOX; although its performance is superior to gemcitabine, its adverse effects are severe (53). The administration of FOLFIRINOX and gem/*nab*-paclitaxel is unlimited until disease progression. Dose modification is necessary when initial adverse effects such as peripheral neuropathy and myelosuppression appear (54). The FDA approved a second-line treatment for those PDAC patients who have progressed on gemcitabine-based therapy, which is the combination of nanoliposomal irinotecan and 5-FU (55). Other therapies aim to target the tumour microenvironment of the pancreas, impact the immune system (i.e. manipulate tumour associated-macrophages, lymphocytic T-cells), induce DNA damage (e.g. PARP inhibitors) and target specific genes involved in carcinogenesis (e.g. p53) (54).

To summarise, FOLFIRINOX is the standard care of PDAC patients receiving neo-adjuvant treatment in the UK, whereas gemcitabine, *nab*-paclitaxel plus gemcitabine and erlotinib are given as adjuvant chemotherapeutics. A combination of chemotherapeutic regimens is given to locally advanced or metastatic PDAC patients. However, despite developments in early diagnosis and progress in understanding the biology and molecular mechanisms of PDAC there is still need for better therapies, specific biomarkers and diagnostic techniques to detect this disease at an earlier stage, particularly to those people at risk of developing pancreatic carcinoma.

1.1.8 Clinical trials for pancreatic cancer

1.1.8.1 Clinical trials for resected pancreatic cancer

Surgical resection is the only effective therapeutic treatment that provides a potential cure to patients with pancreatic cancer. Nevertheless, some patients will follow an adjuvant chemotherapeutic treatment.

In 2004, the European Study Group for Pancreatic Cancer (ESPAC) carried out a multicentre trial (ESPAC-1), in which adjuvant chemotherapy with 5-FU had a significant survival benefit in patients with resected PDAC whilst a deleterious effect on patients' survival was observed when chemoradiotherapy was administered (56).

In 2007, a phase III randomised clinical trial that aimed to address the efficacy and toxicity of gemcitabine in resected PDAC patients was carried out (Charité Onklogie 001 [CONKO-001], ISRCTN34802808) (57). A prolonged overall survival (OS) was observed in patients randomised to adjuvant gemcitabine (22.8 months) in comparison with the control group (20.2 months); showing a 10-year overall survival of 12.2% and 7.7%, respectively, and a 5-year overall survival rate of 20.7% and 10.4%, respectively. The disease-free survival for the gemcitabine group was 13.4 months whereas patients that only underwent a surgical procedure (observation group) had a disease-free survival of 6.9 months. Therefore, these findings supported the use of adjuvant gemcitabine in this setting as it resulted in increased overall survival and disease-free survival in patients with complete resection of the pancreatic tumour.

The failure of adjuvant chemoradiotherapy to enhance survival was reflected in the ESPAC-1 trial, however, the Radiation Therapy Oncology Group proposed to evaluate whether adding gemcitabine to adjuvant fluorouracil chemoradiation might improve survival for patients with resected pancreatic adenocarcinoma in a phase III randomised clinical trial (RTOG-9704, NCT00003216) (58). Nevertheless, despite the

survival benefit was associated to the addition of gemcitabine to adjuvant fluorouracil-based chemoradiation, this improvement was not statistically significant.

In 2016, a Japanese study did a randomised, multicentre phase III clinical trial, JASPAC-01, where patients with resected PDAC were assigned to receive either gemcitabine or fluoropyrimidine S-1 (or S-1). They aimed to verify whether S-1 was superior to gemcitabine as an adjuvant chemotherapeutic agent for patients with resected PDAC given that S-1 is widely used in gastric cancer in Japan and its toxicity profile is different from gemcitabine, considering it to have some positive effects on patient's quality of life (59). A significant improved overall survival was shown with adjuvant S-1 chemotherapy (46.5 months) in resected PDAC patients compared with adjuvant gemcitabine (25.2 months). An association with a higher 5-year survival was observed in the S-1 group (43.6%) than in the gemcitabine group (24.2%). The median relapse-free survival in the gemcitabine group was 11.3 months, whilst the S-1 group showed a relapse-free survival of 22.9 months. Despite the fact that this adjuvant chemotherapy with S-1 has shown a superior effectiveness than gemcitabine, it has only been proven in Japanese patients, hence, it should be evaluated in non-Asian patients.

It has been shown in other clinical trials that a combined therapy using gemcitabine and capecitabine improved survival and tumour response in advanced or metastatic PDAC patients (47). Capecitabine is an orally active tumour-selective fluoropyrimidine carbamate, which provides a prolonged exposure to fluorouracil at lower peak concentrations. Thus, the European Study group for Pancreatic Cancer investigated the use of adjuvant combination therapy, with patients randomly assigned to 6 months of gemcitabine-capecitabine *versus* gemcitabine monotherapy (ESPAC-4, ISRCTN96397434). More than 700 patients were recruited, and the highest median overall survival was 28 months and it was observed in the combined therapy group whereas the gemcitabine group showed a median OS of 25.5 months (60). The

ESPAC-4 clinical trial showed that overall survival significantly increased with combined adjuvant chemotherapy (gemcitabine plus capecitabine) compared to gemcitabine monotherapy after surgical resection of pancreatic cancer. Therefore, gemcitabine plus capecitabine is now one of the adjuvant chemotherapeutic alternatives for patients with PDAC after surgical resection of the pancreas.

Currently, there are some ongoing clinical trials such as the one carried out by the Radiation Therapy Oncology Group (RTOG-0848, NCT01013649) which is currently addressing the value of chemoradiotherapy in patients who have received standard-of-care adjuvant chemotherapy after surgical resection. The ongoing RTOG-0848 clinical trial randomly assigned patients to 6 months of adjuvant gemcitabine-based chemotherapy with or without erlotinib. Furthermore, those patients whose disease has not progressed after a full course of gemcitabine will be randomly assigned to receive chemoradiotherapy (capecitabine plus fluorouracil). Another phase III randomised clinical trial is ATRACT (Nab-paclitaxel and Gemcitabine vs Gemcitabine Alone as Adjuvant Therapy for Patients With Resected Pancreatic Cancer (the "Atract" Study), NCT01964430), which is currently assigning resected PDAC patients to gemcitabine monotherapy for 6 months or to gemcitabine/*nab*-paclitaxel combination therapy. The target accrual is 800 patients, with an estimated completion date of 2020. The efficacy of FOLFIRINOX to treat resectable pancreatic adenocarcinoma is also being evaluated in the ACCORD 24 clinical trial (Trial Comparing Adjuvant Chemotherapy With Gemcitabine Versus mFOLFIRINOX to Treat Resected Pancreatic Adenocarcinoma, NCT01526135). It will compare the difference between adjuvant chemotherapy with gemcitabine *versus* FOLFIRINOX.

1.1.8.2 Clinical trials for locally advanced pancreatic cancer

Chemoradiotherapy was historically used as the primary and initial treatment for patients with locally advanced disease. However, whether radiotherapy improves survival and quality of life is a controversial issue. Over the past several decades,

clinical trials have evaluated concurrent chemoradiotherapy compared with chemotherapy alone in patients with locally advanced disease; however, the results obtained were mixed (61).

Currently, the standard treatment in the UK for locally advanced, non-resectable and non-metastatic PDAC is chemotherapy. Since gemcitabine was introduced as the standard care of treatment for patients with advanced pancreatic cancer in 1996, there has been no improvements in survival and this could be due to numerous phase III clinical trials have failed to statistically demonstrate a significant improve in the OS over gemcitabine, which ranged from 5.7 to 6.8 months (47, 62). For instance, in 1997 Burris and colleagues reported a study in which 160 patients with metastatic PDAC were randomly assigned to receive either gemcitabine or fluorouracil (5-FU). This study demonstrated that gemcitabine conferred a modest survival advantage over 5-FU and it was more effective in alleviating some disease-related symptoms (62). Another randomised trial in which patients were randomly assigned to receive gemcitabine plus capecitabine also failed in showing a clinical benefit over gemcitabine monotherapy (47).

Only two trials where gemcitabine was administered together with either erlotinib or FOLFIRINOX showed a significant improvement in overall survival over gemcitabine alone (6.24 vs 5.91 months, and 11.1 vs 6.8 months, respectively) (53, 63). Erlotinib is an oral tyrosine kinase inhibitor of the human epidermal growth factor receptor type 1 (HER1/EGFR) and FOLFIRINOX is the combination of four drugs including 5-fluorouracil, leucovorin, irinotecan and oxaliplatin. Furthermore, progression-free survival was longer in the erlotinib plus gemcitabine arm and in the FOLFIRINOX group than in the gemcitabine alone arm (53, 63). Both studies demonstrated an improvement in survival of PDAC over gemcitabine monotherapy. Hence, they were considered as new treatment options for advanced PDAC patients.

Despite the mechanism of EGFR activation in pancreatic cancer remains poorly understood, there are potential factors such as gene amplification, ligand overexpression, altered receptor processing and autocrine or paracrine up-regulation, which support the role of EGFR in activating the progression of this malignant disease. Pre-clinical studies confirmed the anti-tumour effect of EGFR inhibition by tyrosine kinase inhibitors (TKIs) in pancreatic cancer (64-67). In two mouse xenograft models of pancreatic cancer where erlotinib was administered alone and in combination with gemcitabine a significantly increased apoptosis was observed as well as a blockage of EGFR phosphorylation (65). Similarly, when HPAC cells treated with erlotinib were implanted into athymic/nude mice, tumours grew slowly and showed a reduced metastatic potential (*in vivo* and *in vitro*) (67). Another study used TKIs that inhibited cell proliferation and phosphorylation of MAPK and EGFR autophosphorylation in pancreatic cell lines (66). In a pancreatic cancer mouse xenograft model, another TKI (PKI-166) exhibited activity alone and together with gemcitabine (64).

Furthermore, erlotinib has been also evaluated in several clinical as a therapeutic target for solid tumours including pancreatic cancer (68, 69). The most recent study upon which the FDA based its approval of erlotinib showed that this therapeutic agent in combination with gemcitabine in patients with advanced pancreatic cancer results in a statistically significant improvement in OS (63). They also assessed the expression of EGFR in tumour samples, but it did not correlate with clinical response. However, the presence or absence of mutant K-ras is a potential molecular predictor of response to EGFR inhibitors given that RAS/MAP kinase is one of the downstream signalling pathways of EGFR. Hence, mutations in any of these signalling components may lead to therapeutic resistance. Moore et. al/ observed that patients with wild-type K-ras treated with erlotinib showed a trend of greater OS; however, this finding was not statistically significant (63). Moreover, it has been observed that patients who developed a skin rash showed improved outcomes but the mechanism by which this

occurs is still unclear. The challenge remains on identifying advanced pancreatic patients most likely to benefit from this therapeutic agent. Therefore, further investigation is needed to discover molecular markers predictive of response to erlotinib among pancreatic cancer patients.

In 2011, a phase I/II clinical trial investigated the efficacy of a first-line gemcitabine plus *nab*-paclitaxel therapy in metastatic PDAC (70). *Nab*-paclitaxel is an albumin-based formulation of paclitaxel. The randomised phase III MPACT clinical trial [NCT00844649] confirmed previous report from 2011, supporting the superior effect of *nab*-paclitaxel plus gemcitabine over gemcitabine alone (71). The treatment of pancreatic cancer patients with *nab*-paclitaxel plus gemcitabine demonstrated a statistically improvement across all endpoints compared to gemcitabine alone, including OS (8.5 months versus 6.7 months) and progression-free survival (5.5 vs 3.7). Therefore, it has been suggested that *nab*-paclitaxel together with gemcitabine could serve as a backbone on which other therapies for patients with pancreatic disease can be added.

1.2 Biliary tract cancer: Cholangiocarcinoma

Biliary tract cancer is an uncommon type of cancer in developed countries and has a poor prognosis. In the UK, approximately 1,200 new cases are diagnosed each year (72). It includes cancer types from the biliary tree such as cholangiocarcinoma, ampullary carcinoma and gallbladder cancer.

Patients with either resectable or locally advanced/metastatic biliary tract cancer have no established standard chemotherapy because individual randomised clinical trials for this rare type of cancer have not been performed. The data regarding first-line therapy in this type of cancers is very limited given that the only data available is from PDAC or hepatocellular carcinoma in which biliary tract cancer patients are included as a sub-group. Therefore, results from these studies should be considered with caution, as they are not sufficiently robust to define a standardised treatment.

Valle and colleagues suggested that biliary tract cancers are chemo-sensitive and, although very little is known about their biology, it seems to be similar to that of gastrointestinal epithelial cancers sharing also similar oncogenic mutations (72).

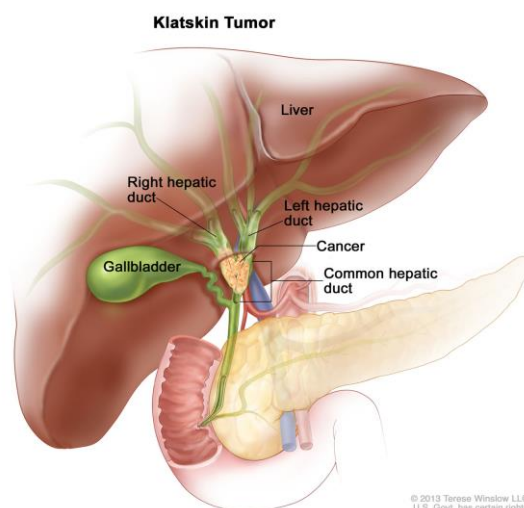


Figure 1.3. Cholangiocarcinoma.

This is a cholangiocarcinoma (CCA), perihilar subtype that occurs in the hepatic duct bifurcation. For the National Cancer Institute © (2018) Terese Winslow LLC, U.S. Govt. has certain rights.

1.2.1 Types of cholangiocarcinoma

Cholangiocarcinoma (CCA) is a relatively rare and heterogeneous tumour that originates from cholangiocytes, epithelial cells lining the biliary tree tract, which suffer a malignant neoplastic transformation. It is formed in the bile ducts that connect the gallbladder, the liver, the pancreas and the small intestine. There is a biological fluid called bile that flows through the bile ducts until it reaches the small intestine.

This type of cancer is the most common malignancy of the biliary tract and the second most common primary hepatic malignancy (73). Adenocarcinoma is the most common type (90-95%) presenting poor to moderate differentiation, high desmoplastic stroma and mucin expression (74).

According to its anatomic location, cholangiocarcinoma tumours are classified into three different subtypes (**Figure 1.4**): intrahepatic (iCCA), perihilar (pCCA) and distal extrahepatic (dCCA); being pCCA the most common – accounting for 80-90% of CCAs, and usually referred to as ‘Klatskin tumour’ (74). Originally, cholangiocarcinoma tumours were divided into intrahepatic and extrahepatic, including this latter the subtypes pCCA and dCCA (75-77). Intrahepatic CCA arises from biliary epithelial cells or hepatic progenitor cells located in the hepatic parenchyma near the secondary branches of the right and left hepatic ducts, whereas pCCA and dCCA arise from biliary epithelial cells and peribiliary glands. iCCA is separated from pCCA and dCCA by the second-order of biliary ducts and these latter two subtypes are separated by the cyst duct. The secondary branches of the right and left hepatic ducts and the common hepatic duct next to the origin of the cyst duct are the boundaries of pCCA tumours. Finally, dCCA is located in the common bile duct without including the Ampulla of Vater and two types of precursor lesions are proposed to arise from it: intraductal papillary neoplasms and biliary intraepithelial neoplasia (74).

Different growth patterns can be observed in intrahepatic and extrahepatic cholangiocarcinomas. In iCCA, four growth types are identified: 1) mass-forming, 2) periductal-infiltrating, 3) intraductal, and 4) undefined; whilst only two can be observed in pCCA: 1) mass-forming and 2) intraductal. Mass-forming is the most common form of iCCA and the combination of mass-forming and periductal-infiltrating subtype has the worst prognosis (73). In pCCA, the intraductal subtype can be further divided into nodular and periductal-infiltrating, being this latter the most common subtype (78).

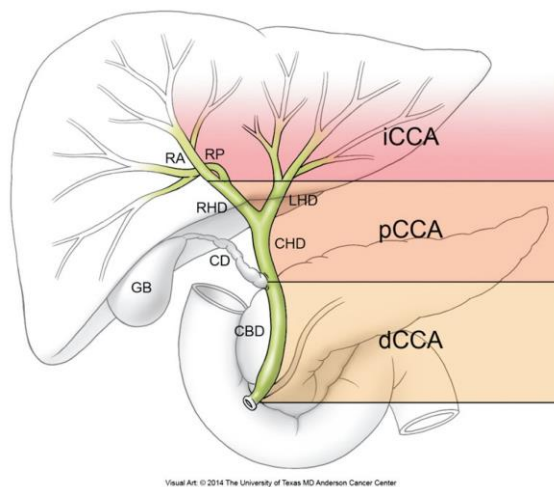


Figure 1.4. Classification of cholangiocarcinoma subtypes.

This classification is based on anatomical location within the biliary tree system into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA). *Reproduced from Blechacz et al., Gut and Liver 1, 13-26 (2017).*

These cholangiocarcinoma tumours differ in their anatomic origin, their biological behaviour, their clinical management and their response to therapy. Therefore, the different cholangiocarcinoma subtypes should be considered as separate entities in regards to its clinical management.

1.2.2 Epidemiology and statistics of cholangiocarcinoma

Generally, CCA accounts for only 3% of all gastrointestinal tumours. The incidence of cholangiocarcinoma presents a wide geographic variability. For instance, Asian countries (e.g. Thailand) show the highest incidence of CCA – probably due to the elevated prevalence of hepatobiliary flukes associated with the ingestion of undercooked fish, while Australia presents the lowest rate (79). In Europe, between 4 to 18 inhabitants per million will be diagnosed with CCA(80). The incidence ratios of intrahepatic cholangiocarcinoma have increased in Europe, particularly in Western Europe, whereas the opposite has occurred with extrahepatic cholangiocarcinoma in which the incidence ratios have been stabilised or decreased (81). The median age of diagnosis is > 65 years and it is slightly more prevalent in males than in females (79).

1.2.3 Aetiology and clinical characteristics of cholangiocarcinoma

Established risk factors associated with the development of CCA include primary sclerosing cholangitis (PSC), hepatobiliary parasites (*Opisthorchis viverrini* and *Clonorchis sinensis*), congenital biliary malformations (Caroli's disease, choledochal cyst), hepatolithiasis and chemical agents (thorotrast). Other possible risk factors such as infectious diseases (Hepatitis B, Hepatitis C, liver cirrhosis), biliary enteric drainage, toxins (i.e. dioxins), diabetes, obesity and smoking could also contribute to cholangiocarcinoma pathogenesis (74, 82).

Generally, cholangiocarcinoma is clinically silent and usually symptoms, although unspecific, appear at an advanced stage.

More than half of perihilar CCA patients have signs of malignancy such as abdominal pain, weight loss, malaise and cachexia. Moreover, it is also very common that patients present with biliary problems, as this type of tumour involves the main bile duct, such

as biliary obstruction with painless jaundice (90%) and cholangitis – although this is less frequent (10%) (73). Clinically, symptoms related to dCCA are similar to pCCA causing cholestasis and cholangitis as well as abdominal discomfort and weight loss (73)

Intrahepatic cholangiocarcinoma presents with unspecific symptoms including abdominal discomfort, night sweats, malaise, fatigue and cachexia (73, 78). Unfortunately the detection of iCCA in asymptomatic patients is sometimes incidental by detecting an abdominal mass during an imaging procedure or a physical examination (76)

1.2.4 Molecular characteristics of cholangiocarcinoma

Chronic inflammation of the biliary epithelium and obstruction of the biliary tree are two common conditions associated to neoplastic transformation of the cholangiocytes and, therefore, risk factors that predispose patients to develop CCA. This malignant transformation of the cholangiocytes is the consequence of a chronic biliary inflammation and obstruction that triggers the production of cytokines and reactive oxygen species that causes cellular stress and irreversible DNA damage (83). These molecular changes contribute to tumour development and survival of malignant cells.

1.2.5 Diagnosis of cholangiocarcinoma

Diagnostic techniques such as computed tomography (CT) and magnetic resonance imaging (MRI) are helpful for evaluating the size of the primary tumour and detecting the presence of satellite lesions (73, 78). However, it is preferable to perform a CT as it might be more appropriate in order to assess the vascular status, identify extrahepatic metastases and predict resectability. Despite both imaging techniques

have low specificity (73), they accurately distinguish iCCA from hepatocellular carcinoma (HCC) in tumours > 2 cm diameter (74). It is important to discern iCCA tumours from HCC as they respond differently to therapy and the outcomes following liver transplantation could be suboptimal in patients with mixed features of both HCC and CCA (84). Positron emission tomography (PET) has a limited diagnostic value. Generally aids in detecting larger iCCAs and metastases (78) and PET/CT showed a very high sensitivity and specificity (95%) but it does not offer a significant advantage over the CT or MRI (85). Thus, histological diagnosis is also necessary.

Serum tumour markers are attractive molecules that can help in CCA diagnosis, however, these markers have not reached adequate specificity. The most common serum tumour marker is the carbohydrate antigen 19-9 (CA 19-9) but its expression levels can also be elevated in other malignancies including gastrointestinal, pancreatic and gynaecological diseases as well as benign cholangiopathies (74). For example, serum levels of 129U/mL or greater in iCCA patients with PSC have a sensitivity and specificity of 79% and 98%, respectively (86). Nevertheless, it only detects iCCA patients with 62% sensitivity and 63% specificity (75). Therefore, it should be used in combination with other diagnostic tests.

Perihilar and distal CCA subtypes have been grouped together as extrahepatic cholangiocarcinoma due to their clinical similarities. Hence, the same diagnostic criteria for pCCA are applied to dCCA.

Endoscopic retrograde cholangiopancreatography (ERCP) is useful in evaluating biliary strictures, sampling epithelial cells for cytology and, in cases of obstruction of the bile ducts, aids in therapeutic dilatation and stent placement (75, 87). In addition, endoscopic ultrasound (EUS) combined with fine-needle aspiration can also help in assessing the biliary tract tree; however, it should be avoided because the tumour may spread to other sites (88). The implementation of PET is limited as it provides false-negative and false-positive results because of a high desmoplastic and inflamed

environment surrounding the tumour (89). Thus, the preferred imaging modality for perihilar CCA is magnetic resonance cholangiopancreatography (MRCP) because it is a valuable diagnostic tool for the identification of this subtype of cancer with an accuracy of 76% and a sensitivity of 89% (90). A combination of MRCP with MRI enables the assessment of the biliary tree and it can also evaluate the extent of the tumour and its resectability with an accuracy of up to 95% (73). Serum levels of CA 19-9 are less specific in pCCA than in iCCA (73). In addition, other conditions such as duct obstruction or cholangitis can also induce an increase in these levels, for this reason testing for serum tumour markers should be used in combination with other diagnostic tools.

It is challenging to distinguish benign from malignant biliary duct strictures; therefore, diagnostic imaging techniques should be performed to determine the extent of the disease and stage it correctly, assess biliary tract obstruction(s) and manage patient therapy prior to surgery and/or biliary interventions. Additionally, histopathology and cytology analysis should be carried out as well as testing the levels of serum tumour markers in CCA patients.

In summary, a multidisciplinary approach combining different diagnostic modalities is required, as it still remains a challenge to diagnose this disease at an early stage.

1.2.6 Staging system for cholangiocarcinoma

Cholangiocarcinoma has been classified using different staging systems depending on the subtype. For instance, intrahepatic cholangiocarcinoma tumours were previously classified based on the TNM classification, which is a pathologic staging system. There are three major staging systems for iCCA: 1) the American Joint Committee on Cancer/Union for International Cancer Control (AJCC/UICC), 2) the Liver Cancer Study Group of Japan (LCSGJ), and 3) the National Cancer Center of

Japan (NCCJ). However, none of them fulfils the criteria of an ideal staging system due to lack of correlation with prognosis. Therefore, a specific staging system has been recently proposed by the 7th edition of the American Joint Committee on Cancer (AJCC) (91).

On the other hand, perihilar cholangiocarcinoma can be classified based on two staging systems including the AJCC/UICC and the Memorial Sloan-Kettering Cancer Center (MSKCC) staging system. The former one uses pathological data and can provide patient prognosis; however, assessment of surgical treatment is not applicable. By contrast, the MSKCC failed in stratifying patient with resectable and unresectable CCA with adequate accuracy (74, 84). Another classification is the Bismuth-Corlette, which is not a staging system, but was created to help surgical management (92). Despite the fact that it does not provide any prediction on resectability, this classification describes the location of the tumour and its spread within the biliary tract tree. A new staging system was proposed by an international CCA group (93) that combines the Bismuth-Corlette classification, the TNM and MSKCC staging systems and includes new prognostic factors. Its purpose is to serve as a more accurate pre-operative staging system to be used in the surgical environment.

Currently, the only staging system for distal extrahepatic cholangiocarcinoma is the 7th edition of the AJCC/UICC staging system, which is solely of prognostic value although it will require further validation (91).

1.2.7 Surgical treatment of cholangiocarcinoma

The preferred treatment option for all the CCAs is surgical resection with negative tumour margins (R0). In some perihilar CCAs, liver transplantation is also a treatment of choice. For those patients presenting unresectable tumours, combination chemotherapy of cisplatin and gemcitabine may be considered, if available. However,

treatment options are limited in the case of intrahepatic CCA and are usually associated to high recurrence rate and short survival times (i.e. months rather than years) (78). Currently, the only strategy with potential curative value is surgery in patients with localised disease (84). Otherwise, chemotherapy and enrolment in clinical trials are alternative options that should be considered if available. As a first-line of treatment, gemcitabine plus cisplatin is given to patients with advanced disease because either the tumour is non-resectable or it has already spread (84).

Surgical resection is the treatment of choice for perihilar CCA in absence of PSC because it provides long-term survival (5-year survival is 20-30%) (84). However, negative tumour margins and absence of nodular involvement increases survival up to 45%. On the other hand, patients with pCCA and PSC might require a liver transplantation if the tumour is localised. Another alternative for early-stage pCCA patients with PSC but presenting non-resectable tumours is neoadjuvant chemoradiation plus liver transplantation (5-year survival of up to 72%) (84). For those patients with pCCA who are not candidates for either surgical resection or liver transplantation, chemotherapy with gemcitabine and cisplatin could be an option to consider (78). Valle and colleagues have proved that gemcitabine in combination with a platinum-based agent (e.g. cisplatin) increased survival of advanced biliary tract cancer patients (72).

Patients with distal CCA often require a Whipple procedure. The overall 5-year survival of dCCA is 27% after resection (R0), with a median survival time of 25 months (94). However, if surgical resection is not possible then improvement of biliary obstruction and administration of a combination of chemotherapeutic agents (cisplatin and gemcitabine) or 5-FU alone is provided (78).

1.2.8 Therapeutic treatment of cholangiocarcinoma

Regarding clinical trials, they were focused on testing gemcitabine and platinum-based agent(s) alone (cisplatin, oxaliplatin) as well as a combination chemotherapy using gemcitabine plus cisplatin or gemcitabine plus oxaliplatin (72, 95, 96). The combined chemotherapy with gemcitabine and a platinum-based agent was proved to be efficient and well tolerated in advanced biliary tract cancer patients so it was assigned as a standard treatment for patients with advanced biliary tract cancers. For that reason, Lee et al. tested this therapeutic combination with the addition of erlotinib, a tyrosine-kinase inhibitor of EGFR (epidermal growth factor receptor), in a phase III clinical trial and even a significant difference in progression-free survival between groups was not observed, it did show anti-tumour activity (97); therefore, this strategy might be another first-line treatment option to consider in patients with advanced CCA. However, these still have some limitations such as lack of population diversity with respect to ethnic origin that will need to be overcome.

Palliative care therapies are other alternatives of treatment offered to those patients with unresectable CCA tumours at the time of diagnosis. Usually this disease is diagnosed in elderly patients who also have comorbidities that preclude surgical resection (76). The purpose of these therapies is to relieve biliary obstruction and to ameliorate symptoms to improve patients' quality of life.

Taken together, there have been advances in the diagnosis and clinical management of CCA. However, it is necessary to develop better staging systems in order to correctly stratify cholangiocarcinoma patients and designed an individualised therapeutic plan given that CCA tumours should be treated differently depending on their location. Moreover, clinical trials still have some limitations that will need to be overcome.

1.3. Biliary tract cancer: Ampullary carcinoma

1.3.1 Epidemiology and statistics

Ampullary cancer or ampullary carcinoma is a rare type of tumour, accounting for less than 1% of gastrointestinal cancers and approximately 7% of periampullary cancers (98). It was first reported by Cattell and Pyrtek in 1950 (99). It derives from the Ampulla of Vater, a region of 1.5 centimetres or less, located in the last part of the common bile duct and the pancreatic duct where it traverses the duodenal wall and opens into the duodenal lumen.

1.3.2 Types of ampullary carcinomas

This type of cancer is often confused with periampullary cancers, which originate in other parts of the body such as the pancreas, the bile duct or the small bowel. The vast majority of ampullary cancers are adenocarcinomas although there is a variety of sub-types including papillary, adenosquamous and mucinous. There are two main histologic subtypes based on the epithelium of origin: 1) intestinal, which derives from the intestinal epithelium, and 2) pancreatobiliary, which originates from the distal common bile duct and distal pancreatic duct (100). This latter presents the worse prognosis.

1.3.3 Molecular and clinical characteristics of ampullary carcinoma

Patients normally present with symptoms related to biliary obstruction (i.e. jaundice) at an early stage when the tumour can be resected, hence, about 50% undergo curative surgery – typically a pancreatoduodenectomy (or Whipple procedure) (101). In general, these cancers have better survival rates than periampullary cancers (32-

68%); however, recurrence rate is high and resected patients will eventually die from this disease (102, 103).

Demeure and colleagues performed a whole-genome sequencing of an ampullary tumour and found genetic alterations in *KRAS*, *SMAD4* and *PTEN*. Mutations in *KRAS* were located in codon 12 as in the majority of PDAC tumours; however, the incidence of these mutations was low in ampullary carcinoma (25 to 37%) compared to PDAC (95%). A somatic mutation in *SMAD4* was observed and has also been previously reported in other studies to be present in ~50% of ampullary cancers. Moreover, *PTEN*, a tumour suppressor gene, was found to be deleted in a focal region in chromosome 10 (104).

1.3.4 Diagnostic of ampullary carcinoma

An accurate preoperative staging is essential and is based on numerous imaging modalities including computed tomography (CT), magnetic resonance (MRI), endoscopic ultrasonography (EUS), extracorporeal ultrasonography (US), magnetic resonance cholangiopancreatography (MRCP) and endoscopic-retrograde cholangiopancreatography (ERCP). The most commonly used imaging techniques are EUS and/or US, CT and MRCP because of their non-invasive technology and their availability (105). For tumour staging either EUS and/or intraductal US following TNM classification are used; however, intraductal US provides a superior resolution. To detect distant metastases is recommended to perform an MRI or a CT scan. It can show the location and extension of the tumour and the presence or absence of metastasis (106). It has a high accuracy in detecting disease of the abdominal cavity (105). Another technique is MRCP, which is highly accurate in detecting an obstruction and dilation of the biliary tract tree. Similarly, ERCP detects malignant biliary obstructions and can retrieve biopsy specimens and brush cytology samples for a

pathological diagnosis. However, this latter is more invasive and cannot distinguish the stage of the tumour (105). In summary, it is difficult to discriminate ampullary carcinoma from periampullary carcinoma and a carcinoma from the head of the pancreas. Therefore, a multimodality approach including standard blood biochemistry and tumour markers (i.e. CA19-9), usage of several imaging techniques such as abdominal US scan followed by CT, MRCP or ERCP should be carried out to diagnose the disease at an early stage.

1.3.5 Treatment of ampullary carcinoma

There is a lack of randomised clinical trials focused on ampullary carcinoma given that they represent a minority of gastrointestinal cancers (gastric and colorectal cancers). Thus, therapeutic treatment options are mainly derived from clinical trials conducted in pancreaticobiliary tract cancers in where a sub-group of patients present ampullary cancer. However, this sub-group is usually heterogeneous because it includes other types of pancreatic and biliary tract cancers and the sample size of each sub-group is too small, therefore, results from these clinical trials should be carefully considered.

Gemcitabine has been used as the standard treatment for pancreatic cancer and is commonly used in biliary tract cancers and advanced ampullary carcinomas. However, its efficacy as a monotherapy remains poor (95).

In 2009, a phase II trial was conducted in which capecitabine plus oxaliplatin (CAPOX) was evaluated in patients with advanced adenocarcinoma of the small bowel or the Ampulla of Vater. The results from this drug combination provided an overall survival (OS) of 50% and a median OS of 20.4 months, showing a 33% of response for ampullary carcinoma. A year later, a phase III clinical trial (ABC-02) investigated the combination of gemcitabine with cisplatin, demonstrating a significant advantage in patients with advanced biliary tract cancers and this therapy was recommended as an

appropriate option (72). Furthermore, another study evaluated the effectiveness of a chemotherapy combination between cisplatin, epirubicin, FU and gemcitabine (PEFG) in which ampullary carcinoma was the most responsive to this chemotherapeutic treatment with a median survival of 12.6 months (107).

The management of the resected ampullary carcinoma is followed by the administration of adjuvant chemotherapy, preferably gemcitabine, because a survival benefit from adjuvant chemoradiation has not been observed (108, 109). However, a new clinical trial called BILCAP evaluated the role of adjuvant chemotherapy with capecitabine after macroscopically complete surgical resection of a biliary tract cancer was performed (110). A total of 447 patients were recruited in the UK and they were randomly assigned to the surgery alone group (observation group) or to the capecitabine group. Capecitabine used as an adjuvant chemotherapeutic treatment following surgical resection of a biliary tract tumour improved overall survival (OS) in patients with BTCs: the median OS for capecitabine was 51 months, whereas for the observation group was 36 months. Therefore, the outcome from the BILCAP clinical trial is that adjuvant chemotherapeutic treatment with capecitabine following surgical treatment could be the standard of care for patients with biliary tract cancer (110).

In the case of locally advanced or metastatic stage, both chemoradiotherapy and chemotherapy are applied although there is no standard treatment yet. Generally, patients with advanced ampullary carcinomas receive an anti-metabolite (fluoropyrimidine and/or gemcitabine) plus a platinum agent (cisplatin, oxaliplatin).

1.4 Bile fluid

1.4.1 Bile fluid

Bile is a greenish yellow fluid produced by hepatocytes in the liver. They produce approximately 1 litre of bile every day, which consists of 95% water in where organic and inorganic complex solutes (5%) are dissolved. This fluid is slightly alkaline, with a pH of 7 to 8.

1.4.2 Bile function

Bile has a main function, which is to emulsify dietary fats and facilitate their intestinal absorption. It also neutralises hydrochloric acid present in the stomach before it passes into the small intestine (111). Nonetheless, there are other few functions which are also essential: 1) protects the organism from enteric infections by secreting inflammatory cytokines, immunoglobulin A (IgA) and stimulates the intestinal innate immune system, 2) it is the major excretory route for many molecules such as potentially harmful exogenous substances (i.e. heavy metals: arsenic, copper, manganese, lead, mercury, selenium, silver and zinc), hormones and cholesterol from the body, and 3) it is an essential component of the cholehepatic and enterohepatic circulation (111).

1.4.3 Bile formation and secretion

Bile is continuously produced and secreted from hepatocytes in the liver into the gallbladder and the common bile duct. The bile secretory system consists of a canalicular network composed by the apical membrane of adjacent hepatocytes and sealed by tight junctions. There are transporters (aquaporins) that create an osmotic

gradient within the bile canalicular lumen allowing the movement of the fluid into the lumen towards the portal tracts, which are bigger in size, that connect with the canals of Hering and the bile ducts (111). Once secreted into the biliary tree, the bile fluid can flow into the gallbladder, where it can be concentrated, stored or directly delivered to the common bile duct and then into the small intestine (duodenum). Hormones including cholecystokinin, gastrin and somatostatin, and also the vagus nerve control the amount of bile secreted into the duodenum.

1.4.4 Components of bile fluid

Bile is a highly complex secretion aqueous fluid, which contains less than 5% of solid (organic and inorganic) contents. For example, bile salts, cholesterol, bilirubin phospholipid, steroids amino acids, enzymes, porphyrins, vitamins, heavy metals, xenobiotics, exogenous drugs and environmental toxins are solid contents found in bile fluid (111). The most prevalent organic solutes are bile salts, which are large, negatively charged ions and are classified into four different types of free bile acids: cholic, chenodeoxycholic (CDCA), deoxycholic and lithocholic acid. The former two are synthesised by the liver and can be conjugated with glycine or taurine to form more complex acids and salts. The latter two are produced by intestinal bacteria which convert cholic acid into deoxycholic acid and CDCA into lithocholic acid. Bile fluid contains water, chloride, sodium and bicarbonates (111).

Phosphatidylcholine and cholesterol are the two most predominant phospholipid and sterol in bile fluid, respectively. Moreover, many proteins including albumin, haemoglobin/haptoglobin complexes, apotransferrin and FGF19 (fibroblast growth factor 19) are relatively abundant in bile. They access the bile via vesicular exocytosis at the canalicular membrane. Peptides and amino acids (glycine, cysteine and glutamic acid), vitamins (e.g. vitamin D, folic acid, pyridoxine and transcobalamin) as

well as hormones (oestrogens, insulin and steroids) are also found in bile. Furthermore, traces of other substances such as mucus, lecithin, fatty acids, serum proteins, neutral fats and urea can be found in bile fluid (111).

1.4.5 Studies based on bile composition

In the last decade, few studies have been carried out in which researchers investigated the composition of the bile fluid by performing proteomic and genome analysis. For example, Kristiansen and colleagues showed the first comprehensive proteomic study of human bile fluid using liquid chromatography and mass spectrometry (112). They identified a total number of 87 unique proteins in bile, which include proteins synthesised by hepatocytes such as enzymes (e.g. γ -glutamyl transferase), epithelial glycoproteins, transport proteins (e.g. transferrin, lactoferrin, ceruloplasmin) and proteins in the coagulation cascade (e.g. anti-thrombin and fibrinogen). Moreover, other known and unknown cancer-associated proteins were also found: mucin 16 and mucin 2; Mac-2, lipocalin 2 and DMBT1 (deleted in malignant brain tumours 1), respectively (112). In a different study, Chapman and colleagues carried out a whole genome RNA expression profiling in bile samples from cholangiocarcinoma patients. They discovered that bile contains high levels of mRNA expression, which include genes such as HOXA10, PU5F1, PVT1 and LEF1 (113).

These studies provide more information to the current knowledge of bile fluid components, suggesting that bile could be used as fluid for biomarker discovery and that gene expression profiling can be performed using RNA from bile. Bile fluid could serve as a source for early diagnosis of cancer in individuals with high-risk of developing biliary tract cancers (e.g. Hispanic communities and American Indians).

1.5 MicroRNAs

Non-coding RNAs (ncRNAs) are a class of RNA molecules that do not code for proteins (114) and have emerged as attractive therapeutic targets and diagnostic tools in cancer. There are different classes of ncRNAs that comprise a broad range of functions including splicing, chromatin architecture, translational inhibition and messenger RNA (mRNA) degradation such as ribosomal RNAs (rRNAs), transfer RNAs (tRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and yet-to-be discovered small regulatory RNAs (114). They also have important roles in epigenetic, developmental and physiological processes as well as in pathological conditions such as cancer (114).

MicroRNAs are small non-coding RNAs of 20-25 nucleotides (nt) in length that regulate gene expression at the post-transcriptional level by cleaving the mRNA or repressing its translation (115). MiRNAs are involved in RNA silencing also called RNA interference (RNAi), a physiologically conserved process that regulates gene expression (116, 117). This process is commonly shared by miRNAs and small interfering RNAs (siRNAs), where they function as guides of endonucleolytic cleavage of their RNA targets (115, 118). Several miRNAs can bind and regulate a single mRNA target; however, it is also feasible that a single miRNA binds to and controls many different mRNA targets (119).

Taken together, there is consensus in classifying a small RNA as a miRNA when these criteria are met: 1) the mature form of a miRNA can range between 20-24 nucleotides in length, 2) it has the ability to bind to duplex RNAs (i.e. mRNA) in an imperfect complementary manner (see section [1.5.4](#)), 3) its sequence should be evolutionary conserved, 4) it is expressed endogenously, and 5) it is expressed in particular cell types (120). Also, confirmation of its expression should be carried out by performing

molecular techniques such as reverse transcription quantitative polymerase chain reaction (RT-qPCR) or microarray, which are frequently used in this field (121).

1.5.1 The discovery of miRNAs

MicroRNAs were first discovered in 1993 by Victor Ambros and his colleagues Rosalind Lee and Rhonda Feinbaum. They discovered that *lin-4*, a gene known to control the timing of *Caenorhabditis elegans* (*C. elegans*) development, instead of coding for a protein produces a pair of small RNAs (22 and 61 nucleotides in length, respectively) (122). These small RNAs are complementary to a repeated sequence in the 3' untranslated region (UTR) in *lin-14*, resulting in a decrease in LIN-14 protein between the first and the second larval stage. Therefore, *lin-4* RNAs might downregulate *lin-14* by an antisense mechanism.

After the discovery of *lin-4* RNA there was no evidence of any similar non-coding RNAs in other species upon until *let-7* was identified by Reinhart and colleagues in 2000 (120). It was another *C. elegans* gene which encodes a small RNA of 21 nucleotides in length and that is complementary to elements in the 3'UTR of *lin-14*, *lin-28*, *lin-41*, *lin-42* and *daf-12* genes. They have proved that complementary sites in the 3'UTR of *lin-41* bind to *let-7* RNA during late and adult larval stages downregulating the activity of *lin-41* gene and, therefore, repressing the synthesis of LIN-41 protein (120). Moreover, homologs of *let-7* gene were found in human and fly genomes as well as *let-7* RNA (123). One difference between *let-7* and *lin-4* is that the former is conserved across bilateral organisms whereas the latter is only present in nematodes (123). Before being named microRNAs, Pasquinelli proposed to call small temporal RNAs (stRNAs) these new RNAs generated by *lin-4* and *let-7* but this was until over a hundred additional new genes for small non-coding RNAs were discovered just a year after: 20 new genes in flies (*Drosophila*), 30 in human and 60 in nematodes (124, 125).

Reinhart and colleagues discovered that plants also present miRNAs. They initially described 16 *Arabidopsis* miRNAs of 20-25 nucleotides in length detected by Northern blot and predicted that plant miRNA precursors are longer than animal miRNAs. They also suggested that the presence of miRNAs in plants indicates that they arose early in eukaryotic evolution (126).

1.5.2. The biogenesis of miRNAs

Previous evidence from earlier investigations indicated that based on their genomic locations, miRNA genes can be grouped as follows: 1) exonic miRNA located in non-coding transcription units, 2) intronic miRNA in non-coding transcription units, and 3) intronic miRNA located in protein-coding transcription units (121, 124). The vast majority of animal miRNA genes are transcribed by RNA polymerase II (Pol II), although Pol III can also transcribe a small group of miRNAs associated with Alu repeats (127, 128). This process occurs inside the nucleus and results in long primary miRNAs (pri-miRNAs) that contain a hairpin stem-loop structure, which are in turn processed by a multiprotein complex called the Microprocessor whose core components are the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha (129).

RNase III proteins are dsRNA-specific endonucleases classified into three classes based on their domain organisation. Class I enzymes contain one conserved RNase III domain and an adjacent dsRNA-binding domain and are generally found in yeast and bacteria. Class II enzymes include Drosha and its homologues and contain a tandem RNase III domains and one dsRBD. Class III has a tandem nuclease domain, a dsRBD, a PAZ domain and a putative helicase domain. Dicer enzyme is included in this latter class (130).

1.5.2.1 MicroRNA transcription and processing by Drosha

Drosha is a large protein (130-160 kDa) that possesses two tandem RNase III domains (RIIIDA and RIIDB) and a dsRBD (121). It interacts with a dsRNA binding protein called DGCR8 (DiGeorge syndrome critical region gene 8), in mammals, and Pasha in *Drosophila* and *C. elegans* (131). There has not been found any Drosha and/or DGCR8 homologues in plants nor in yeast (126).

DGCR8 and Drosha are components of a multi-protein machine named the Microprocessor (~ 500 kDa in *D. melanogaster* and ~ 650 kDa in humans) and are required in processing pri-miRNAs into pre-miRNA (131). The DGCR8 subunit is a protein encoded by the *DGCR8* gene whose deletion was originally identified in the DiGeorge syndrome chromosomal region 8 (DGCR8) in the human chromosome 22q11 (132, 133). DGCR8 is an evolutionary conserved protein of ~ 120 kDa that has two dsRBD near the C-terminal and a WW domain, which interacts with proline peptides, near the N-terminal (129, 131). The WW domain interacts with the surface of the amino terminus (N-terminal) domain of Drosha, which contains proline-rich peptides (129).

The two RNase III domains of Drosha (RIIIDA and RIIDB) form an intramolecular dimer where the two catalytic sites are in close proximity to each other. The C-terminal domain of DGCR8 interacts with the central region of Drosha and, seemingly, this complex orientates itself and recognises the terminal loop of the pri-miRNA. The RIIIDA cleaves the 3' strand whereas the RIIDB cuts the 5' strand, generating an intermediate transcript known as precursor miRNA (pre-miRNA) of 60-70 nucleotides in length. This precursor has an overhang of 2-3 nucleotides which seems to be a signature motif for miRNA processing (121, 134, 135).

1.5.2.2 Nuclear export by exportin-5

Next, the pre-miRNA is transported to the cytoplasm via a Ran-GTP mechanism in which Exportin-5 recognises this signature motif characteristic of the RNase III cleavage (130). Once in the cytoplasm, the pre-miRNA is cleaved by another RNase III enzyme termed Dicer, which is a cytoplasmatic multi-domain protein of ~ 200 kDa and it is highly conserved in all eukaryotic organisms (121). Dicer contains two RIIIDs, one dsRBD and a long N-terminal section that has a DEAD-box RNA helicase domain, a central RNA binding PAZ domain and a DUF283 domain (134, 135). The PAZ domain possesses a binding pocket that joins to protruding nucleotides at the 3' end of small RNAs (136).

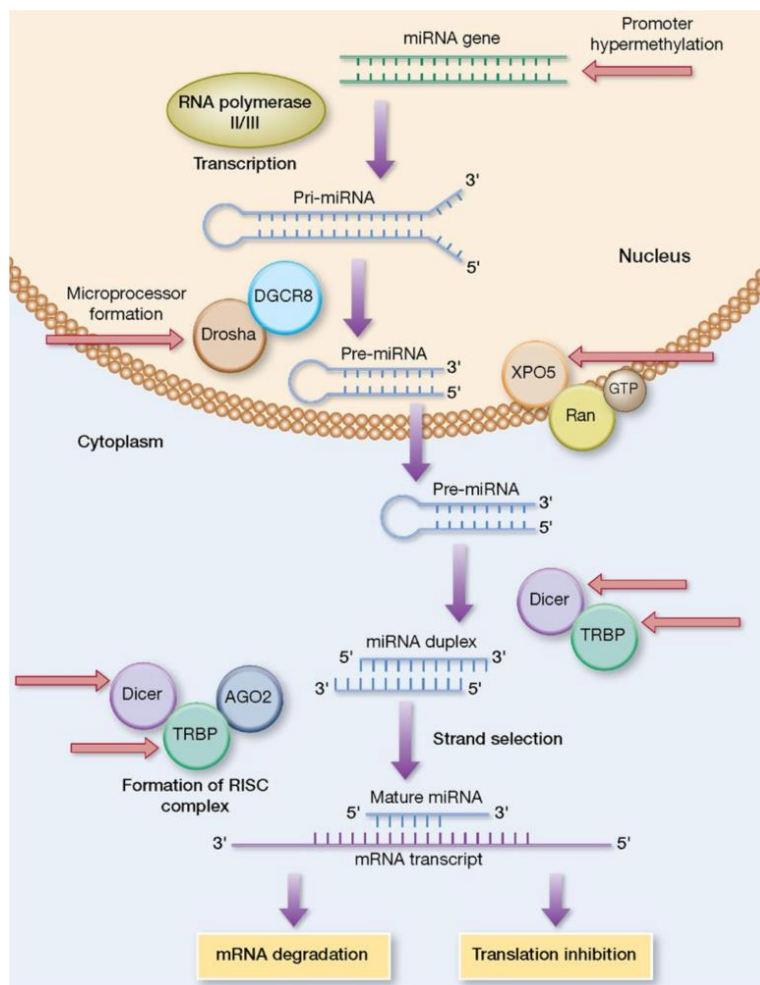


Figure 1.5. MicroRNA biogenesis in animals.

The primary microRNA (miRNA) is transcribed by the RNA polymerase II and the microprocessor (Drosha-DGCR8) cleaves it inside the nucleus, resulting in a precursor miRNA

(pre-miRNA) that is transported into the cytoplasm through Exportin-5. Once in the cytoplasm, Dicer in complex with TRBP cleaves the pre-miRNA generating a duplex miRNA. The passenger strand of the miRNA duplex is degraded and the functional strand is recruited by Ago2 and loaded into the RISC complex, which guides the RISC to silence target mRNA through mRNA degradation or inhibition of translation. *Reproduced from Mulrane et al., Cancer Research 73, 6554-6562 (2013).*

1.5.2.3 MicroRNA processing by Dicer

Dicer interacts with dsRBD proteins TRBP/Loquacious (TAR RNA-binding protein) and PACT (protein activator of PKR), which in turn recruit the Argonaute-2 protein (Ago2). All these proteins are associated together (Dicer-TRBP-Ago2) and in absence of ATP hydrolysis form a trimeric complex (RISC loading complex, RLC) that initiates the assembly of the RNA-induced silencing complex (RISC) (137). This complex, particularly RNase III Dicer, recognises the double-stranded pre-miRNA with special affinity for the 5' phosphate and the 3' overhang at the base of the stem loop (115). At this point, the pre-miRNA can follow two fates: 1) Dicer RIIIDa cuts the 3' strand whilst the RIIIDb cleaves the 5' strand of the precursor, producing a mature miRNA duplex (miRNA:miRNA*) of approximately 22 nucleotides (134) or 2) prior to Dicer cleavage of the pre-miRNA, Ago2 cuts in the middle of the 3' arm of the pre-miRNA (prospective passenger strand, miRNA*) generating a nicked ac-pre-miRNA that will be subsequently cleaved by Dicer eliminating the stem loop hairpin (132).

1.5.2.4 MicroRNA binding to the RISC complex

The active form of RISC, a ribonucleoprotein complex, only contains a single-stranded miRNA that guides it to the target mRNAs. Therefore, one strand (guide strand) of the RNA duplex is incorporated into the RISC complex due to its complementarity to the mRNA target, while the passenger strand (miRNA*) is subsequently degraded. This preference relies on the thermodynamic stability of the miRNA which determines which strand will be loaded into the RLC. Basically, the strand with relatively lower stability

of base-pairing at its 5' end will be loaded into RISC (118). Both strands are separated in the absence of ATP hydrolysis (115, 138, 139) and it is thought that the unwinding and removal of the unselected strand is mediated by an RNA helicase activity (135). The guide strand associated with Ago2 leads the RLC (RISC Loading Complex) to the RNA target and RISC recognises the target mRNA based on complementarity between the miRNA and the mRNA transcript (139). In cases of perfect or near-perfect complementarity to the miRNA, target mRNAs are cleaved and degraded; whereas in cases of imperfect base-pairing the translation is repressed (140).

1.5.3 MicroRNA biogenesis in plants

On the other hand, the biogenesis of plant miRNAs appears to differ from animals. First, mature miRNAs in plants seem to be produced from longer primary transcripts. Second, a Drosha homolog in plant has not been found yet. Third, mature miRNAs are processed inside the nucleus. Fourth, most of the plant miRNAs cleave their target mRNAs (141). Studies carried out by Reinhart, Papp and Hin Le (126, 141, 142), suggested that DCL1, one of the four Dicer family members in plants, could function as Drosha and Dicer-like homologues during the cleavage steps for the maturation of miRNAs in plants. The first step involves cleaving the pri-miRNA which generates a pre-miRNA of ~ 70 nucleotides. Next, a Dicer homolog (DCL1 or another enzyme) present in the nucleus makes a second cut resulting in the miRNA:miRNA* duplex that will be then exported into the cytoplasm by a plant ortholog of Exportin-5 (HASTY) (115). After these cleavage steps, plant and animal miRNAs share the same RNA silencing pathway steps.

1.5.4 MicroRNA target recognition

It is known that animal and plant miRNAs derive from endogenous, conserved genes and are processed from one arm of stem-loop precursors by Dicer or Dicer-like enzymes (126). However, they differ in their biogenesis and mode of target recognition. The mechanism whereby miRNAs pair with their target mRNAs to inhibit translation is different. In plants, miRNAs base pair to mRNA with a perfect or nearly perfect complementarity, whilst most animal miRNAs display an imperfect base pairing (143). Moreover, complementary sites in plants are mainly located within open reading frames (ORFs) and in 5'UTR regions but also in 3'UTRs, whereas in animals these sites are only within 3'UTR regions and present multiple sites within the same target mRNA (143).

In animals, the mechanism of target recognition between miRNAs and mRNAs might result in a perfect or imperfect base pair complementarity, which is only found on 6-8 nucleotides at the 5' end of the miRNA. This region is known as the miRNA *seed sequence* and comprises nucleotides 2 to 7. Sites that are complementary to the seed sequence are known as *canonical sites* and are evolutionary conserved across species (144). There are seven types of miRNA target sites divided into three groups: canonical sites, marginal sites and atypical sites (145).

Four types of miRNA target sites are seed-match sites that are selectively conserved; three of them belong to the canonical group (8mer, 7mer-m8 and 7mer-A1) and one to the marginal site group (6mer) (146). Canonical sites match the seed sequence but also pair with position 8 of the miRNA which is supplemented by a Watson-Crick pairing (7mer-m8) or have an adenine opposite position 1 of the miRNA (7mer-A1) or both (8mer). Marginal sites (6mer and offset 6mer site) match the seed region with only 6 nucleotides and generally have a reduced efficacy (147). When miRNA-mRNA interaction occurs at the 3' portion of the miRNA and it is centred on nucleotides 13-

1.5.5 Bioinformatic prediction and validation of miRNA targets

The functional characterisation of miRNAs relies on the identification of miRNA target genes. However, these mRNA targets are difficult to characterise given that each miRNA has multiple mRNA targets and *vice versa*. Despite the correct identification of miRNA-mRNA interactions remains a challenge; this problem can be address through experimental validation and computational analysis (148).

1.5.5.1 Experimental techniques for miRNA binding and target recognition

Experimental methods include genetic screening, RNA sequencing (RNA-seq) and techniques based on AGO crosslinking and immunoprecipitation (AGO-CLIP). Genetic screening is a general approach to characterise the functionality of a gene and has the advantage that the targets identified are already linked to a phenotype (120, 122). The only disadvantage is that it can provide direct and indirect targets and this method is difficult to perform in animals. Hence, complimentary approaches should be used. For instance, measuring the gene expression by RNA-seq (145) or identifying proteins affected by changes in the expression of miRNAs with an approach named SILAC (stable isotope labelling by amino acids in cell culture) (149, 150). Nevertheless, as with genetic screenings, SILAC methodology also produces direct and indirect miRNA targets and is not very precise on where are located the miRNA-binding sites: it does not identify a direct correlation and functionality. Another technique relies on the immunoprecipitation (IP) of AGO proteins to detect miRNA-mRNA interactions leading to discover individual miRNA target sites up to single nucleotide resolution (147). This technique comprises different AGO- and CLIP-based methods including AGO-IP (151), AGO-CLIP (152), PAR-CLIP (153), HITS-CLIP (154, 155), iCLIP (156) and CLASH (crosslinking, immunoprecipitation and sequencing of hybrids) (157). However, these approaches also have drawbacks and computational models from CLIP data such as microMUMMIE, PARma and MIRZA are still needed to certainly infer the specific

interaction between miRNAs and their targets (147). Furthermore, once miRNA/mRNA targets are identified the individual interaction of miRNA/mRNA has to be verified by performing experimental validation techniques including RT-qPCR, luciferase reporter assays and western blot as the most commonly used (158).

1.5.5.2 Computational techniques for miRNA target prediction

Computational prediction tools provide a rapid method to identify putative miRNA targets and require a miRNA sequence dataset when searching for miRNA-mRNA interactions (148). Thus, the more sequences added to this dataset, the more robust the tool will be to predict effective miRNA-mRNA interactions. Databases include Rfam, miRBase, TarBase or miRecords, which contain sequences from different species. In 2003, an up-to-date database called miRNA Registry (159) was created to store the RNA family sequences in which all new identified and published miRNAs from various species were annotated. This database assigns unique names to different miRNAs before they are published and provides a comprehensive and searchable catalogue available to the scientific community. Since then, the number of miRNAs annotated in, for instance, miRBase (<http://www.mirbase.org/>) rose from only a few hundreds to more than 38,000 miRNAs entries (160). However, it is important to bear in mind that not all sequences have been experimentally validated (only 25%) and the precise number of miRNAs is not established yet (148).

1.5.5.3 Types of bioinformatic tools

Bioinformatic tools can be classified into three groups according to the platform used: 1) web-based, which are more user-friendly allowing a simple data entry with few options, 2) downloaded programmes, which are also user-friendly and allow more options for processing data, and 3) packages, which are more complex requiring a knowledge in bioinformatics (148). Generally, computational methods are based on the seed region as the key determinant of miRNA specificity for miRNA target

prediction. These methods search for multiple conserved regions of miRNA complementarity within 3'UTRs and include TargetScan (119), EIMMo (161), miRbase (162), PicTaR (163), EMBL (<http://www.mirnas.ruselllab.org/>), miRanda (164) and miRMap (165). However, other bioinformatic methods use their own criteria for base pairing. For example, DIANA Tools is a web service that offers access to data resources for miRNA analysis and considers miRNA-recognition elements (MREs) for miRNA:mRNA binding (166). It is clear that each computational method has its own advantages and disadvantages. For instance, using packages and scripts offers a more precise target prediction for each particular case (148). Hence, it is recommended to use more than one computational method.

Taken together, the use of bioinformatics tools to find miRNA:mRNA target regulators is of fundamental interest amongst researchers. This could provide specific information to enable the construction of a complex regulatory network of mRNA-miRNA targets and highlight new genes that might be involved in tumourigenesis.

1.5.6 MicroRNA studies in cancer

It is known that pancreatic cancer and cancers of the bile duct are lethal diseases and present significant challenges, including lack of efficient diagnostic techniques and limited treatment options, which leads to a late detection and poor survival rates. Therefore, further investigation is needed to improve the detection process and the design of specific molecular therapeutic strategies. With the discovery of microRNAs and their involvement in the initiation and progression of human cancers, a wide range of studies focused on miRNA expression profiling have been reported with the aim of identifying signatures associated with staging, progression, diagnosis, prognosis and response to treatment.

1.5.6.1 MicroRNA profiling studies in cancer

A very ambitious project aimed to characterise the expression patterns of all miRNAs known until that moment. For the first time the expression of miRNAs across all cancers would be determined. Nonetheless, to carry out this study an accurate and inexpensive profiling method had to be designed. In 2005, Lu and colleagues developed a bead-based profiling method in where oligonucleotide-capture probes complementary to miRNAs of interest were coupled to carboxylated 5-micron polystyrene beads impregnated with variable mixtures of two fluorescent dyes, each representing a single miRNA (167). The beads were analysed with a flow cytometer capable of measuring bead colour and phycoerythrin intensity. Moreover, this platform allows the addition of new miRNA capture beads to enable the detection of novel discovered miRNAs. From this study, a differential expression of almost all known miRNAs across cancer types was observed as well as the clustering of miRNA profiles into two major branches according to their developmental origin (epithelial and haematopoietic), supporting the theory that patterns of expression of miRNAs codify the developmental history of human cancers. For instance, samples from pancreas, liver, colon and stomach clustered together, mirroring their common derivation from embryonic endoderm tissues. A further investigation led to the conclusion that miRNAs are generally downregulated in tumours compared to normal tissues (167).

A more challenging diagnostic differentiation was to address the cellular origin of tumours with uncertain diagnostic or histology. Successfully, Lu *et al.* classified poorly differentiated cancers and established the correct diagnosis with modest accuracy using miRNA expression profiles (167). They believed in the feasibility and potential diagnostic utility of monitoring the expression of miRNAs in human cancers given their extraordinary level of diversity across cancers.

Later, Volinia *et al.* performed a large-scale miRNA microarray analysis in a total number of 540 samples from six distinct solid tumours (168), concluding that miRNAs

control the expression of oncogenes and tumour suppressor genes, and are also involved in cancer pathogenesis.

Similarly, Bloomston and colleagues aimed to determine a miRNA expression pattern in pancreatic cancer (PC) and compare it with chronic pancreatitis (CP) patients and those with normal pancreas (169). They used a miRNA microarray to identify a global pattern of miRNA expression in PDAC. A common miRNA in all three groups was not observed, although chronic pancreatitis and normal pancreas showed a very similar expression pattern with a tendency of clustering together. These findings showed several up- and down-regulated miRNAs in PDAC compared to normal pancreas and chronic pancreatitis, meaning that a distinct miRNA expression pattern exists which can discriminate patients with PC versus CP and normal pancreas with an accuracy of 93% and 90%, respectively. Impressively, 95% accuracy was observed when comparing PDAC samples with both normal pancreas and chronic pancreatitis as a control condition.

The first comprehensive study on miRNA expression profiles in tissues from chronic pancreatitis, normal pancreas and PDAC was conducted by Szafranska *et al.* in order to identify miRNA candidates with a potential clinical use (170). The combination of microarray profiling and statistical analyses enabled the identification of differentially expressed miRNAs in PDAC compared to normal and/or cell line samples. In a different study, Szafranska and co-workers aimed to demonstrate the feasibility of using fine-needle aspirate (FNA) biopsies of PDAC to detect specific miRNAs able to distinguish malignant from benign pancreatic tissues (171). Previous data showed that miR-217 was found to be expressed in pancreas but low or absent expression was seen in pancreatic carcinoma, whereas miR-196 was directly related to neoplastic processes and was upregulated in PDAC (170). Interestingly, Szafranska *et al.* validated previous results in this study in where they observed an opposite expression of these two particular miRNAs (171). A healthy pancreas accounts for 90% of acinar

cells, so it has been suggested that miR-217 is produced in acinar cells due to its high expression in healthy pancreas, whilst miR-196 is only produced in ductal adenocarcinoma cells and not in healthy acinar or ductal cells. Therefore, they proposed a two-miRNA classifier: an acinar-cell specific marker (miR-217) and a PDAC-specific marker (miR-196a) to measure the proliferation ratio of ductal adenocarcinoma cells versus acinar cells. These results indicate that miR-196a correlates with a carcinoma or an intermediate/high-risk early lesion. Hence, this miRNA classifier could serve to assess the pathological behaviour of the pancreas in suspicious cases to obtain a certain diagnosis.

Ampullary carcinoma is a heterogeneous type of pancreatobiliary adenocarcinoma originating from the ductal epithelium and from the intestinal mucosa of the papilla. Despite the fact that ampullary carcinomas are biologically different from PDAC, Schultz and colleagues aimed to define the differences in the expression patterns of miRNAs between PDAC, ampullary carcinoma, chronic pancreatitis and normal pancreas using non-microdissected FFPE tissues (172). Additionally, another goal was to discover novel diagnostic miRNAs and miRNA combinations in cancer tissue. A commercial microRNA assay from TaqMan was used to carry out the miRNA profiling and, as a result, a 19-miRNA classifier was generated to distinguish pancreatic and ampullary carcinomas from chronic pancreatitis and normal pancreas, which exhibited a high sensitivity and accuracy. Furthermore, a three-combination of two miRNAs (miR-198 and miR-411, miR-122 and miR-614, miR-93 and miR-614) significantly differentiates PDAC and ampullary carcinoma from CP and normal pancreas (172). Nevertheless, these combinations and the miRNA classifier model should be validated prospectively.

Cholangiocarcinoma and pancreatic adenocarcinoma are two types of cancers that have overlapping immunohistochemical profiles and are indistinguishable from the clinical and histopathological point of view. Knowing this, Collins and colleagues

addressed for the first time the difference between cholangiocarcinoma and PDAC by developing a miRNA profile in order to be able to discriminate these two types of cancers at the molecular level (173). In contrast to the study carried out by Schultz and colleagues (172), they used micro-dissected paraffin-embedded tissues from both tumour types and matched benign tissue. They discovered several deregulated miRNAs in cholangiocarcinoma and pancreatic adenocarcinoma relative to the adjacent normal tissue in the bile duct epithelium and the pancreas, respectively. These findings expose that both cancers have a distinct miRNA profile, which supports previous studies where comparisons between PDAC and other types of cancers also showed a different miRNA expression profile (170, 172, 174).

Recently, a PCR-based multiomics molecular platform that simultaneously analyses mRNA and miRNA expression patterns has been used to differentiate benign from malignant pancreatobiliary cancers using a minimal amount of biopsy material (175). This platform is based on the TaqMan microfluidic array technology and contains customised mRNAs and miRNAs including 79 mRNA markers, 5 mRNA reference genes, 9 miRNA markers and 2 small RNA reference genes (176). The results from this study indicated that this technology might serve as a suitable molecular diagnostic tool to differentiate patients with a suspect solid pancreatic mass from benign lesions in a very accurate manner, presenting an excellent sensitivity, specificity and accuracy (91.7%, 94.5% and 93%, respectively). Furthermore, the combination of mRNAs and miRNAs as biomarker classifiers was superior to previous reported miRNA markers alone.

To summarise, an extensive characterisation of the miRNA expression profile across all cancers was performed. Poorly differentiated cancers were also classified according to this. Different types of cancers displayed a distinct miRNA expression profile, which allowed discriminating with high accuracy, sensitivity and specificity

PDAC from pancreatobiliary malignancies such as cholangiocarcinoma and ampullary carcinoma.

1.5.6.2 MicroRNA expression profiles in pancreatic cystic lesions and in PanIN lesions

There have been several studies demonstrating the potential of miRNAs in differentiating between benign and malignant pancreatic cyst tumours which include MCNs and IPMNs (174, 177-179), suggesting that miRNA signatures could accurately diagnose pancreatic cysts with a higher sensitivity and specificity than performing a cytological analysis. For instance, Lubezky *et al.* found that up-regulation of miR-21, miR-155 and miR-708 and decrease of miR-217 occurred during IPMN malignant transformation (174). However, these miRNA signatures have not been translated into the clinical setting yet.

Similarly, a few studies assessing the expression of miRNAs in early pancreatic neoplasia have been carried out. For example, Du Rieu and Yu performed a miRNA profile study where they found that miR-21 was overexpressed with increasing PanIN grade lesions (180, 181). Moreover, Xue and colleagues discovered that miR-148a and miR-217 are down-regulated in PanIN 2-3 whereas miR-10b and miR-196 are up-regulated (182). It seems that a miRNA signature that characterises high-grade PanIN lesions is emerging (180-182).

1.5.6.3 Biomarker discovery using miRNAs from liquid biopsies for pancreatic cancer

Currently, the only biomarker routinely used for the management of PC is CA 19-9, however 5% of the population do not express this marker (42), it has a variable specificity and sensitivity (85%) to detect PC and due to low prevalence of the disease it is not applicable for screening nor relevant to confirm diagnosis. Therefore, sensitive and specific biomarkers for early detection and diagnosis of pancreatic cancer are

essential due to the limited value of CA 19-9 and its inability to discriminate malignant from benign disease. During the last decade, the discovery of novel blood-based diagnostic biomarkers for cancer detection has been investigated. For instance, in 2008 Mitchell *et al.* demonstrated the feasibility of using a blood-based miRNA biomarker for cancer detection using both clinical serum samples from patients with prostate cancer and a xenograft mouse model (183). Furthermore, they confirmed the presence of circulating miRNAs in plasma. They profiled the expression of 365 miRNAs in human prostate cells using a microfluidic TaqMan low-density microRNA array. Two miRNAs were identified in human prostate cells but mouse homologues were not found, thus, it was hypothesised that they might be tumour-specific. The results revealed that these two miRNAs (miR-629 and miR-660) were detectable in all xenografted mice but not in control mice and the levels of these miRNAs in plasma correlated with tumour mass in each mouse. Moreover, they discovered that miR-141, miR-200b and miR-200c were expressed in prostate epithelial cells but were absent in prostate stromal cells. Additionally, serum levels of miR-141 can discriminate patients with prostate cancer from healthy individuals with significant specificity and sensitivity. In a different study, Wang *et al.* (2009) addressed the accuracy of developing a blood-based biomarker for pancreatic carcinoma that could distinguish PDAC patients from healthy subjects using a panel of four miRNAs (miR-21, miR-155, miR-196a and miR-210) that were previously reported in other studies to be implicated in PDAC (169, 170, 184). Nonetheless, these previous studies used tissue samples instead of biological fluids to evaluate the utility of a miRNA signature for screening and early detection of pancreatic cancer. Results from the study carried out by Wang and colleagues indicated that this panel of four miRNAs in plasma differentiate healthy individuals from PDAC patients with a moderate sensitivity and specificity. Despite limitations in the sample size of this study, these findings provided support towards the development of a specific and sensitive blood-based miRNA biomarker for PDAC.

Previous studies from Lee (2007) and Szafranska (2007) have reported variations in miRNA expression in samples from pancreatic tumour, chronic pancreatitis and normal pancreas (170, 185). Following this, Bauer and colleagues assessed the power of miRNA signatures for diagnosis in blood samples compared to pancreatic tissue samples (186). A total of 245 samples were profiled: 129 samples from tissue and 116 samples from blood, distributed in three groups of patients (PDAC, CP and control). The results showed an overlap of differentially expressed miRNAs in blood and tissue samples, indicating a functional relationship between them. MicroRNAs in both blood and tissue exhibited an excellent accuracy in discriminating healthy individuals and patients with CP and PDAC. However, discrimination between PDAC patients and patients with CP was low in blood (Area Under Curve (AUC) < 0.70) compared to tissue (AUC > 0.80). These findings demonstrated that effective blood-based miRNA biomarkers distinguish malignant lesions from benign disease; nonetheless, biomarkers that differentiate between pancreatic lesions are still needed.

Another study designed by Schultz and colleagues (2014) analysed whole blood from patients with PDAC, CP and healthy individuals with the purpose of describing differences in their miRNA expression as well as discovering a panel of miRNAs to be used in pancreatic cancer diagnosis (187). In this large case-study, two novel panels of miRNAs (index I and index II) were developed for pancreatic cancer diagnosis, which are composed of 4 or 10 miRNAs, respectively. These indexes can discriminate, to a certain degree, PDAC patients from healthy controls, although they did not provide a significant improvement over serum marker CA19-9. However, a combination of both indexes with CA19-9 could be useful in cases where CA19-9 is normal.

A few years ago, a Japanese group analysed the expression of more than 2,500 miRNAs in serum samples from patients with PDAC, biliary-tract cancer, other digestive cancers, patients with non-malignant disease and healthy participants using a highly sensitive microarray called “3D-Gene” (188). Two different cohorts were

established (test and training cohorts) and with the expression profiles of the ten most significant miRNAs a diagnostic index was developed. As a result, a high sensitivity, specificity and accuracy was reached by combining eight miRNAs (80.3%, 97.6% and 91.6%, respectively). This combination was able to identify pancreatobiliary patients from patients with other types of cancer or non-malignant abnormalities and healthy subjects with a very good performance.

Recently, Puik and colleagues conducted a study focused on profiling plasma samples from patients with distal CCA and healthy individuals to identify a panel of miRNAs capable of accurately diagnosing and differentiating distal CCA from PDAC. They have discovered a three-miRNA plasma-based signature that can discriminate malignant from benign disease with high discriminatory power (AUC = 0.881). Curiously, two of these miRNAs could also distinguish distal CCA from PDAC with an AUC of 0.814 (189). These results suggest the possibility of using a combined panel of plasma miRNAs to diagnose distal CCA and aid in decision-making for those patients with pancreatobiliary diseases.

It has been observed that biological fluids such as blood (187), serum (190), plasma (183, 191, 192), saliva (193, 194), urine (195), cerebrospinal fluid (196) (CSF), breast milk and seminal fluid (197) carry miRNA species that might be specific for a particular type of cancer. The discovery of miRNA panels that distinguishes benign from malignant pancreatic lesions with high accuracy is of special importance. Hence, developing a sensitive and specific miRNA signature for PDAC that could serve as a potential biomarker for early diagnosis is essential.

1.5.6.4 MicroRNA expression profiles using bile fluid in pancreatic ductal adenocarcinoma and biliary-tract cancers

The vast majority of the studies investigated the potential utility of a sensitive and specific blood test as a non-invasive biomarker for early detection of pancreatic cancer

as it would be very valuable given that it is challenging to obtain useful tissue biopsies from patients with suspicious pancreatic cancer. It is known that circulating miRNAs can be detected in blood, however, they can originate from distant sites/organs and it has not been proved that circulating miRNAs found in blood are originated from patients with a pancreatic malignancy. Therefore, developing a blood-based test for a particular type of cancer, concretely for PDAC, might be less specific as if, for example, another biological fluid such as bile is taking into consideration. Therefore, other researchers assessed the capability of bile fluid as a potential biomarker for early diagnosis of PDAC given its proximity to the malignant lesion.

For instance, Gregory Cote and co-workers carried out a study where the utility of miRNAs as diagnostic biomarkers to discriminate PDAC from CP and healthy subjects was assessed in plasma and bile samples (191). A selection of ten candidate miRNAs was based on previous studies where an association to PDAC was described. Eight miRNAs (miR-10b, miR-30c, miR-106b, miR-155, miR-181a, miR-181b, miR-196a and miR-212) were significantly different across groups and between individuals with CP and PDAC in plasma samples, whereas only five miRNAs were significantly different across groups in bile samples (miR-10b, miR-30c, miR-106b, miR-155 and miR-212) and between PDAC and controls two more miRNAs were significantly different (miR-181a and miR-196). These five common miRNAs in plasma and bile (miR-10b, miR-30c, miR-106b, miR-155 and miR-212) showed an excellent accuracy in differentiating PDAC from CP and controls. Thus, these five miRNAs were selected to create a PDAC signature panel in plasma and bile that was further validated in a training cohort, in where this panel exhibited an excellent accuracy and specificity to diagnose PDAC. Therefore, these findings indicated that a plasma and bile miRNA signature could be used as a non-invasive diagnostic test for PDAC or for evaluating and treating pancreatobiliary strictures after an ERCP procedure, respectively (191).

Similarly, Voigtländer *et al.* evaluated the miRNA profile of patients with primary sclerosing cholangitis (PSC) and patients with cholangiocarcinoma (CCA) in serum and bile (190). They performed a global miRNA profiling and discovered significant differences in serum and bile miRNAs between PSC patients and CCA patients (miR-26a, miR-30b, miR-122, miR-126 and miR-1281; and miR-412, miR-640 and miR-1537 and miR-3189 respectively). Although the sensitivity to discriminate between patients with PSC and CCA was low (50-67%), both panels of serum and biliary miRNAs showed a good accuracy (AUC > 0.80). Nonetheless, these results need to be validated in larger cohorts before using them for diagnostic purposes.

In another study, Li and colleagues aimed to characterise human biliary extracellular vesicles and their miRNA content (198). This study was the first investigating the presence of extracellular vesicles (EVs) in human bile, although other researches have hypothesised about it in previous studies given the remarkable stability of miRNAs in biological fluids. Their results concluded that EVs contained miRNAs and a five-miRNA panel (miR-191, miR-486, miR-1274, miR-16 and miR-484) could be used to diagnose cholangiocarcinoma as it exhibited a superior performance than CA 19-9 and any current diagnostic methodology used in clinical practice.

Taken together, these studies showed the feasibility of using bile fluid as a source of miRNAs for developing a miRNA-signature capable of differentiating pancreatic and biliary tract cancers from benign disease. This bile-based biomarker could be used in a clinical setting to evaluate and treat strictures in the biliary tract system and classify those patients with an uncertain diagnosis for PDAC or biliary-tract cancers.

1.6 Exosomes

1.6.1 Extracellular vesicles

Originally, the term exosome was used to name vesicles of 40-1,000 nm in size released by a diversity of cultured cells that carried 5'-nucleotidase activity (199). However, this terminology was adopted in 1983 by both Rose Johnstone's group (200) and Philip Stahl's group (201) who were investigating the maturation of reticulocytes into red blood cells. They described the existence of extracellular vesicles (EVs) that could fuse with the plasma membrane and release their contents. The term exosomes was proposed later, in 1987, to define these EVs of endosomal origin generated by the fusion of multivesicular endosomes (MVEs) or multivesicular bodies (MVBs) with the plasma membrane (202). Almost a decade later, Raposo and Zitvogel showed that immune cells such as B lymphocytes and dendritic cells (DCs) also release exosomes (203, 204).

Extracellular vesicles are formed of a lipid bilayer that contains transmembrane and cytosolic proteins, lipids and nucleic acids (i.e. RNA). Their size range from 40 to 500 nm (up to 2,000 nm) and are highly heterogeneous. Currently, extracellular vesicles are divided into two main groups: exosomes and microvesicles, which are subdivided into different subtypes (**Figure 7**).

1.6.2 Exosomes

Exosomes are intraluminal vesicles (ILVs) generated by inward budding of the endosomal membrane during maturation of MVBs and are secreted by fusing with the plasma membrane of the cell (205). Their size ranges from 30 nm to 150 nm and are involved in intercellular communication between distinct types of cells.

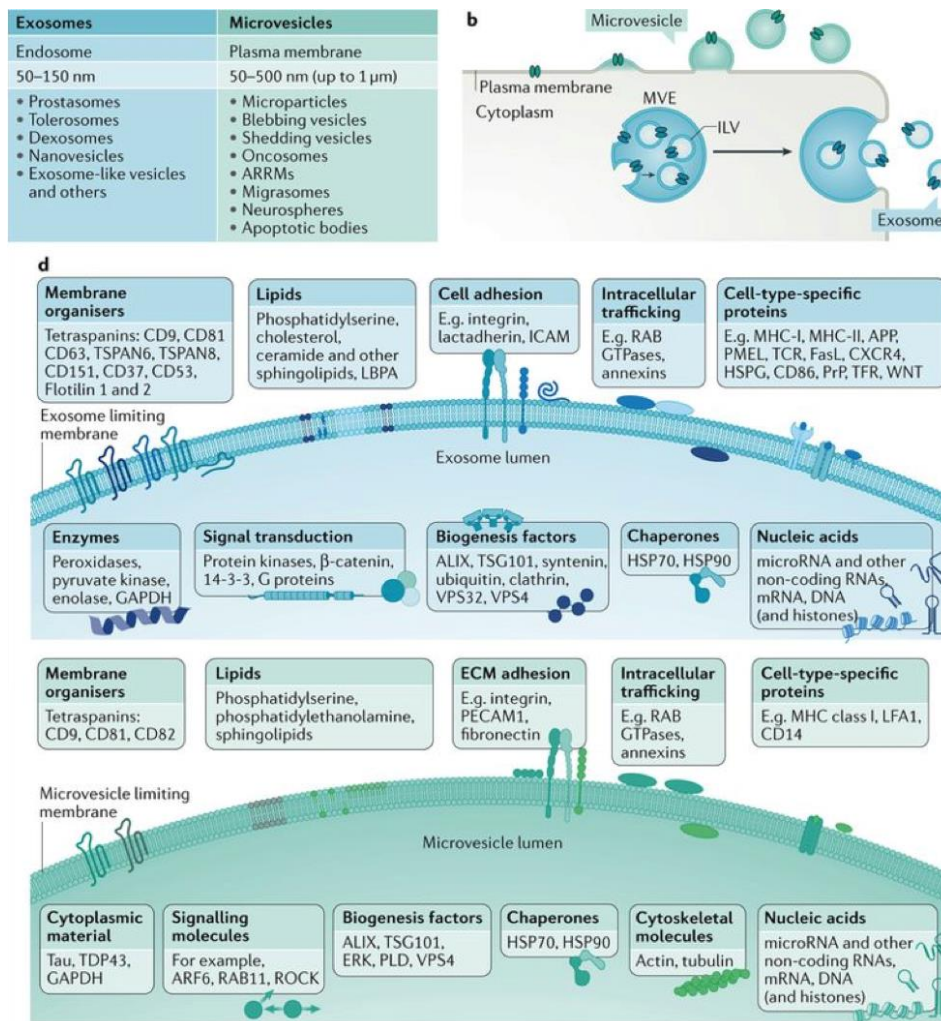


Figure 1.7. Characteristics of extracellular vesicles: exosomes and microvesicles.

Extracellular vesicles (EVs) are a heterogeneous population of membrane vesicles of diverse origin. Their size varies from 30-50 nm to 500 nm or even larger (1-10 µm). EVs are divided into two classes: exosomes and microvesicles. **B)** Formation of extracellular vesicles. **D)** Composition of extracellular vesicles. They contain a diversity of cargoes which include proteins, lipids and nucleic acids. *Reproduced from Van Niel et al., Nature Reviews Molecular Cell Biology 19, 213-228 (2018).*

1.6.2.1 Composition of exosomes

They contain in their cargoes proteins, lipids and nucleic acids (e.g. DNA, mRNA and miRNA). They are enriched in lipids including sphingomyelin, phosphatidylserine, cholesterol and ceramide. Several studies have reported that exosomes contain specific sequences of mRNA and miRNA that are preferentially retained inside the

cells or secreted/exported and this can affect gene expression in distant cells (206, 207). Furthermore, exosomes also contain specific groups of proteins depending on the type of cell. Nevertheless, the most predominant proteins belong to endosomes, the plasma membrane and the cytosol, whilst other proteins from the nucleus, the Golgi complex or the endoplasmic reticulum are less abundant (208).

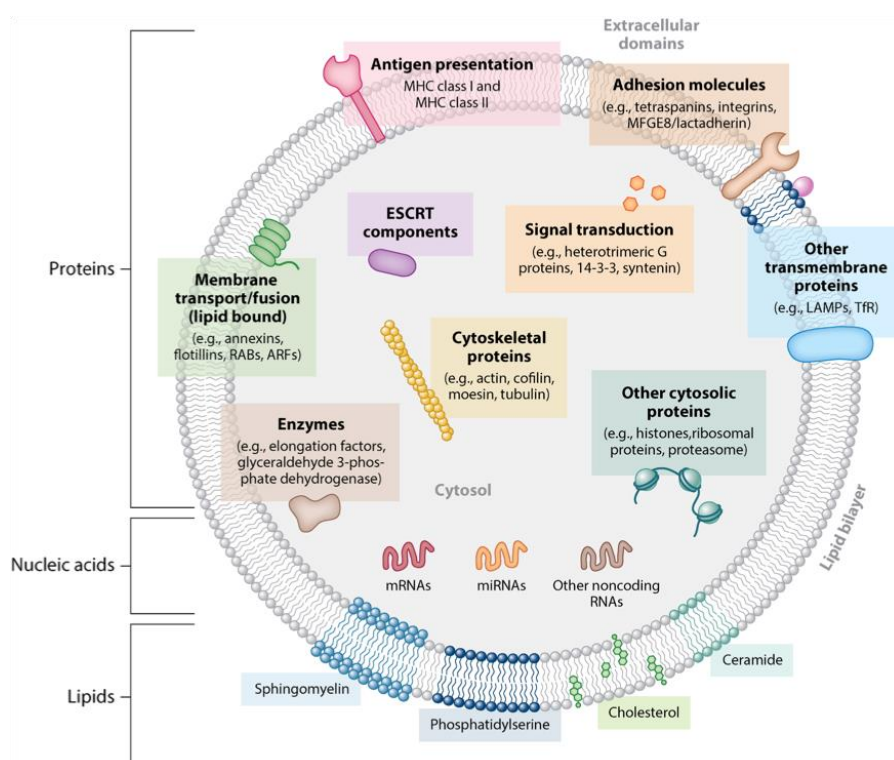


Figure 1.8. Composition of extracellular vesicles.

Schematic representation of the composition and membrane orientation of extracellular vesicles. *Reproduced from Colombo et al., Annual Review of Cell and Developmental Biology* **430**, 255-289 (2014).

1.6.2.2 Biogenesis of exosomes

Exosomes are a specific subtype of extracellular vesicles formed in endosomal compartments (i.e. MVBs). The formation of exosomes involves inward budding of MVBs, which form small internal vesicles (exosomes) that contain cellular proteins, lipids and nucleic acids that are free floating in the cytoplasm. When these MVBs fuse with lysosomes their contents will be degraded; however, if the fusion occurs with the

plasma membrane, these internal vesicles (exosomes) are released and can travel until they reach their destination(s), generally determined by specific binding to ligands on the cellular surface of target cells. This binding could occur by releasing EVs contents' directly into the cytoplasm of the target cell or by an endocytic mechanism in which the target cell incorporates these EVs inside (209).

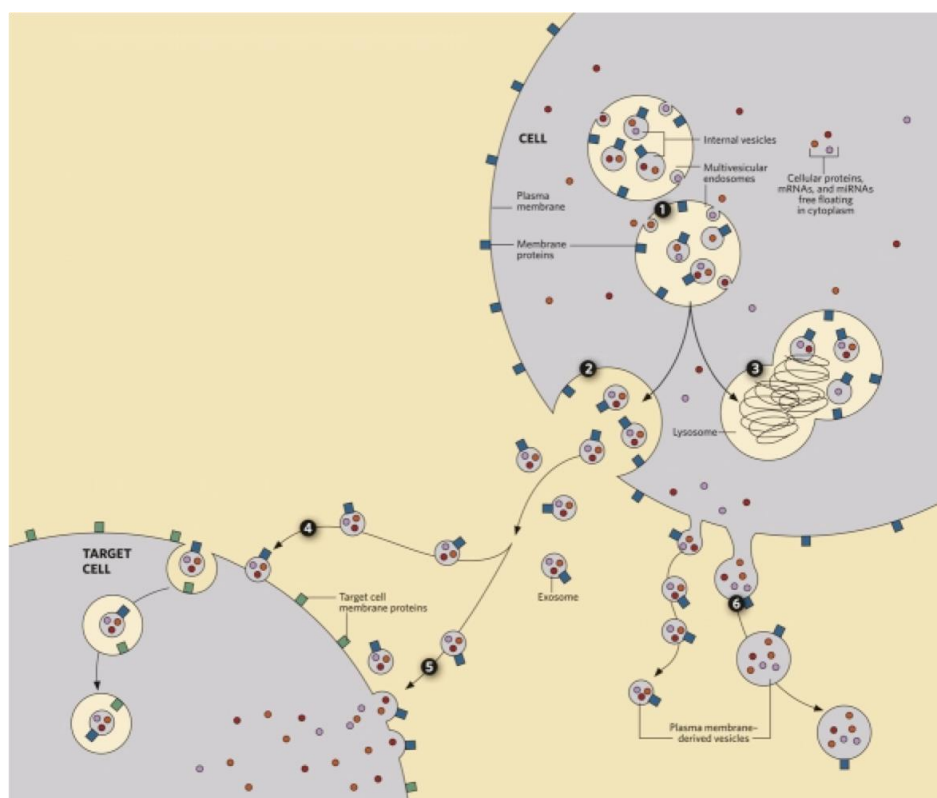


Figure 1.9. Biogenesis of exosomes.

Exosomes are small membrane vesicles secreted by most cell types. Inward budding of multivesicular endosomes (MVE) form small internal vesicles that contain proteins, nucleic acids and lipids from the cytoplasm. MVE can fuse with lysosomes or alternatively, these internal vesicles (exosomes) can be released when MVEs fuse with the cell membrane. Then exosomes can enter target cells via endocytic pathway or by fusing with the target cell membrane and releasing its content. *Reproduced from Thery et al., F1000 Biology Reports 3, 1-8 (2011).*

The process of biogenesis of exosomes requires particular sorting machineries, in which the endosomal sorting complex required for transport (ESCRT) is the main player (210). ESCRT contains four complexes (ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III) and additional proteins associated. ESCRT-0 is the complex responsible

for recognising and recruiting ubiquitinated lipids and transmembrane proteins (cargoes) in the endosomal limiting membrane. ESCRT-0 is connected to ESCRT-I and together assemble cargoes in membrane microdomains of the limiting membrane of MVBs. ESCRT-II helps in the recruitment of ESCRT-III, a dynamic complex that promotes membrane budding followed by a vesicle neck constriction which drives vesicle scission through the assembling of individual subunits to the limiting membrane of MVBs. The accessory protein VPS4 ATPase allows dissociation and recycling of the ESCRT machinery (211, 212). In contrast, other studies revealed that exosomes could also be generated via an ESCRT-independent mechanism in which lipids, tetraspanins or heat shock proteins are involved (213). However, ESCRT-III is required for releasing exosomes into the MVB lumen, therefore, this complex is shared by both mechanisms.

In summary, both ESCRT-dependent and ESCRT-independent mechanisms contribute in the biogenesis of exosomes.

1.6.2.3 Secretion of exosomes

Proteins of the Rab family are regulators of intracellular vesicle trafficking and are involved in exosome secretion. They control different stages of intracellular vesicle transport to the plasma membrane. SNARE proteins (soluble N-ethylmaleimide-sensitive fusion attachment protein (SNAP) receptor) together with other SNAPs create a complex that enables the fusion of both the MVB membrane and the plasma membrane, resulting in the secretion of exosomes (214). RAB11 seems to be associated to recycling, whereas RAB35 acts in early endosomal sorting, and RAB27A and RAB27B are associated to late endosomal and secretory compartments, respectively (215). Therefore, different RABs are involved in different steps of endosomal maturation along the secretory pathway.

1.6.3 Methodology to purify and isolate exosomes

It is important to bear in mind that there is no standardised methodology to purify, isolate and characterise exosomes. Each method has its own advantages and disadvantages and researchers choose the preferred technique(s) based on the type of starting sample material and the downstream experiments that will be performed afterwards.

The starting sample material can be cell cultured medium or a biological fluid (blood, serum, plasma, ascites fluid, urine, saliva, bile, cerebrospinal fluid, milk and seminal fluid). Each sample will have different viscosities and will require an adaptation of the standard protocol because the rate and the duration of some steps will affect the efficiency of the technique. For instance, high-viscosity fluids such as plasma, serum, semen and bile should be diluted to decrease their viscosity; otherwise, they will have a lower sedimentation efficiency (216).

1.6.3.1 Exosome isolation techniques

Different methods to isolate extracellular vesicles have been described, being the following the most common: differential centrifugation (plus optional filtration step), density gradient, immunoaffinity-based technique (coated magnetic or latex beads), microfluidic devices and commercial kits.

The gold standard method to purify exosomes is differential centrifugation. This technique is characterised by two initial low-speed centrifugation steps to remove cells, dead cells and debris (300 x *g* for 10 minutes at 4°C and 2,000 x *g* for 10 minutes at 4°C, respectively). An optional filtration step is followed using a 0.2 µm or 0.8 µm filter, especially in those cases where RNAs are planned to be analysed – mainly to avoid contamination with large particles or additional debris. Then, a final low-speed centrifugation step at 10,000 x *g* for 30 minutes at 4°C is carried out. The final supernatant is then ultra-centrifuged at 100,000 x *g* for 1.5-2 hours at 4°C to separate

small and large extracellular subpopulations. The pellet obtained in this ultracentrifugation contains exosomes and contaminating proteins, therefore, and additional centrifugation at the same high speed and multiple washings with PBS (Phosphate Buffered Saline) are recommended (217). Especial attention to viscous biological fluid mentioned before where higher centrifugation force and time plus increasing dilutions in PBS should be performed (218).

One disadvantage of using ultracentrifugation to isolate exosomes from body fluids is that they contain EVs from multiple cell types and differential centrifugation does not differentiate EVs subtypes and cells of origin (219). Thus, immunoaffinity-based techniques can be helpful for this particular aim. Basically, specific antibodies are coupled with magnetic beads allowing the isolation of a more homogenous exosomal profile (220). Similarly, sucrose density gradient centrifugation is another technique to purify exosomes and other extracellular vesicles from contaminants effectively, especially when immunoaffinity capture is limited because of antibody availability and suitability of exosomes markers (221). The density range at which exosomes have been found to float is 1.15 g/ml to 1.19 g/ml (217). Additionally, microfluidic devices might overcome the disadvantages of the current exosome isolation techniques including immunological separation, trapping and filtration. These devices showed a higher efficiency, specificity, purity and simplicity (222).

Rekker and colleagues did a comparison between two techniques to isolate exosomes from serum: ultracentrifugation and a commercial kit called ExoQuick (Systems Biosciences, SBI). They concluded that both methodologies are suitable to perform an exosomal miRNA profiling using serum from patients (223). Commercial kits are an alternative to isolate exosomes from small volumes of samples using chemical reagents, particularly when there is a limitation with the starting sample material.

1.6.3.2 Exosomes characterisation

Once exosomes are isolated it is important to verify that the purified material are exosomes and no other extracellular vesicles, therefore, assessment of their content in terms of proteins, nucleic acids, lipids as well as extracellular protein surface markers is performed.

There are various techniques that can characterised exosomes based on their shape and size including electron microscopy (EM), atomic force microscopy (AFM), nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) analysis and qNANO GOLD (216). Furthermore, there are several methods available to differentiate exosomes from other extracellular vesicles based on exosome markers which include liquid chromatography, mass spectrometry, immune-blot analysis, flow cytometry, western immune-blotting and ELISA technique (216). These molecular tools assess the presence of specific molecules from the exosome limiting membrane and/or from the exosome lumen such as tetraspanins (CD9, CD63, CD81, Flotilin1 and 2), lipids (cholesterol, sphingolipids, ceramide), integrins (Ep-CAM), chaperons (HSP70, HSP90), enzymes, biogenesis factors (ALIX, TSG101, VPS4), nucleic acids (miRNAs, mRNAs, DNA) and cytoskeletal molecules (205). However, other extracellular vesicles (i.e. microvesicles) display a common composition in their luminal and extracellular space. For that reason, it is crucial to detect those molecules that are solely specific for a subtype of extracellular vesicles.

1.6.4 The role of exosomes in cancer

Caby and co-workers showed for the first time the presence of exosomes in plasma blood from patients (224). These results provide strong evidence that blood cells secrete exosomes and that its isolation from blood fluids is possible. It also supports the hypothesis that exosomes are involved in intercellular communication. Later,

Valadi *et al.* published a remarkable study demonstrating exchange of nucleic acids between cells via exosomes (225). They were the first to discover that different miRNAs were present in exosomes, suggesting that exosomes transport both mRNA and miRNA when they are released from host cells. They transferred mouse exosomal RNA into human mast cells and they found that new mouse proteins were generated in recipient cells, which supports the hypothesis that exosomes are involved in intercellular communication, delivering RNA and modulating the activity of other recipient cells.

Melo *et al.* discovered that circulating exosomes (crExos) expressing Glypican-1 (GPC1+) could serve as an accurate biomarker for early detection of PDAC. They reported that crExos GPC1+ discriminate PDAC from benign pancreatic disease and healthy controls with a near perfect sensitivity and specificity (226). In contrast, Lai and co-workers exposed that an exosomal miRNA signature comprising elevated levels of miR-10b, miR-21, miR-30c miR-181a and low levels of let-7a was superior to exosomal GPC1 or plasma CA 19-9 levels in establishing a diagnosis for PDAC as well as discriminating between PDAC and CP (227). Furthermore, Frampton *et al.* have not observed a significant difference in GPC1 levels between normal pancreas and PDAC tissue. They were unable to differentiate PDAC from benign pancreatic disease using circulating exosomes that expressed GPC1 (228). However, a significant decrease in crExos expressing GPC1 was exposed when comparing pre-operative and post-operative plasma samples from PDAC patients. Additionally, patients with high levels of crExos containing GPC1 showed significantly larger PDAC tumours, and these results are in line with findings by Melo and colleagues (226, 228). Thus, high expression of GPC1 in crExos might determine tumour size and disease burden.

In 2010, Webber and co-workers led a study about the effect of exosomes on target cell responses and they discovered that exosomes derived from tumour cells may be

implicated in inducing pre-metastatic changes resulting in the conversion of fibroblasts into myofibroblasts (229). They were the first to evidence that fibroblast differentiation into myofibroblasts was due to tumour-derived exosomes expressing TGF- β 1. Related to the formation of a pre-metastatic niche, Peinado *et al.* identified exosomes derived from melanoma cells as the factors that lead metastatic niche formation through a process of educating bone marrow progenitor cells towards a pro-vasculogenic and pro-metastatic phenotype, resulting in tumour progression and metastasis (230). They showed that exosomes are responsible for the pro-metastatic behaviour of bone marrow progenitor cells due to an increased expression level of MET, an oncoprotein. They also defined an exosome signature specific for melanoma with therapeutic and prognostic value, which may be helpful as an indicator of metastatic disease and tumour burden. They suggested that tumour-derived exosomes communicate with bone marrow progenitor cells through the delivery of genetic material that enables the progression and formation of a metastatic niche.

Furthermore, O'Brien and colleagues supported the hypothesis that exosomes from triple-negative breast cancer cells concede phenotypic traits to other breast cancer recipient cells causing an aggressive behaviour to the latter (231). It has been demonstrated that exosomes have the ability to impact on recipient cells functionality as they are key players in intercellular communication and modulating the tumour microenvironment.

In summary, exosomes are extracellular vesicles involved in intercellular communication that can modulate the environment of neighbouring cells. They are present in biological fluids and can induce the formation of pre-metastatic niches in distant organs. Moreover, it has been reported that exosomes can promote changes in the cells' behaviour.

1.6.5 Exosomes and miRNAs involved in cancer

A study led by Skog *et al.* reported that primary human glioblastoma cells are highly enriched in miRNA, mRNA and angiogenic proteins in comparison with donor cells (232). They showed that glioblastoma cancer cells released microvesicles that can stimulate the formation of tubules, supporting the hypothesis that glioblastoma-derived microvesicles have a role in initiating an angiogenic phenotype in brain endothelial cells. EGFRvIII, a tumour-specific protein, was detected in serum microvesicles in glioblastoma patients (232). Therefore, given that mRNA and some miRNAs may be detected in serum exosomes from glioblastoma patients, a blood test where these microvesicles can be identified might provide diagnostic information and help in therapeutic decisions for cancer patients.

Taylor and colleagues were the first in demonstrating the presence of small RNA species associated with circulating tumour exosomes (233). This and other previous studies reported aberrant expression profiles of miRNAs in ovarian cancer cells and other tumour cells, respectively. This abnormal miRNA expression was used to establish cancer-specific signatures and it appeared in both cellular and exosomal compartments of ovarian cancer patients. They observed a high degree of correlation between miRNAs from the tumour and the corresponding exosomes. Benign disease exhibited low or inexistent levels of exosomes and/or exosome small RNA, whereas exosomal miRNAs were significantly elevated in malignant disease. However, differences in exosomal miRNA expression between early and late stage ovarian cancer patients were not observed (233). These results only proved that specific miRNAs associated with circulating exosomes could be used in screening in order to identify the disease at an early stage given that malignant and benign disease seem to be distinguishable based on a panel of 8 specific miRNAs for ovarian cancer. Moreover, Fabbri *et al.* identified a mechanism in which miRNAs interact with toll-like receptors (TLRs) and modify the tumour microenvironment (234). They showed that

miR-21 and miR-29a are secreted by lung cancer cells via exosomes and these miRNAs activate a pro-metastatic inflammatory response by binding to TLRs in immune cells. This process might modify the tumour microenvironment driving towards tumour progression and metastasis in which exosomes are key players in the preparation of the cellular microenvironment for tumour invasion. This exemplifies the importance of exosomes as potential biomarkers for cancer.

A Japanese study led by Umezu *et al.* exposed that higher levels of exosome production were observed in hypoxia-resistant multiple myeloma (HR-MM) cells compared to parental cells under normal levels of oxygen (normoxia) or acute hypoxia conditions (235). Exosomal expression levels of miR-135b were high in HR-MM-derived exosomes and the formation of tubes was enhanced by targeting the HIF-1/VEGF signalling pathway. An interesting study carried out by Zhang and colleagues (2015) demonstrated that exosomes secreted by astrocytes transferred miRNAs that silence tumour suppressor gene *PTEN* in metastatic breast cancer cells that have spread to the brain (236). As a consequence, proliferation and metastatic niche formation is enhanced in brain metastatic cells. Nevertheless, once these cells (disseminated breast tumour cells) leave the brain and are no longer exposed to astrocytic exosomes, they expressed PTEN again. This evidenced the existence of a bi-directional cross-talk between metastatic tumour cells and their tumour microenvironment (236).

Taken together, the presence of miRNAs in exosomes has been verified in several studies. They are involved in cancer progression and the formation of metastasis. Therefore, exosomal miRNAs could serve as cancer-specific signatures that can be used in early detection to discriminate benign and malignant disease.

1.6.6 Exosomes and miRNAs in pancreatic cancer

It has been shown that the formation of pre-metastatic niches depends on tumour-derived exosomes (230). In 2015, Costa-Silva and co-workers showed that in naïve mice the release of exosomes from PDAC cells induces the formation of a pre-metastatic niche and, as a consequence, an increase in liver metastatic burden occurs (237). They demonstrated that exosomes derived from malignant pancreatic lesions are key players in the formation of a pre-metastatic niche in the liver. Exosomes can activate fibrotic signalling pathways resulting in the establishment of a pro-inflammatory environment that favours metastasis. They detected increased levels of MIF (Macrophage Migration Inhibitory Factor) in exosomes derived from plasma in mice with pre-tumoural pancreatic lesions (either PDAC lesions or PanIN lesions). Furthermore, high exosomal levels of MIF were present in plasma from early stage PDAC patients, before liver metastases were formed. This indicated that MIF derived from tumour exosomes is responsible for pre-metastatic niche formation during PDAC metastasis. Therefore, MIF has the potential to be used as a biomarker of PDAC liver metastasis. In parallel, Madhavan *et al.* found a panel of exosomal proteins and miRNAs in serum that could be helpful in the diagnosis of pancreatic cancer (238).

Despite that extracellular vesicles were identified in most cellular types and bodily fluids, the existence of EVs in human bile was not examined yet. Li and colleagues were the first to establish and characterise the presence of extracellular vesicles in human bile fluid (198). They supported the hypothesis that bile fluid enables cell-cell communication between hepatic and biliary tree cells through the transport of EVs containing miRNAs. They indicated that human biliary EVs contain miRNA species and proposed a biliary vesicle miRNAs' panel for CCA diagnosis that showed a sensitivity and specificity of 67% and 96%, respectively, in comparison to the current available diagnostic methods (198). Li *et al.* reported the clinical potential of this novel bile-based 5-miRNAs EVs' panel for the diagnosis of patients with CCA. Furthermore, in another

study carried out by the same research group, a miRNA (miR-195) was found to be down-regulated in both CCA and neighbour stromal cells, whereas the overexpression of it in stroma cells inhibited tumour growth, migration and metastasis in adjacent CCA cells (198). They also observed that injection of EVs containing miR-195, an inhibitor of stromal pathways, in a rat model of CCA triggered the inhibition of CCA tumour growth and prolonged its survival. These results suggested that delivering EVs containing a therapeutic agent to CCA cells *in vitro* and *in vivo* might prevent cancer cell growth and extend survival.

Recently, Richards et al. showed that cancer-associated fibroblasts (CAFs), when exposed to chemotherapy, exhibit an active role in modulating tumour survival and proliferation in cancer cells (239). They discovered the mechanism by which CAFs regulate proliferation and drug resistance in pancreatic cancer cells. They found that CAFs are intrinsically resistant to gemcitabine (GEM) and when they are exposed to it a significantly increase in the release of exosomes occurs. These exosomes contain chemo-resistance factors (mRNAs and miRNAs) that are transferred into recipient cells promoting proliferation and drug resistance. Epithelial pancreatic cancer cells treated with GEM-treated CAF exosomes exhibited high levels of Snail, an augmented proliferation and drug resistant. Conversely, when GEM-exposed CAFs were treated with an inhibitor of exosome release (GW4869), a significant suppression of chemo-resistant capabilities of CAF cells was observed. Therefore, prevention of CAF exosomes to be secreted might be a promising therapeutic strategy for patients receiving gemcitabine-based treatments.

Taken together, the use of exosomes to deliver therapeutic molecules into tumour cells to induce an immune response, reduce proliferation and suppress metastatic behaviour, is a promising approach, especially in the treatment of PDAC. However, many properties and mechanisms of extracellular vesicles' biology are still elusive.

1.7 Aims and Objectives

This project is focused on investigating the role of non-coding RNAs, especially miRNAs, as biomarkers in bile fluid to detect biliary tract cancers (BTCs) and pancreatic ductal adenocarcinoma (PDAC) at an early stage, as very little is known on this. Furthermore, discovering a bile-based miRNA-signature may serve as a classifier to stratify patients and subsequently improve diagnosis by personalising their treatment.

Therefore, the aim of this research work is to profile microRNAs from bile samples to be able to discriminate PDAC and BTCs from benign disease. This miRNA expression profiling may provide further insights into the tumour biology of the pancreas and the biliary tract system, helping on the discovery of a miRNA-signature that could serve as a valuable predictive or prognostic biomarker in PDAC and/or BTCs.

The **first objective** is to collect a large number of bile samples from PDAC and BTCs patients as well as individuals with benign disease (i.e. cholelithiasis and chronic pancreatitis) in order to perform a microRNA microarray to determine a miRNA expression pattern across these types of cancers.

The **second objective** is to validate the performance of the candidate miRNAs found previously in this study that may play a role in differentiating benign from malignant lesions.

The **third objective** is to determine whether miRNAs previously discovered in bile fluid samples from PDAC and BTCs patients are also expressed in exosomes (*in vitro*). Additionally, determine whether exosomal miRNAs could be used to distinguish malignant from benign disease.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials used

2.1.1 Consumable plastic ware, chemicals, commercial kits and reagents

Table 2.1. List of consumable plastic ware

Consumable item	Company	Catalogue number
Micro-centrifuge tubes: 0.5 ml 1.5 ml 2 ml	StarLab	S1605-0000 S1615-5500 S1620-2700
0.22 µm filters	Sartorius	16534
0.2 ml 48-well plate (PCR)	Thermo Fisher Scientific	AB-0648
8-cap strips	Molecular Bio Products	3418-C
Fast Optical 96-well reaction plate (PCR)	Applied Biosystems	4346906
Optical adhesive film	Applied Biosystems	4311971
Serological pipettes (plastic wrapped): 5 ml 10 ml 25 ml	Corning	CORN4051 CORN4101 4489
T25 flask and T75 flasks	Corning	430639 430641
30 ml container bottle	VWR	215-0321
15 ml falcon tubes and 50 ml falcon tubes	VWR	525-0401 525-0402
250 ml filter system 500 ml filter system	Corning	513-3354 513-3355

Table 2.2. List of chemicals

Chemical	Company	Catalogue number	Storage conditions
2- propanol	Sigma-Aldrich	I9516-500ML	Room temperature
Ethanol absolute	VWR Chemicals	20821.330	Room temperature
4% paraformaldehyde	Santa Cruz Biotechnology (ChemCruz)	SC-281692	+4°C
Karnovsky fixative	Solmedia	HST408S-C	-20°C
Bovine Serum Albumin (BSA)	Sigma	A3912	+4°C
Chloroform	VWR Chemicals	22711.324	Room temperature

Table 2.3. List of commercial kits

Commercial kit	Company	Catalogue number	Storage conditions
TRIzol Reagent (200 ml)	Invitrogen	15596018	Room temperature
TRIzol LS Reagent	Invitrogen	10296028	Room temperature
TaqMan® MicroRNA Reverse Transcription kit (1,000 reactions)	Life Techonologies (TaqMan)	4366596	-20°C
TaqMan® Universal Master Mix II, no UNG (1 x 5 ml)	Life Techonologies (TaqMan)	4440040	-20°C
RNA 6000 Nano assay kit	Agilent Technologies	5067-1511	Room temperature, +4°C and -80°C
RNA 6000 Pico assay kit	Agilent Technologies	5067-1513	Room temperature, +4°C and -80°C
ExoQuick-TC (50 ml)	Systems Biosciences (SBI)	EXOTC50A-1	Room temperature
Exosome ELISA Complete Kit (CD9 detection)	Systems Biosciences (SBI)	EXOEL-CD9A-1	+4°C and -20°C
Exosome ELISA Complete Kit (CD63 detection)	Systems Biosciences (SBI)	EXOEL-CD63A-1	+4°C and -20°C

Exosome ELISA Complete Kit (CD81 detection)	Systems Biosciences (SBI)	EXOEL-CD81A-1	+4°C and -20°C
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Table 2.4. List of reagents

Reagent	Recipe	Company	Catalogue number	Storage conditions
RNase ZAP	Sodium lauryl sulfate	Ambion	AM9780	Room temperature
75% ethanol	For 1 litre: Add 750 ml of ethanol to 250 ml of ddH ₂ O	VWR Chemicals	22711.324	Room temperature
80% ethanol	For 1 litre: Add 800 ml of ethanol to 200 ml of ddH ₂ O	VWR Chemicals	22711.324	Room temperature
Phosphate Buffered Saline solution (PBS)	For 1 litre: Add 800 ml of ddH ₂ O Add 8g of NaCl Add 0.2 g of KCl Add 1.44 g of Na ₂ HPO ₄ Add 0.24 g of KH ₂ PO ₄ Adjust pH to 7.4 with HCl Add ddH ₂ O to a total volume of 1 litre Sterilise by autoclaving (20 min at 121°C, liquid cycle)	-	-	Room temperature or +4°C
0.5 sodium cacodylate buffer				
5% bacteriological disinfectant (distel)	For 1 litre: Add 50 ml of detergent to 950 ml of ddH ₂ O	Tristel Solutions	TM305	Room temperature
hEGF (stock: 1mg/ml)	Add 200 µl of 10mM acetic acid to 0.2 mg of hEGF Final concentration: 1mg/ml Aliquots of 5 µl will be stored at -20°C	Sigma-Aldrich	E9644-0.2MG	-20°C

Hydrocortisone (stock: 1mg/ml)	<p>Add 1 ml of 100% EtOH (ethanol) to 1 mg of hydrocortisone</p> <p>Final concentration: 1mg/ml</p> <p>Aliquots of 20 µl will be stored at -20°C</p>	Sigma-Aldrich	H088-1G	Room temperature (aliquots at -20°C)
Apo-transferrin (stock: 2.5 mg/ml)	<p>Add 10 ml of ddH₂O to 25 mg of apo-transferrin</p> <p>Final concentration: 2.5 mg/ml</p> <p>Aliquots of 1 ml will be stored at -20°C</p>	Sigma-Aldrich	T1147-100MG	-20°C
Tri-iodothyronine (stock: 0.1 µg/ml)	<p>Add 10 ml of DMSO to 1 mg of tri-iodothyronine</p> <p>Take 10 µl from above and add 990 µl of DMSO</p> <p>Final concentration: 0.1 µg/ml</p> <p>Aliquots of 2.5 µl will be stored at -20°C</p>	Sigma-Aldrich	T0281-10MG	-20°C
O-phosphoryl ethanolamine (stock: 20mg/ml)	<p>Add 1 ml of ddH₂O to 20 mg of O-phosphoryl ethanolamine</p> <p>Final concentration: 20 mg/ml</p> <p>Aliquots of 50 µl will be stored at -20°C</p>	Sigma-Aldrich	P0503-10MG	-20°C
cOmplete™ protease inhibitor cocktail	25 tablets	Sigma-Aldrich	11836153001	+4°C

2.1.2 microRNAs

Table 2.5. List of microRNAs

microRNA name	TaqMan miRNA assay ID
hsa-miR-15a-5p	000389
hsa-miR-15b-5p	478313_mir
hsa-miR-16-5p	477860_mir
hsa-miR-21-5p	477975_mir
hsa-miR-23a-3p	478532_mir
hsa-miR-25-3p	477994_mir
hsa-miR-29b-3p	478003_mir
hsa-miR-30d-5p	478606_mir
hsa-miR-93-5p	478210_mir
hsa-miR-125b-5p	477885_mir
hsa-miR-148a-3p	477814_mir
hsa-miR-194-5p	477956_mir
hsa-miR-181c-3p	477933_mir
hsa-miR-200b-3p	477963_mir
hsa-miR-451a	478107_mir
hsa-miR-575	479056_mir
hsa-miR-663a	479445_mir
Cel-miR-39	000200
U6 snRNA	001973

2.1.3 Pancreatic cancer cell lines

Table 2.6. List of pancreatic cancer cell lines

Cell name	Cell type	Company	Catalogue number
PANC-1	Human pancreatic epithelial cancer cell	American Type Culture Collection (ATCC)	ATCC-CRL-1469
MiaPaCa-2	Human pancreatic epithelial cancer cell	American Type Culture Collection (ATCC)	ATCC-CRL-1420
HPNE	Human pancreatic ductal epithelial cell line	–	–
HPDE_H6c7	Human pancreatic ductal epithelial cell line (H6c7)	Kerafast	ECA001
TKCC-02	Human pancreatic epithelial cell line derived from mouse xenografts initiated from a moderately differentiated adenocarcinoma taken from a pancreatic resection	Australian Pancreatic Cancer Genome Initiative (APGI)	–
TKCC-10	Human pancreatic epithelial cell line derived from mouse xenografts initiated from a poorly differentiated adenocarcinoma biopsy	Australian Pancreatic Cancer Genome Initiative (APGI)	–

2.1.4 Tissue culture materials: cell growth media and supplements

Table 2.7. Composition of cell culture growth media of PANC-1 and Mia-PaCa-2.

PANC-1 and MIA-PaCa-2		
Cell culture media	Volume (ml)	
DMEM	500 ml	
Supplements	Volume to be added	Final concentration
Foetal Bovine Serum (FBS), exosome-depleted	50 ml	10X
L-glutamine (200mM)	5 ml	1X (2mM)
Penicillin / Streptomycin	5 ml	1X

Table 2.8. Composition of cell culture growth media of HPDE and H6c7.

HPDE and H6c7		
Cell culture media	Volume (ml)	
Keratinocyte SFM	500 ml	
Supplements	Volume to be added	Final concentration
Antibiotic antimycotic (100X)	5 ml	1X

Table 2.9. Composition of cell culture growth media of TKCC-02.

TKCC-02		
Cell culture media	Volume (ml)	
RPMI-1640	500 ml	
Supplements	Volume to be added	Final concentration
Foetal Bovine Serum (FBS), exosome-depleted	50 ml	10 %
L-glutamine (200mM)	5 ml	1 % (2mM)
Penicillin / Streptomycin (100X)	5 ml	1 % (1X)
hEGF (stock: 1mg/ml)	10 µl	20 ng/ml

Table 2.10. Composition of cell culture growth media of TKCC-10.

TKCC-10		
Cell culture media	Volume (ml)	Final volume (ml)
HAM F-12	250 ml	500 ml
M199	250 ml	
Supplements	Volume to be added	Final concentration
Foetal Bovine Serum (FBS), exosome-depleted	37.5 ml	7.5 %
L-glutamine (200mM)	5 ml	1 % (2mM)
Penicillin / Streptomycin	5 ml	1 %
hEGF (stock: 1mg/ml)	5 µl	10 ng/ml

Hydrocortisone (stock: 1mg/ml)	20 µl	40 ng/ml
Apo-transferrin (stock: 2.5 mg/ml)	1 ml	5 µg/ml
Tri-iodothyronine (stock: 0.1 µg/ml)	2.5 µl	0.5 pg/ml
O-phosphoryl ethanolamine (stock: 20 mg/ml)	50 µl	2 µg/ml
Insulin (100 IU/ml)	500 µl	0.1 IU/ml
Glucose solution	6 ml	0.12 %
Gentamicin (1000X)	500 µl	1X
HEPES (100X)	7.5 ml	15 mM
MEM vitamins	5 ml	1 %

Table 2.11. List of tissue culture growth media, supplements and reagents

Name	Company	Catalogue number	Storage conditions
DMEM (Dulbelco's Modified Eagle Medium)	Sigma-Aldrich	D5546-500ML	+4°C
RPMI-1640	Sigma-Aldrich	R5886-500ML	+4°C
DMEM/F-12	Gibco	11320074	+4°C
HAM F-12	Gibco	21765029	+4°C
M199	Gibco	31150022	+4°C
DMEM (with 4.6g/l of glucose)	Lonza	BE12-604F	+4°C
Keratinocyte SFM (+ supplements)	Gibco	17005042	+4°C / -20°C
Fetal Calf Serum (FCS)	First Link UK	02-00850	50 ml aliquots at -20°C
Fetal Bovine Serum (FBS), exosome-depleted	Gibco	A2720801	50 ml aliquots at -20°C
HEPES	Gibco	15630080	+4°C
Phosphate Buffered Saline (PBS)	-	-	Room temperature or +4°C
Versene solution	Gibco	15040066	+4°C

Trypsin	Sigma-Aldrich	T4549-100ML	5 ml aliquots at -20°C
EDTA	Sigma-Aldrich	E8008-100ML	+4°C
L-glutamine (200mM)	Invitrogen	25030-024	5 ml aliquots at -20°C
Penicillin / Streptomycin	Invitrogen	15070-063	5 ml aliquots at -20°C
Antibiotic antimycotic (100X)	Gibco	15240-062	5 ml aliquots at -20°C
Hydrocortisone	Sigma-Aldrich	H088-1G	20 µl Aliquots at -20°C
hEGF	Sigma-Aldrich	E9644-0.2MG	10 µl aliquots at -20°C
Insulin (100UI/ml)	Sigma-Aldrich	I9278-5ML	+4°C
Glucose (10%)	Gibco	G8644	+4°C
Gentamicin (1000X)	Sigma-Aldrich	G1397-10ML	+4°C
Apo-transferrin	Sigma-Aldrich	T1147-100MG	1ml aliquots at -20°C
Tri-iodothyronine	Sigma-Aldrich	T0281-10MG	2.5 µl aliquots at -20°C
O-phosphoryl ethanolamine	Sigma-Aldrich	P0503-10MG	50 µl aliquots at -20°C
MEM vitamins	Gibco	11120052	5 ml aliquots at -20°C

2.1.5 Antibodies

Table 2.12. List of primary and secondary antibodies.

PRIMARY ANTIBODIES				
Antibody	Dilution	Company	Dilution buffer	Storage conditions
Exosomal surface marker CD9	1:100	SBI – part of Exosome ELISA complete kit (CD9)	1X Blocking buffer	+4°C
Exosomal surface marker CD63	1:100	SBI – part of Exosome ELISA complete kit (CD63)	1X Blocking buffer	+4°C

Exosomal surface marker CD81	1:100	SBI – part of Exosome ELISA complete kit (CD81)	1X Blocking buffer	+4°C
Monoclonal anti-CD63	1:20	Abcam (ab23792)		-20°C
SECONDARY ANTIBODIES				
Antibody	Dilution	Company	Dilution buffer	Storage conditions
Goat anti-Rabbit HRP	1:5,000	SBI – part of Exosome ELISA complete kit (CD81)	1X Blocking buffer	+4°C
Goat anti-mouse IgG Fc specific (10 nm) gold	1:50	BBI Solutions (EM.GAM10)		+4°C

2.1.6 Patient bile samples

Following written informed consent, bile specimens were obtained from individuals with a benign condition such as cholelithiasis or chronic pancreatitis and from individuals who underwent tumour resection for a known PDAC, CCA, ampullary carcinoma or IPMN carcinoma between 2013 to 2018 at three different institutions: Hammersmith Hospital Imperial College London NHS Trust (London, UK), VUMC Amsterdam Center (Amsterdam, The Netherlands) and University Hospital of Pisa (Pisa, Italy).

In the first cohort, called the discovery cohort, 57 bile samples were assessed from patients with pancreatic or biliary tract lesions and were classified into seven groups as follows: cholelithiasis and chronic pancreatitis ($n = 14$); pancreatic ductal adenocarcinoma (PDAC) primary stage IIa ($n = 8$); PDAC primary stage IIb ($n = 14$); PDAC advanced stage III/IV ($n = 5$); cholangiocarcinoma (CCA, $n = 6$), ampullary cancer ($n = 3$) and invasive intraductal papillary mucinous neoplasia (IPMN, $n = 7$). Patients with cholelithiasis and chronic pancreatitis composed the benign group,

whereas the malignant group involved PDAC, CCA, ampullary cancer and IPMN patients.

The second cohort or validation cohort consisted of a total of 71 bile samples from patients with a benign condition (cholelithiasis) or those who underwent tumour resection for a known PDAC, CCA or ampullary carcinoma. Bile samples were obtained from the same international institutions mentioned above and patients' samples were divided into four groups: benign disease (cholelithiasis, $n = 34$), PDAC stage I ($n = 1$), stage IIa/b ($n = 14$), stage III/IV ($n = 12$); CCA ($n = 7$) and ampullary carcinoma ($n = 3$). Patients with PDAC, CCA and ampullary carcinoma conformed the malignant group.

Inclusion criteria included adult patients (> 18 years) with resectable disease, whereas patients with bad performance status and/or (no obvious) metastatic disease were not suitable for resection, and therefore, were excluded.

Complete clinicopathological, follow-up and recurrence data were available from prospectively maintained databases. Further detailed clinicopathological information about the patients is provided [in Appendix section A](#).

2.2 Methods used

2.2.1 Ethical approval

Analysis of miRNAs in human specimens of bile was approved by a UK national research ethics committee (Camden & Islington REC, London; 09/H0722/77) and by Imperial College Healthcare Tissue Bank (R17048, ICHTB HTA licence 12275, Wales REC 17/WA/0161) in the UK and the ethics committee at the University Hospital of Pisa in Italy and The VUMC Cancer Center in The Netherlands.

2.2.2 Collection of patient samples

All three European institutions followed the same standard procedure to collect bile. During surgery, bile was aspirated from each patient from either the gallbladder (patients with benign lesions) or the common bile duct (performing a Whipple procedure in patients with malignant lesions) using a medical syringe. After collection of the sample, this was stored at -80°C until further use.

2.2.3 RNA extraction

2.2.3.1 RNA isolation from human bile specimens

An adapted protocol from Yan and Patel (240) was followed when performing RNA extraction in bile samples. Generally, 1 ml of bile was centrifuged (Sigma 1-14K) at low speed (3,000 x g) for 10 minutes at +4°C and the supernatant was then filtered with 0.22 µm Minisart® filters. Ideally 700-500 µl of bile were eluted after the filtration.

To extract RNA from bile, TRIzol® LS Reagent protocol was followed according to manufacturer's instructions although a few modifications were applied:

1. After adding TRIzol to homogenise the sample, 5 µl of a spike-in control (cel-miR-39) was added.
2. After adding chloroform and centrifuging the sample, three phases are observed. The aqueous phase is transferred into a new micro-centrifuge tube and an equal volume of chloroform is added. The sample is centrifuged again at the same speed as stated in the protocol and the upper phase is transferred to a new tube.
3. The sample is stored at -80°C after adding isopropanol for an overnight incubation.
4. The following step after isopropanol overnight incubation was a centrifugation. This centrifugation step was performed at high speed (16,500 x g) for 30 minutes at +4°C.
5. Ethanol concentration is at 80% (for 1 litre: 80 ml of 100% ethanol plus 20 ml of ddH₂O) and washes are performed twice.

After re-suspending the pellet in RNase-free water, concentration of RNA is measured with a spectrophotometer (NanoDrop 1000, Thermo Scientific) at 260 nm and 280 nm wavelengths, with special attention to the absorbance ratios A₂₆₀/A₂₈₀ and A₂₆₀/A₂₃₀ to assure a good RNA quality: the A₂₆₀/A₂₈₀ should range between 1.8 and 2.0, and the A₂₆₀/A₂₃₀ should be greater than 1.5.

2.2.3.2 RNA isolation from cell culture exosomes

After precipitating exosomes from cell culture media using ExoQuick-TC, the pellet was re-suspended in 100-500 µl of PBS. TRIzol® LS Reagent protocol was followed according to manufacturer's instructions although a few modifications were applied (see section [2.2.3.1](#)).

Exosomal RNA concentration was measured using a spectrophotometer as well as a microfluidics-based platform named Bioanalyzer for checking not only the concentration but also the RNA integrity of the samples.

2.2.4 Agilent RNA Nano and Pico Assay Bioanalyzer

To determine the integrity and the concentration of total and small RNA from bile samples an Agilent 2100 Bioanalyzer device was used. It is a microfluidic chip-based technology, which separates RNA samples using an electrophoretic separation and detects RNA via laser-induced fluorescence. The software generates an RNA profile of each sample, which is displayed both as an electropherogram and a gel-like image. Additionally, the results expose the RNA concentration and the ribosomal ratio. There are different types of RNA assay/chips for RNA quality assessment. The RNA Nano assay/chip offers a sensitivity range from 25 ng/μl to 500 ng/μl and loading a total of 12 samples, whereas the RNA Pico assay provides a range of sensitivity from 0.1 ng/μl to 5 ng/μl and a total loading of 11 samples.

Depending on RNA concentrations observed with the spectrophotometer (NanoDrop 1000, Thermo Scientific), RNA samples were analysed with either the RNA 6000 Nano or Pico assay kit following protocol instructions.

A novelty from Agilent Bioanalyzer is the introduction of the RNA Integrity Number (RIN), a new tool for RNA quality assessment. It is an algorithm that assigns integrity values to RNA measurements. This algorithm permits the classification of eukaryotic total RNA based on a numbering system from 1 to 10, being 1 the most degraded and 10 the most intact (241).

2.2.5 MicroRNA Reverse Transcription quantitative real-time Polymerase Chain Reaction (RT-qPCR)

2.2.5.1 cDNA synthesis

A reverse transcription using TaqMan MicroRNA Reverse Transcription Kit was performed following the manufacturer's instructions. A total volume of 5 µl of RNA sample (10 ng) was mixed with 7 µl of master mix and 3 µl of 5X reverse transcription miRNA TaqMan® primer in a 48-well PCR plate and it was spun down to bring the solution to the bottom of the well. The samples were incubated in a PCR thermal cycler (Veriti, Applied Biosystems) at 16°C for 30 minutes to allow primer annealing, followed by 30 minutes at 42°C for the elongation step and 5 minutes at 85°C to inactivate the reverse transcriptase enzyme.

Table 2.13. Master mix components of the reverse transcription

Components	Master mix volume (µl) per 15 µl single reaction
100 mM dNTPs (with dTTP)	0.15 µl
MultiScribe™ Reverse Transcriptase, 50 U/ µl	1.00 µl
10X Reverse Transcription Buffer	1.50 µl
Rnase Inhibitor, 20 U/ µl	0.19 µl
Nuclease-free water	4.16 µl
Total volume	7.00 µl

A list of miRNA-specific primers used in the reverse transcription is provided in section [2.1.3](#). This selection of miRNAs was chosen based on bioinformatic analysis carried out after a microRNA expression assay was performed.

MiRNA-181c was chosen as an endogenous control in the discovery and validation cohort because it was found to be consistently expressed across all RNA samples from the discovery cohort.

2.2.5.2 Quantitative real-time PCR (qPCR)

The PCR amplification process is based on repeated thermal cycles involving three sequential steps: denaturation (95°C), annealing (50°C to 56°C) and extension (72°C). During denaturation the double-stranded template DNA is separated into two single strands. At this point, the temperature is lowered to allow annealing to the template DNA. Finally, the extension phase permits the elongation of the DNA strand by the polymerase enzyme. These three stages are repeated 30-40 times, increasing the number of copies of DNA each time.

TaqMan probes or hydrolysis probes consist of a dual-labelled oligonucleotide with two fluorescent moieties: a reporter fluorophore and a quencher fluorophore at the 5' and 3' end, respectively. As long as both fluorophores are in close proximity the probe will remain intact: the quencher dye will absorb the fluorescence emitted by the reporter fluorophore.

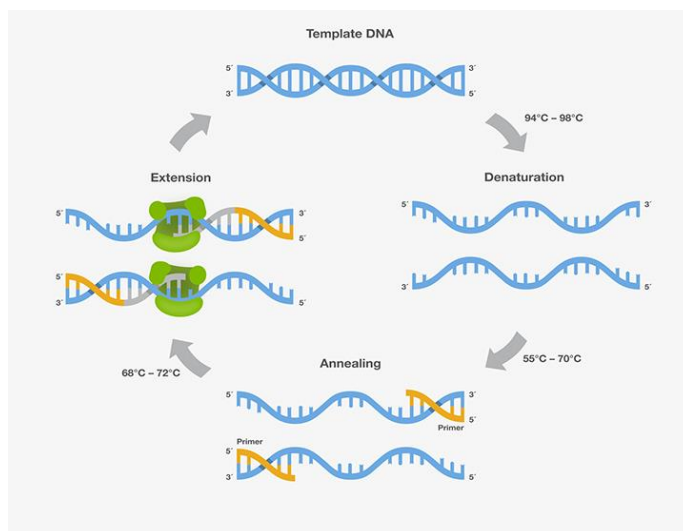


Figure 2.1. Polymerase chain reaction steps.

Main steps in PCR including denaturation, annealing and extension of the target sequence from a template DNA. *Reproduced from ThermoFisher Scientific.*

A mixture of forward and reverse primers and a dual-labelled hydrolysis probe are used to amplify and detect the cDNA target. Both the primer and the hydrolysis probe bind to the complementary DNA strand during the annealing (or amplification phase) and extension phase, immediately after the denaturation step. After hybridisation and during the amplification phase, the *Thermus aquaticus* (Taq) polymerase recognises and binds to the double-stranded molecule and proceeds along the template, adding dNTPs that are complementary to the target on the 3' end of the primer strand (242). However, when the polymerase enzyme encounters the hydrolysis probe this is degraded through the 5'-nuclease activity of the enzyme, cleaving the reporter fluorophore at the 5' end and separating it from the hydrolysis probe and the quencher. As a result, the reporter dye emits a fluorescence signal which will be detected by the real-time PCR device and which is directly proportional to the amplification product. The fluorescence will increase consecutively as PCR cycles progresses, accumulating and exponentially increasing the intensity of the light emitted.

To amplify mature miRNAs, the master mix for a single reaction was prepared for each miRNA (or TaqMan® assay) by combining 10 µl of 2X TaqMan Universal Master Mix II with 1 µl of 20X TaqMan® Assay (i.e. hydrolysis probe) in a micro-centrifuge tube (Fisher Scientific mySPIN6). The cDNA template from each sample, which was previously generated in the reverse transcription, was diluted in 1:10 with RNase-free H₂O and 4.4 µl were added to the appropriate well in a Fast Optical 96-well reaction plate (Applied Biosystems). Then 11 µl of master mix plus 4.6 µl of RNase-free H₂O, making a final volume of 20 µl, were added to the appropriate well in the reaction plate. Next, the plate was sealed with an adhesive film (Applied Biosystems) and centrifuged (Sigma 1-14K) at 2,000 x g for less than 1 minute at room temperature.

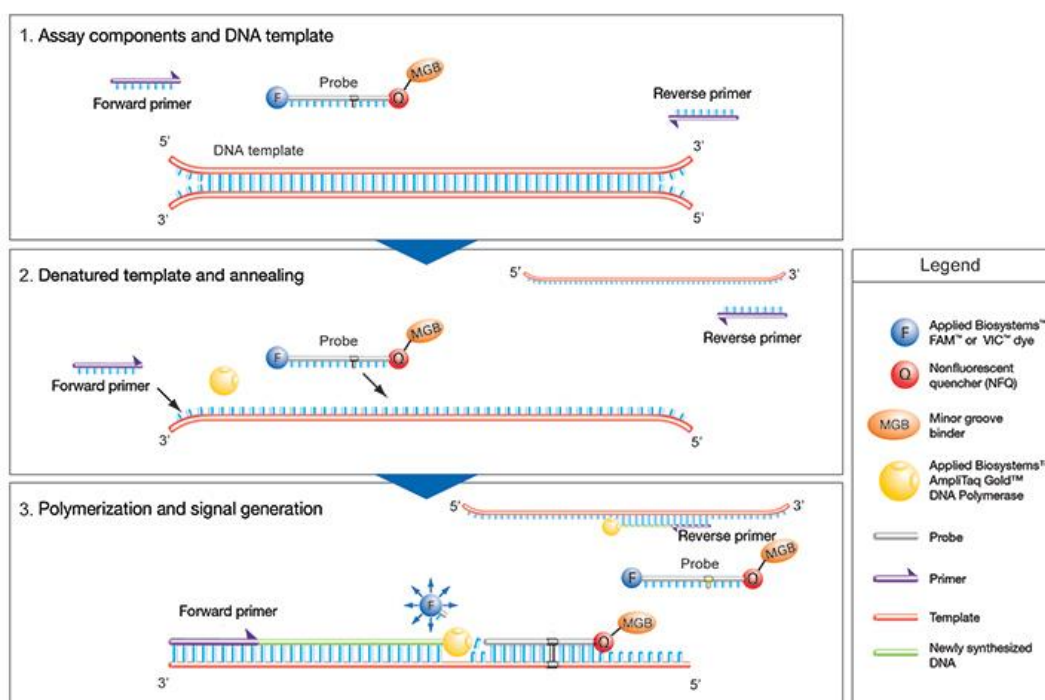


Figure 2.2. TaqMan mechanism.

The first step is to raise the temperature to denature the double-strand of the cDNA (template). At this point the fluorescent dye at the 5' end and the non-fluorescent dye (hydrolysis probe) at the 3' end are bound together. Next, the temperature is lowered to enable the primers and the probe to anneal to their specific target sequences. Finally, the Taq polymerase synthesises new strands using until it encounters the hydrolysis probe, which is degraded and the fluorophore at the 5' end is cleaved from the hydrolysis probe. This results in a fluorescence signal detected by the PCR device which is directly proportional to the amplified PCR product. *Reproduced from ThermoFisher Scientific, TaqMan®*

Quantitative real-time PCR (qPCR) was performed with a StepOne Plus real-time PCR System (Applied Biosystems) and the thermal cycling conditions were as follow: a first stage of 2 minutes at 50°C and a second stage of 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 60 seconds at 60°C.

The list of miRNA TaqMan® assays is provided in section [2.1.5](#). Relative expression levels of each miRNA were calculated using the $2^{-\Delta C_t}$ method, where C_T is the comparative threshold cycle or the number of cycles necessities for the fluorescent signal to cross the threshold in the real-time PCR. To calculate the ΔC_T value, the

average of comparative threshold cycle (C_T) value of the target miRNA and the miRNA control (normaliser) were subtracted: **$\Delta C_T = \text{Average miRNA target } C_T \text{ value} - \text{Average miRNA control (normaliser) } C_T \text{ value}$**

2.2.6 miRNA microarray expression profiling

A microRNA expression profiling using the nCounter® Human v3 miRNA Expression Assay was performed in 58 RNA patients samples (discovery cohort) by NanoString Technologies. This platform is able to quantify the expression of 800 mature miRNAs derived from miRBase and it also includes 20 assay controls, 5 mRNA probes and 5 spike-in controls. The assay controls are as follows: 6 positive controls and 8 negative controls, 3 ligation positive controls and 3 ligation negative controls.

Reference mRNA controls are probes that recognise endogenous mRNA targets usually expressed in tissues. They can determine cellular contamination, failure of ligation, etc. These mRNAs should not be used as reference genes to normalise samples.

Positive (ligation) and negative (ligation) controls are probes that recognise synthetic mRNA targets included or not included in the assay, respectively. Positive controls are used to confirm linear response to input amounts and confirm that low input signal is above background. Negative controls are used to determine background signal independent of the success of the ligation. It can be used to set the threshold to define the expression of miRNA(s). Positive and negative ligation controls monitor ligation efficiency and non-specific ligation, respectively.

Spike-in controls are probes that recognise exogenous miRNA targets that are not included in the assay to monitor sample processing steps such as RNA isolation or purification prior to the NanoString assay protocol.

This assay does not use a reverse transcription or amplification methodology to detect miRNAs, instead, molecular barcodes and single molecule imaging to quantify up to 800 transcripts in a single reaction are used. The steps involved in the preparation of the assay include a multiplexed annealing of specific tags to the miRNAs, a ligation reaction and an enzymatic purification step to remove unligated material. The miRNAs are tagged and ready to be analysed using the nCounter Analysis System. This technology is based on the direct molecular barcoding and digital detection of target molecules using a colour-coded probe pair. The probe pair consists of two probes: a Reporter Probe, which carries signal on its 5' end, and a Capture Probe, which carries a biotin on its 3' end.

During the overnight hybridisation reaction, there is a large excess of probe pairs to guarantee that target nucleic acids find a probe pair. After hybridisation, the excess of probes is removed and probe(s)/target(s) are aligned and immobilised on a cartridge for data collection. Each miRNA is identified by their own colour-code generated by the Reporter Probe.

The complexity of the colour codes (four colours in six positions) enables a large diversity of targets present in the same sample to be individually resolved and identified during data collection.

This digital quantification provides high sensitivity, precision and reproducibility.

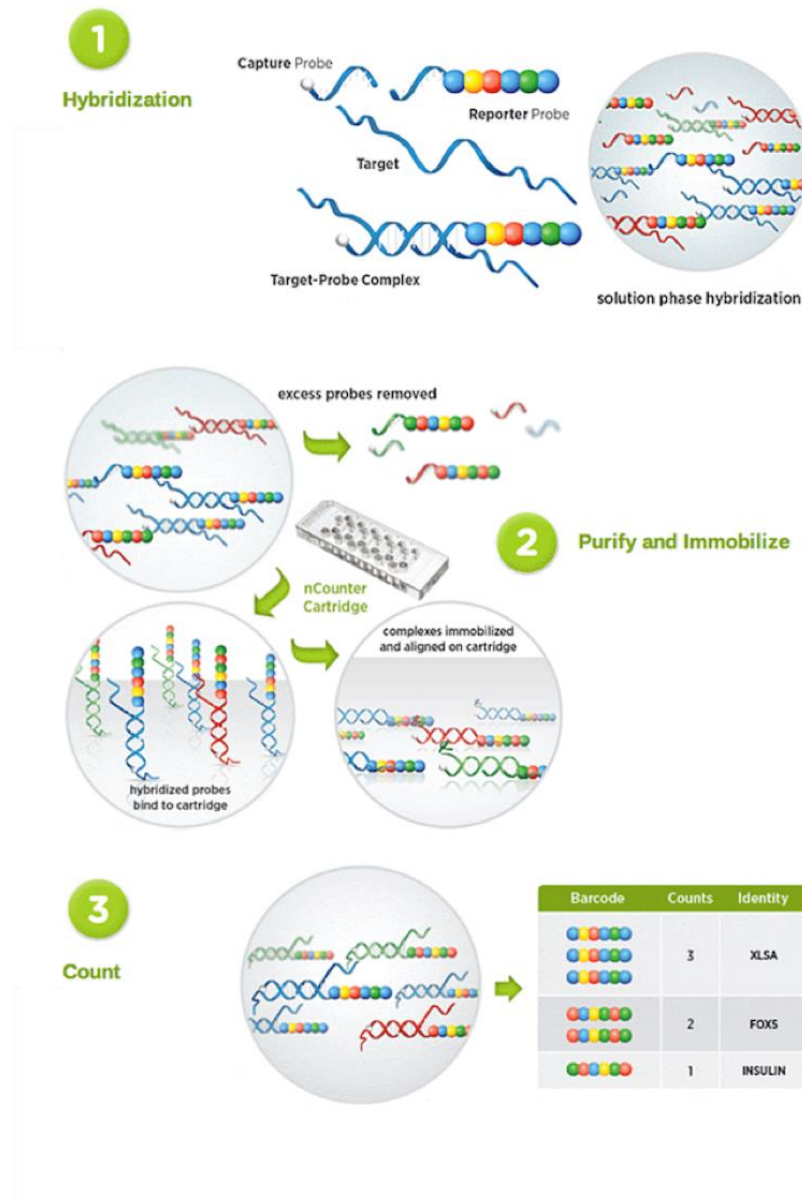


Figure 2.3. nCounter microRNA expression technology.

This platform enables the performance of highly multiplexed, digital quantification of hundreds of different nucleic acids in a single reaction. Each gene of interest requires a corresponding capture probe and a reporter probe. **1)** The sample material is mixed with an excess of reporter and capture probes. The reporter probe contains the fluorescent barcode signal and the capture probe is coupled to a biotin tag that immobilises the hybridised complex. **2)** The excess of capture and reporter probes are removed. **3)** Sample cartridges are placed in the Digital Analyser device for data collection. This machine counts the fluorescent barcodes and a total running of each target is tabulated. *Reproduced from NanoString Technologies nCounter System.*

2.2.7 Bioinformatic analysis

A supervised clustering analysis using R program (DESeq2) was performed to determine differences between groups:

- Benign vs all malignant cases (PDAC, CCA, ampullary carcinoma and IPMN).
- Benign vs PDAC.
- Benign vs CCA.
- PDAC vs CCA.
- PDAC vs other malignant (CCA, ampullary carcinoma and IPMN).
- CCA vs other malignant (PDAC, ampullary carcinoma and IPMN).
- Early PDAC vs late PDAC
- Early PDAC vs CP
- Late PDAC vs CP
- PDAC vs CP

2.2.8 Cell culture and cell lines

2.2.8.1 Passaging for pancreatic cell lines

The cell lines used for exosome experiments were PANC-1, MiaPaCa-2, HPNE, H6c7, TKCC-02 and TKCC-10. All these cell lines are adherent cells and were routinely cultured in T75 flasks and incubated at 37°C in a humidified incubator with a 5% CO₂ atmosphere. Each pancreatic cell line was cultured in different cell culture conditions (see section [2.1.4](#)). All cell culture work was performed under sterile conditions.

When 70-80% confluence was reached, cells were sub-cultured. Therefore, the cell medium was removed and the cells were rinsed with 3 ml of PBS. Afterwards, PBS solution was removed and the addition of 3 ml of EDTA-Trypsin allowed the cells to detach from the flask. After incubating the cells with this solution for 10-15 minutes, the same volume of PBS was added to inactivate the EDTA-Trypsin solution and avoid cells from dying. The contents of the flask(s) were then transferred into a 30 ml container tube and a centrifugation step was performed at 1500 rpm for 5 minutes. The supernatant was aspirated and the pellet was re-suspended in growth culture medium. Cells were transferred into new T75 cell culture flasks and grown to desired cell density. Finally, the cell culture flasks were incubated at 37°C in a humidified incubator with a 5% CO₂ atmosphere.

This process was repeated as demanded by the growth characteristics of the cell line, usually 2 times a week for TKCC-10, HPNE and MiaPaCa-2 and 3-4 times a week for PANC-1, H6c7 and TKCC-02.

2.2.8.2 Collection of exosome-depleted medium from pancreatic cells in growth

Once pancreatic cells reached 80-90% confluence and were growing in the exosome-depleted growth media for at least 24-48 hours, 15 ml of exosome-depleted media was filtered with a 0.22 µm filter and transferred to a 15 ml container tube. One tablet of protease inhibitor cocktail was added to the container before storing the exosome-depleted medium at -80°C until further use.

2.2.9 Precipitation and isolation of exosomes

Pancreatic cell lines were growing in exosome-depleted medium for 24-48 hours until they reached 80-90% confluence. Then, 15 ml of exosome-depleted media was collected from pancreatic cells and filtered using 0.22 µm filters. Next, centrifugation

at 3,000 x *g* for 15 minutes to remove cells and cell debris was performed and the supernatant was transferred to a new collection tube. Subsequently 3 ml of ExoQuick-TC was added to the tube and the mixture was mixed well by inverting or flicking the tube prior to overnight refrigeration at +4°C. The tube(s) remained upright during the overnight refrigeration (at least 12 hours) and were not rotated or mixed. After the incubation period, the mixture was centrifuged at 1,500 x *g* for 30 minutes (room temperature or +4°C). After this, exosomes appeared at the bottom as a white/beige pellet. Next, the supernatant was removed and the tube was centrifuge again at 1,500 x *g* for 5 minutes to remove any residual traces of fluid. After that, aspiration of any residual traces of fluid and re-suspension of the pellet in 100-500 µl of PBS or in a specific buffer was done. RNA content was measured with a spectrophotometer (NanoDrop 1000, Thermo Scientific) and RNA quality was assessed with a microfluidics-based platform (2100 Bioanalyzer, Agilent Technologies).

2.2.10 Enzyme-linked Immunosorbent Assay (ELISA)

An enzyme-linked immunosorbent assay (ELISA) using the Exosome ELISA complete kit for CD9, CD63 and CD81 was performed following the manufacturer's instructions.

After precipitating exosomes from cell culture media using ExoQuick-TC, the pellet was re-suspended in 200 µl of Exosome Binding Buffer and vortex for 15 seconds. Prior to centrifuge the re-suspended pellet at 1,500 x *g* for 5 minutes, an incubation step at 37°C for 20 minutes was done. Then, the supernatant was transferred to a new tube and it was placed on ice.

A standard curve was prepared by performing serial dilutions of the ExoElisa protein standard using the Exosome Binding Buffer.

Next, 50 µl of prepared protein standards and 50 µl of exosome protein sample was added to the appropriate well in the micro-titer plate. Then, the plate was sealed and incubated overnight at 37°C. After the incubation period, the plate was inverted to empty all contents. The plate was washed 3 times for 5 minutes each time with 100 µl of 1X Wash buffer (this washing step was also followed after incubation with primary and secondary antibodies).

Exosome-specific primary antibodies for CD9, CD63 and CD81 were diluted 1:100 in 1X Blocking buffer and 50 µl of were added to each well before incubating the plate for 1 hour at room temperature (with shaking). After the incubation step, the plate was washed. Next, an exosome-validated secondary antibody was diluted in 1:5,000 with 1X Blocking buffer and 50 µl of were added to each well before incubating the plate for 1 hour at room temperature (with shaking). After the incubation, the plate was washed. Subsequently, 50 µl of Super-sensitive TMB ELISA substrate (warmed to room temperature) were added in each well and the plate was incubated (with shaking) for 15-45 minutes at room temperature. Immediately after, 50 µl of Stop buffer solution was added and the plate was read with a spectrophotometric plate reader (Optimax) at 450 nm wavelength of absorbance.

2.2.11 Transmission Electron Microscopy (TEM) and Immunogold labelling

Exosomes were isolated from pancreatic cell culture media using Exo-Quick TC kit as described in section [2.2.9](#) and then were fixed in 4% paraformaldehyde (PFA) for 60 minutes. Then, 10 µl of fixed exosomes were dropped onto a formvar carbon-coated grid for 60 minutes. Three droplets of 0.5 M sodium cacodylate buffer (pH 7.4) were added to each grid followed by 5 minutes incubation each. Next, one droplet of 1% bovine serum albumin (BSA) was added and incubated for 30 minutes. Subsequently,

the grids were incubated for 60 minutes with 10 µl of anti-CD63 (ab23792) at 1:10 dilution. Unbound antibody was removed by blocking the grid with 1% BSA for 5 minutes. For immuno-gold staining, the grids were labelled with a secondary antibody, goat anti-mouse IgG Fc specific (10 nm), conjugated to gold particles (EM.GAM10) at 1:50 dilution for 60 minutes followed by 3 washes in phosphate buffer saline (PBS) and a final wash in distilled water. Then, the grid was stained with 2% uranyl acetate for 3 minutes and washed in distilled water. The excess of liquid was removed and the grid was dried at room temperature for 10 minutes. Observations at 100,000 and 150,000 magnification were carried out using a TEM and digital micrographs were taken using Gatan digital micrograph.

2.2.12 Statistical analysis

Statistical analysis was performed using GraphPad Prism, software version 7 (GraphPad Software, Intuitive Software for Science, San Diego, CA). MiRNA expression levels between groups were compared using a parametric *t*-test for normally distributed data across all samples. Furthermore, for each group of patient samples the mean $\pm$ SD (standard deviation) was presented.

Statistical significance was established at *p-values* <0.05 and 95% CI (confidence interval) are presented. The level of significance was set at *p-value* <0.05, as indicated by asterisks: * *p-value* <0.05, ** *p-value* <0.01, *** *p-value* <0.001, and **** *p-value* <0.0001.

CHAPTER 3. MicroRNAs in bile fluid discriminate between benign and malignant pancreatic and biliary-tract lesions

3.1. Background

It is known that PDAC and BTC are lethal diseases and present significant challenges, including lack of efficient diagnostic techniques and limited treatment options, which leads to a late detection and poor survival rates.

Non-coding RNAs (ncRNAs) are a class of RNA molecules that do not code for proteins (114) and have emerged as attractive therapeutic targets and diagnostic tools in cancer. There are different classes of ncRNAs, which include microRNAs: small endogenous non-coding RNAs of 17-25 nucleotides in length that regulate gene expression at the post-transcriptional level (115). They are thought to be involved in haematopoiesis, cell proliferation and cell death (134, 243). Indeed, their deregulation contributes to cancer development (168). They are stable and evolutionary conserved molecules that can be detected in tissue biopsies (171, 172) and bodily fluids such as plasma (183, 187) and bile (190, 191, 198). Given their stability and tissue specificity, miRNAs are exceptional molecules that could be used in the detection of PDAC (169, 173).

Therefore, finding novel miRNAs in bile fluid associated with PDAC or BTC would be a considerable discovery due to the close proximity of this biological fluid to the malignant lesion.

3.2 Results section

3.2.1 Bile samples collection and RNA isolation from the discovery and validation cohort

3.2.1.1 Study population

In this study a total of 128 individuals were included, with 57 subjects grouped into the discovery cohort and 71 distributed into the validation cohort (**Figure 3.1**). Bile fluid samples were collected from patients with benign lesions (cholecystitis, cholelithiasis and chronic pancreatitis), PDAC (stage IIa, IIb and III/IV), CCA, ampullary carcinoma (AC) and IPMN (**Figure 3.1**). Histopathological data is shown in [Appendix Section A](#).

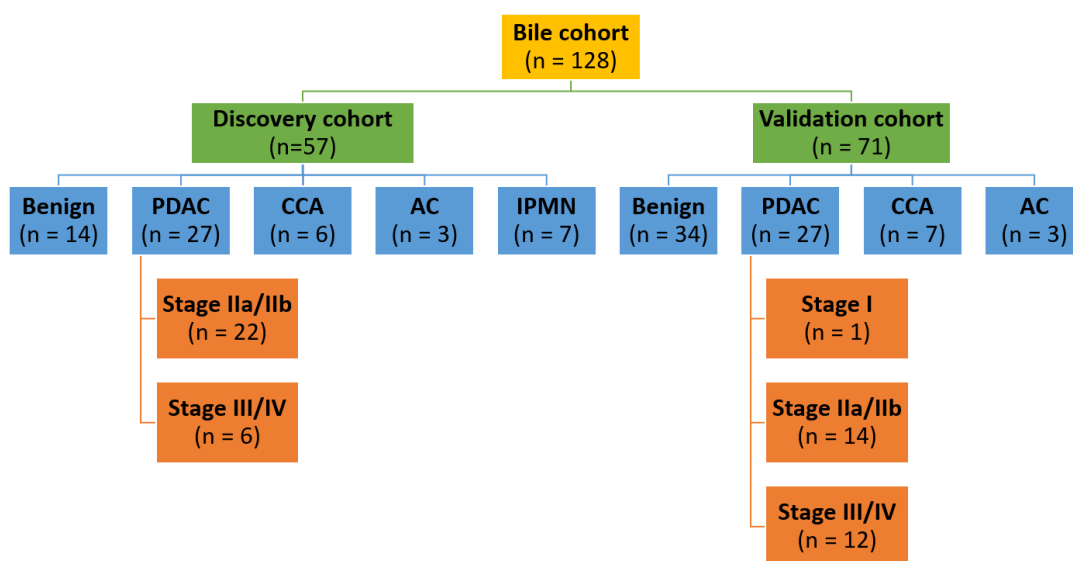


Figure 3.1. Study population.

This bile study contains 128 individuals divided into two different cohorts (discovery, n=57; and validation, n=71). Each group has a benign group and four and/or three different types of cancers, including PDAC, CCA, AC and IPMN carcinoma, respectively.

3.2.2 MicroRNA microarray profiling identifies miRNAs differentially expressed in benign and malignant pancreatic lesions

3.2.2.1 Bioinformatic analyses revealed up-regulated and down-regulated miRNAs in malignant lesions compared to benign lesions

In order to discriminate between benign lesions and malignant pancreatic and biliary lesions, miRNA expression profiling using the nCounter technology (Nanostring) was performed. A total of 800 miRNAs were screened across all samples from the discovery cohort. The miRNA microarray aimed to discover differential expression between pancreatic cancers (i.e. PDAC and IPMN carcinoma) and BTC, as well as comparing these groups to benign lesions. Bioinformatic analyses were carried out using R programme (DESeq2) and the comparisons were as follow:

- All malignant tumours (PDAC, CCA, AC and IPMN) vs benign controls.
- PDAC vs benign controls.
- CCA vs benign controls.
- PDAC vs other malignant tumours (CCA, AC and IPMN).
- CCA vs other malignant tumours (PDAC, AC and IPMN).
- CCA vs PDAC.
- Early PDAC vs late PDAC.
- PDAC vs CP.
- Early PDAC vs CP.
- Late PDAC vs CP.

Tables 3.1 to 3.10 show all the top miRNAs differentially expressed across all the comparisons performed with R program DESeq2. Venn diagrams were also generated (see **Figure 3.2** and **Figure 3.3**). Furthermore, a threshold of significance has been established in an adjusted *p*-value of 0.01 – 0.05 and a fold change of 1.5 – 2.

Malignant tumours vs benign controls

Only one miRNA (miR-148a) was significantly differentially expressed between malignant tumours (PDAC, CCA, AC and IPMN carcinoma), and benign controls with an adjusted *p*-value = 0.0172 and fold change of 1.97. This miRNA was observed to be up-regulated in malignant lesions compared to benign lesions (**Table 3.1**).

PDAC vs benign controls

Similar results were observed when PDAC was compared to benign controls, with miR-148a being the only significantly differentially expressed miRNA (**Table 3.2**).

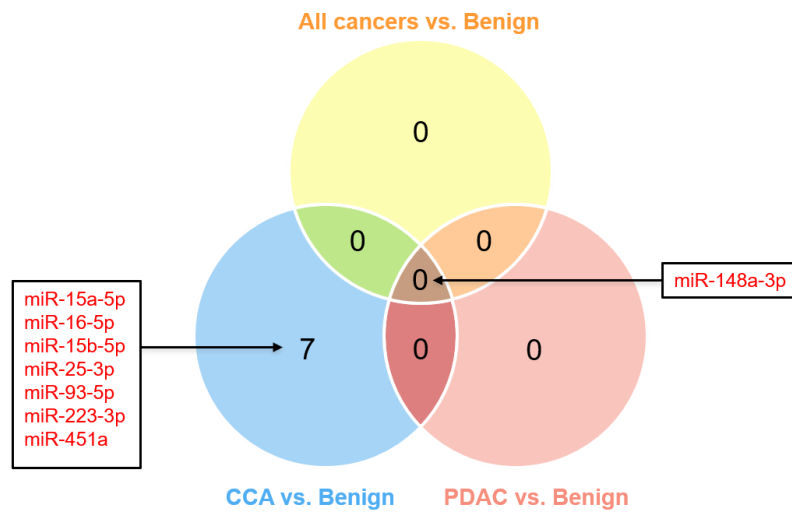
Table 3.1. MicroRNAs differentially expressed in all tumours compared to benign

All malignant tumours versus benign				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-148a-3p	0.9792	0.0004	0.0172	1.9715

Table 3.2. MicroRNAs differentially expressed in PDAC compared to benign

PDAC versus benign				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-148a-3p	0.92849	0.0029	0.1124	1.9032

A)



B)

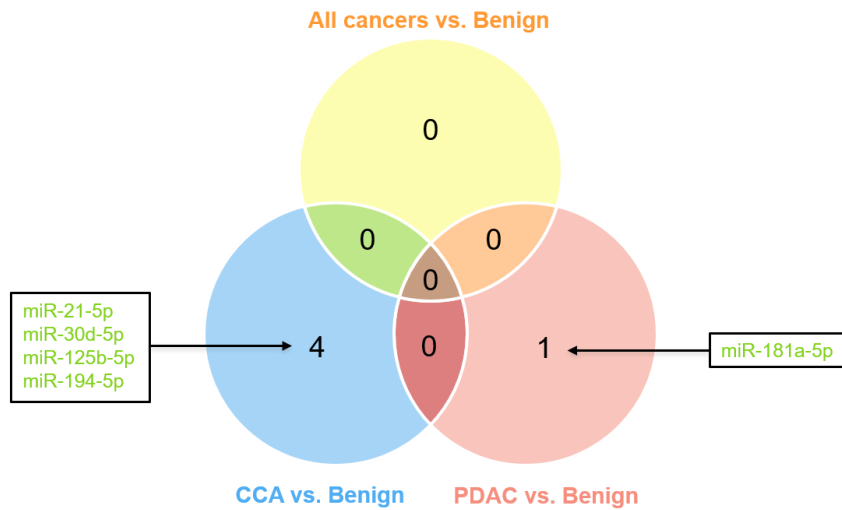


Figure 3.2. Venn diagrams showing up-regulated and down-regulated miRNAs in all malignant tumours compared to benign.

From top to bottom, the expression of different miRNAs is (A) up-regulated (red) or (B) down-regulated (green) in malignant tumours, including PDAC, CCA, AC and IPMN carcinoma lesions, compared to benign lesions.

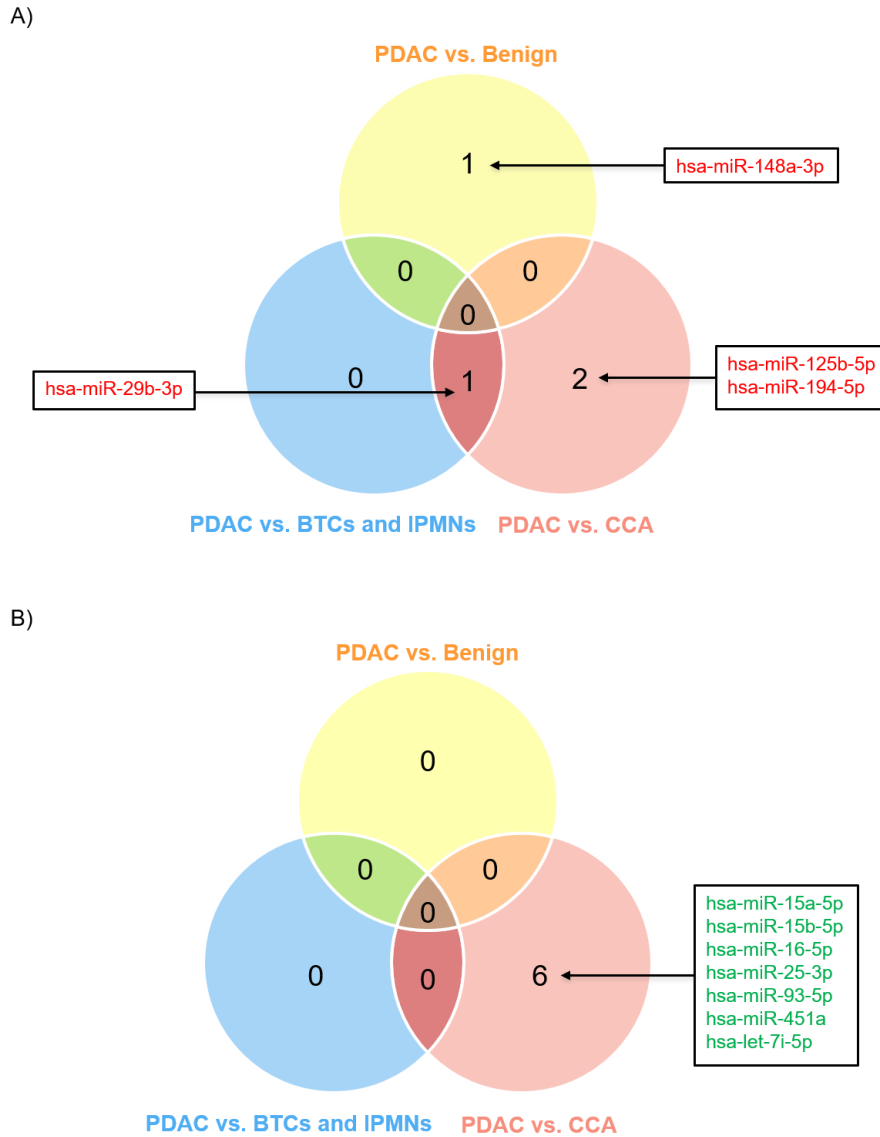


Figure 3.3. Venn diagrams showing up-regulated and down-regulated miRNAs in PDAC.

The expression of different miRNAs is (A) up-regulated (red) or (B) down-regulated (green) in PDAC compared to benign lesions, CCA and other tumours including AC, CCA and IPMN carcinoma lesions.

CCA vs benign controls

Four miRNAs were observed to be significantly differentially expressed between CCA and benign subjects. Three of these miRNAs (miR-15b, miR-25 and miR-93) were found to be up-regulated with a 4- and 3-fold change, respectively, and an adjusted p -

value < 0.05. Conversely, miR-194 was the only significant down-regulated miRNA (adjusted p -value < 0.05), although the fold change was minimal (**Table 3.3**).

Moreover, two miRNAs (miR-15a and miR-16) showed a tendency towards a higher level of expression with a 2.5- and a 3-fold change, respectively, and an adjusted p -value of 0.0511. Another miRNA (miR-30d) showed a tendency towards a lower level of expression (**Table 3.3**).

Table 3.3. MicroRNAs differentially expressed in CCA compared to benign

CCA versus benign				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-15b-5p	2.1976	< 0.0001	0.0014	4.5873
hsa-miR-93-5p	1.6402	0.0002	0.0034	3.1180
hsa-miR-194-5p	-1.8387	0.0028	0.0270	0.2795
hsa-miR-25-3p	1.6277	0.0039	0.0279	3.0904
hsa-miR-15a-5p	1.3430	0.0109	0.0511	2.5369
hsa-miR-16-5p	1.6238	0.0098	0.0511	3.0819
hsa-miR-30d-5p	-1.4395	0.0131	0.0524	0.3686

PDAC vs other malignant tumours

One miRNA (miR-29b) was found up-regulated in PDAC compared to other malignant tumours which include CCA, AC and IPMN carcinoma (adjusted p -value 0.0513 and with a fold change under 2, **Table 3.4**).

Table 3.4. MicroRNAs differentially expressed in PDAC compared to all malignant tumours

PDAC versus all malignant tumours				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-29b-3p	0.9783	0.0013	0.0513	1.9702

CCA vs all malignant tumours

Nine miRNAs were significantly differentially expressed between CCA and other malignant tumours including PDAC, AC and IPMN carcinoma (adjusted p -value < 0.05). Seven miRNAs (miR-15a, miR-15b, miR-16, miR-25, miR-93, miR-451a and let-7i) were up-regulated, showing a fold change between 2.0 and 4.0; whilst miR-125b and miR-194 were down-regulated (adjusted p -value < 0.05) (**Table 3.5**).

Table 3.5. MicroRNAs differentially expressed in CCA compared to all malignant tumours

CCA versus all malignant tumours				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-93-5p	1.7225	< 0.0001	< 0.0001	3.3000
hsa-miR-15b-5p	1.8266	0.0003	0.0040	3.5470
hsa-let-7i-5p	1.0385	0.0005	0.0051	2.0541
hsa-miR-16-5p	1.7823	0.0011	0.0078	3.4398
hsa-miR-451a	2.0191	0.0031	0.0167	4.0535
hsa-miR-25-3p	1.4445	0.0050	0.0226	2.7217
hsa-miR-15a-5p	1.3261	0.0059	0.0231	2.5072
hsa-miR-125b-5p	-1.4293	0.0091	0.0302	0.3712
hsa-miR-194-5p	-1.3007	0.0100	0.0302	0.4059

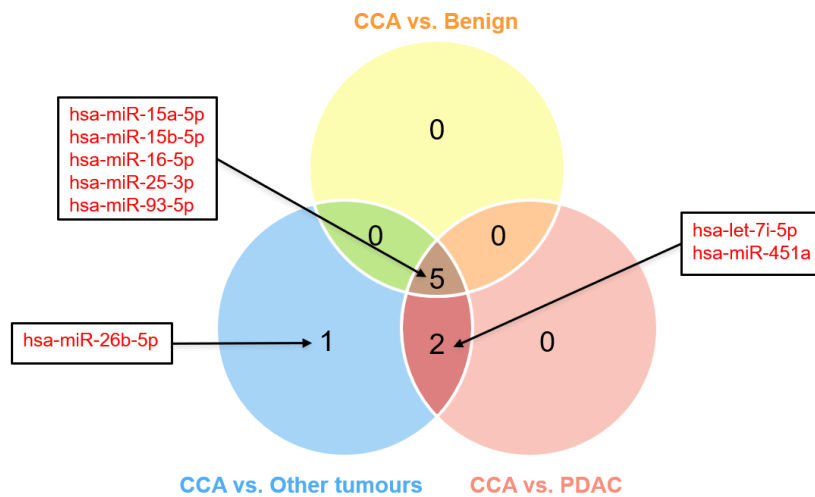
CCA vs PDAC

A comparison between CCA and PDAC was performed and eight miRNAs were observed to be significantly different expressed. Five miRNAs were up-regulated showing a 3-fold change and an adjusted p -value < 0.05. The other three miRNAs (miR-125b, miR-194 and let-7i) were down-regulated (**Table 3.6**). Moreover, miR-15a and miR-29b showed a tendency towards a higher and a lower level of expression, respectively (**Table 3.6**).

Table 3.6. MicroRNAs differentially expressed in CCA compared to PDAC

CCA versus PDAC				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-93-5p	1.6480	< 0.0001	0.0008	3.1340
hsa-miR-25-3p	1.7962	< 0.0001	0.0010	3.4731
hsa-miR-15b-5p	1.8022	0.0004	0.0045	3.4876
hsa-miR-16-5p	1.7776	0.0010	0.0073	3.4285
hsa-let-7i-5p	0.8495	0.0045	0.0224	1.8019
hsa-miR-451a	1.8903	0.0048	0.0224	3.7072
hsa-miR-125b-5p	-1.3564	0.0079	0.0317	0.3905
hsa-miR-194-5p	-1.1579	0.0103	0.0362	0.4481
hsa-miR-15a-5p	1.0797	0.0213	0.0597	2.1135
hsa-miR-29b-3p	-1.1664	0.0194	0.0597	0.4455

A)



B)

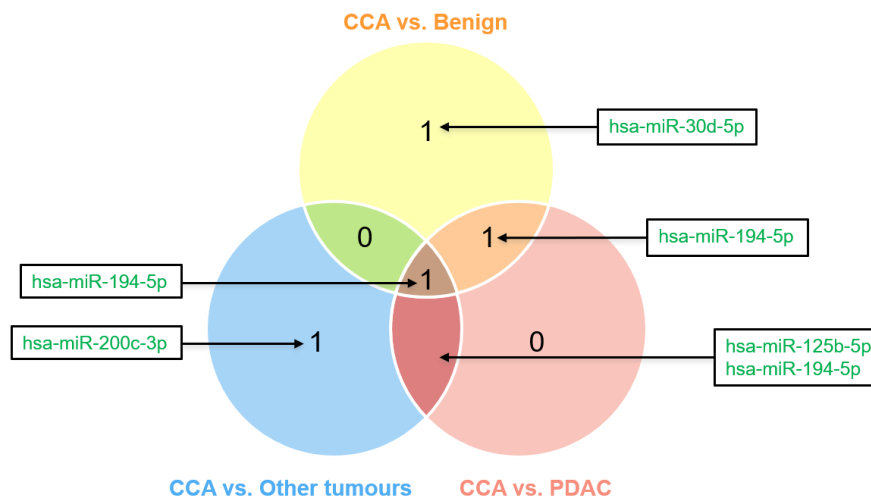


Figure 3.4. Venn diagrams showing up-regulated and down-regulated miRNAs in CCA.

The expression of different miRNAs is (A) up-regulated (red) or (B) down-regulated (green) in CCA compared to benign lesions, PDAC and other tumours including AC, PDAC and IPMN lesions.

Early PDAC vs late PDAC

Four miRNAs exposed a tendency towards a higher (miR-29b and miR-200b) and a lower level of expression (miR-25 and miR-93) when comparing early PDAC and late PDAC (adjusted p -value = 0.1677, **Table 3.7**).

Table 3.7. MicroRNAs differentially expressed in early PDAC compared to late PDAC

Early PDAC versus late PDAC				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-200b-3p	1.0852	0.0197	0.1677	2.1216
hsa-miR-25-3p	-1.1978	0.0178	0.1677	0.4359
hsa-miR-29b-3p	1.6445	0.0163	0.1677	3.1265
hsa-miR-93-5p	-1.1260	0.0070	0.1677	0.4581

PDAC vs CP

In **Table 3.8**, four miRNAs were observed to be significantly different between PDAC and CP (adjusted p -value < 0.05). Three of them (miR-200b, miR-23a and miR-29b) were up-regulated exhibiting a fold change ranging from 3.0 to 5.0, whereas miR-575 was down-regulated.

Early PDAC vs CP

Similarly results were observed when early PDAC was compared to chronic pancreatic (**Table 3.9**). Additionally, miR-148a showed a tendency towards a higher level of expression when comparing early PDAC *versus* CP (adjusted p -value = 0.058 and near a 3-fold change).

Late PDAC vs CP

As shown in **Table 3.10**, only miR-663 was observed to be significantly different when comparing PDAC vs CP (adjusted p -value = 0.01).

Table 3.8. MicroRNAs significantly differentially expressed in PDAC compared to chronic pancreatitis (CP)

PDAC versus CP				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-575	-4.5951	0.0004	0.0164	0.0413
hsa-miR-200b-3p	1.6455	0.0035	0.0364	3.1286
hsa-miR-23a-3p	1.8060	0.0042	0.0364	3.4967
hsa-miR-29b-3p	2.3165	0.0036	0.0364	4.9813

Table 3.9. MicroRNAs differentially expressed in early PDAC compared to chronic pancreatitis (CP)

Early PDAC versus CP				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-200b-3p	1.7912	0.0011	0.0186	3.4632
hsa-miR-575	-4.3676	0.0011	0.0186	0.0484
hsa-miR-29b-3p	2.4913	0.0017	0.0190	5.6229
hsa-miR-23a-3p	1.8238	0.0057	0.0460	3.5401
hsa-miR-148a-3p	1.5246	0.0091	0.0582	2.8771

Table 3.10. MicroRNAs differentially expressed in late PDAC compared to chronic pancreatitis (CP)

Late PDAC versus CP				
miRNA	Log2FC	p-value	p-adj value	FC
hsa-miR-663a	-4.7943	0.0003	0.0120	0.0360

3.2.3 Validation of miRNA microarray data by Reverse Transcription quantitative PCR (RT-qPCR)

To confirm the miRNA microarray results, RT-qPCR was performed and miRNA expression levels were normalised using miR-181c. This miRNA was selected using a program called NormFinder (<http://moma.dk/normfinder-software>), which identifies the optimal normalisation miRNA according to their expression stability among a set of candidates.

3.2.3.1 Up-regulated and down-regulated miRNAs in pancreatobiliary malignancies compared to benign lesions

MiRNA-148a was found to be significantly up-regulated (p -value < 0.0001) in malignant pancreatobiliary lesions and IPMN lesions compared to benign lesions (mean = 17.05 ± 16.33 (SD) vs. 5.66 ± 3.21). This miRNA was also significantly up-regulated in PDAC compared to benign lesions (p -value = 0.0004; 15.07 ± 12.42 vs. 5.66 ± 3.21). Moreover, miR-29b was found to be significantly up-regulated in PDAC (p -value = 0.0038; 53.87 ± 70.55 vs. 12.42 ± 12.72) in comparison to CCA (**Figure 3.5**) and also when PDAC was compared with BTCs and IPMN lesions (p -value = 0.0111; 53.87 ± 70.55 vs. 17.66 ± 27.81). Furthermore, miR-125b and miR-194 were found to be significantly down-regulated in CCA and when compared to benign lesions (miR-125b: p -value = 0.0150, 54.86 ± 37.64 vs. 110.60 ± 71.11 ; miR-194: p -value = 0.0032, 17.99 ± 11.87 vs. 175.50 ± 181.20) (**Fig. 3.5**). These two miRNAs were also significantly down-regulated in CCA in comparison to PDAC (miR-125b: p -value = 0.0096, 54.86 ± 37.64 vs. 126.30 ± 130.80 ; miR-194: p -value = 0.0011, 17.99 ± 11.87 vs. 56.96 ± 55.96) and CCA vs. malignant lesions (miR-125b: p -value = 0.0121, 54.86 ± 37.64 vs. 111.60 ± 117.00 ; miR-194: p -value = 0.0011, 17.99 ± 11.87 vs. 62.76 ± 79.17). Contrary to the bioinformatic analysis, in where miRNA-16 was proving up-regulation in CCA compared with PDAC and compared to other malignant tumours

(see **Table 3.5** and **3.6**), this miRNA was found to be down-regulated instead (see **Fig. 3.5**). The results from the validation of the miRNA microarray data revealed that miR-16 was significantly down-regulated in CCA compared to benign lesions, and also when compared to other malignant lesions (see Appendix Section B.1, **Figure B.1.1**).

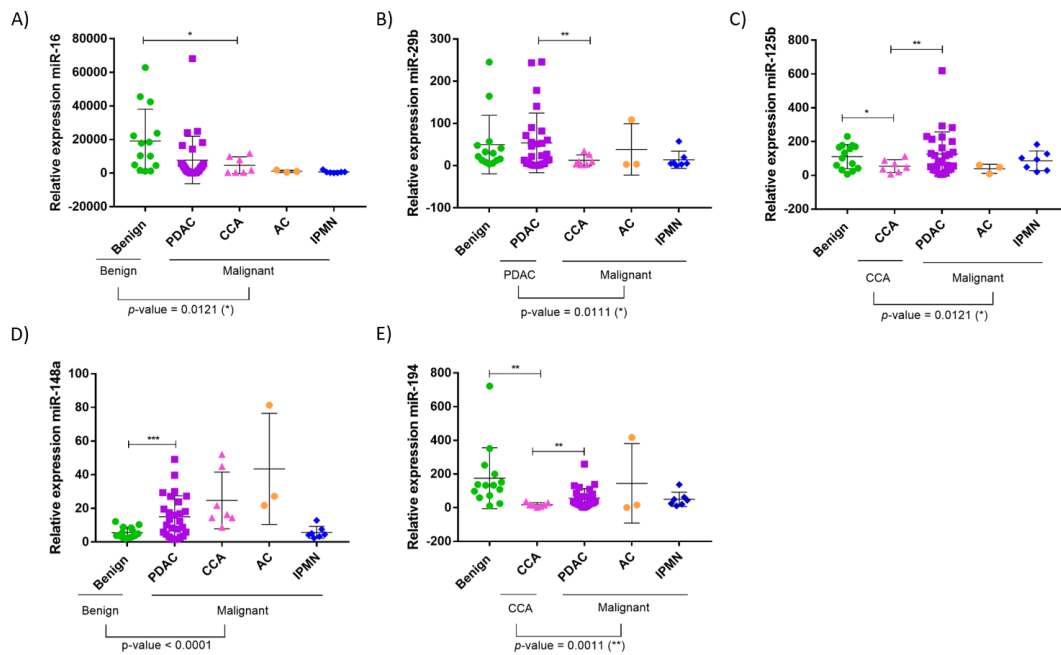


Figure 3.5. Expression levels of miRNAs in different pancreatic and bile duct malignancies (discovery cohort).

A) miR-16, **B)** miR-29b, **C)** miR-125b, **D)** miR-148a and **E)** miR-194.

It is important to mention that most of the miRNA expression levels for the IPMN group are low (**Figure 3.5**), therefore, a positive control for IPMN would be necessary to confirm these results.”

3.2.4 Performance of bile miRNA candidates found in the discovery cohort

The performance of several miRNAs was assessed by Receiver Operating Characteristic (ROC) curve analysis and Area Under Curve (AUC) measurement to determine the discriminatory power of these miRNAs in differentiating between benign and malignant disease and between pancreatic and biliary-tract cancers (BTCs).

3.2.4.1 MiRNA candidates found in the discovery cohort are able to discriminate malignant tumours from benign disease

It can be observed in **Figure 3.6** that miRNA-148a has an AUC higher than 0.75 in discriminating malignant tumours (PDAC, CCA, AC and IPMN lesions) from benign disease (AUC = 0.7532; CI: 0.63 - 0.88; p -value = 0.0046). Moreover, miR-148a differentiated PDAC and BTCs (CCA, AC) from benign disease with an AUC near to 0.80 (see Appendix Section B.1, **Figure B.1.2**). Furthermore, miR-148a exhibited an AUC of 0.7302 in discriminating PDAC from benign disease (see Appendix Section B.1, **Figure B.1.2**)

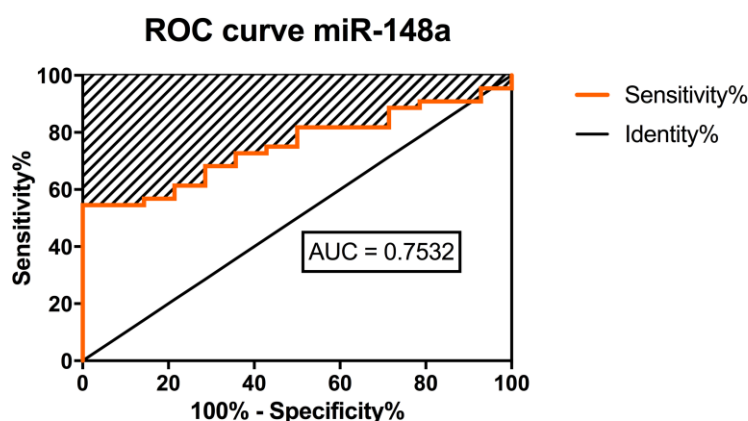


Figure 3.6. ROC curve analysis of miR-148a in discriminating benign from malignant lesions.

MiRNA-148a differentiates malignant lesions (PDAC, BTCs and IPMN lesions) from benign disease with discriminatory power of 75.32%.

Moreover, miR-125b showed an AUC higher than 0.70, whereas miR-194 showed an AUC higher than 0.90 in differentiating CCA from benign controls (Appendix Section B.1, **Figure B.1.3**). Likewise, miR-16 exhibited an AUC > 0.75 in differentiating CCA from benign controls (Appendix Section B.1, **Figure B.1.4**).

3.2.4.2 MiRNA candidates from the discovery cohort are able to distinguish malignancy within pancreatobiliary tract cancers and IPMN lesions

In **Figure 3.7**, miR-194 displayed an AUC > 0.75 in differentiating CCA from PDAC (AUC 0.7884; CI: 0.63 – 0.95; p -value = 0.0203), while miR-125b showed an AUC < 0.70 (AUC = 0.6720, CI: 0.49 – 0.86, p -value = 0.1663). Additionally, miR-29b showed an AUC < 0.70 in distinguishing PDAC from CCA (see Appendix Section B1, **Figure B.1.5**).

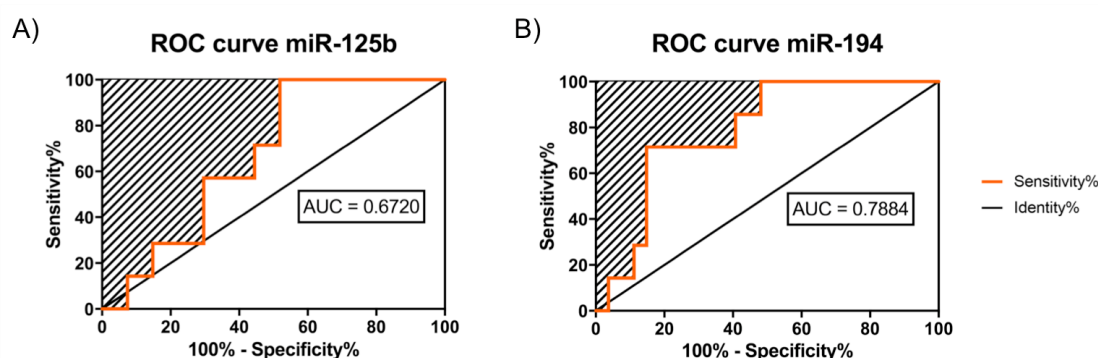


Figure 3.7 ROC curve analysis of different miRNAs discriminating CCA from PDAC.

A) miR-125b, and **B)** miR-194.

Furthermore, miR-125b and miR-194 discriminated CCA from other malignant lesions, which include PDAC, AC and IPMN lesions, (see Appendix Section B1, **Figure B.1.6**) with an AUC < 0.70 and AUC > 0.75, respectively. Moreover, the discriminatory power of miR-29b in distinguishing PDAC from other biliary tract cancers (i.e. CCA and AC) and IPMN lesions was lower than 70% (see Appendix Section B1, **Figure B.1.7**).

3.2.5 Validation of bile miRNA biomarker candidates in an independent cohort by RT-qPCR

3.2.5.1 MicroRNAs are deregulated in pancreatobiliary tract cancers compared to benign controls

To confirm previous results from the discovery cohort, RT-qPCR was performed in an independent cohort of samples and miRNA expression levels were normalised using miR-181c.

MiRNA-148a has been confirmed to be significantly up-regulated in malignant disease compared to benign lesions (p -value = 0.0015; 57.94 ± 78.44 vs. 15.57 ± 22.14) (**Figure 3.8**). Additionally, it was also up-regulated between PDAC and benign disease (see Appendix Section B.2, **Figure B.2.1**).

Furthermore, miR-125b and miR-194 were confirmed to be significantly down-regulated (**Fig. 3.8**) in CCA compared to PDAC (miR-125b: p -value = 0.0030, 82.06 ± 97.38 vs. 243.60 ± 195.50 ; miR-194: p -value = 0.0547, 127.30 ± 65.87 vs. 397.60 ± 838.90). They were also significantly down-regulated in CCA as compared with pancreatobiliary tract cancers (miR-125b: p -value = 0.0028, 82.06 ± 97.38 vs. 373.10 ± 501.30 ; and miR-194: p -value = 0.0164, 127.30 ± 65.85 vs. 599.00 ± 1147).

Contrary to bioinformatic analysis, but in concordance with the results from the discovery, miR-16 was confirmed to be down-regulated in CCA (**Fig. 3.8**) compared to PDAC (p -value = 0.0208, 819.30 ± 880.40 vs. 3364 ± 5965).

Three miRNAs including miR-16, miR-125b and miR-194 were observed to be down-regulated in CCA in comparison to benign lesions (see Appendix Section B.2, **Figure B.2.1**). Additionally, miR-16 was confirmed to be down-regulated in CCA compared to pancreatobiliary tract cancers (see Appendix Section B.2, **Fig. B.2.1**). In the bioinformatic analysis and validation of these data in the discovery cohort, miR-29b

was found to be significantly up-regulated in PDAC as compared to CCA. However, this was not confirmed in the validation cohort. Here, miR-29b was not significantly up-regulated in PDAC compared with CCA (p -value = 0.3223, 10.60 ± 11.60 vs 8.42 ± 10.60); instead a down-regulation was observed when comparing PDAC vs. benign controls (p -value = 0.0146, 10.60 ± 11.60 vs 32.90 ± 55.75), and also between CCA and benign lesions (p -value = 0.0116, 8.42 ± 10.60 vs 32.90 ± 55.75). (**Fig. 3.8**).

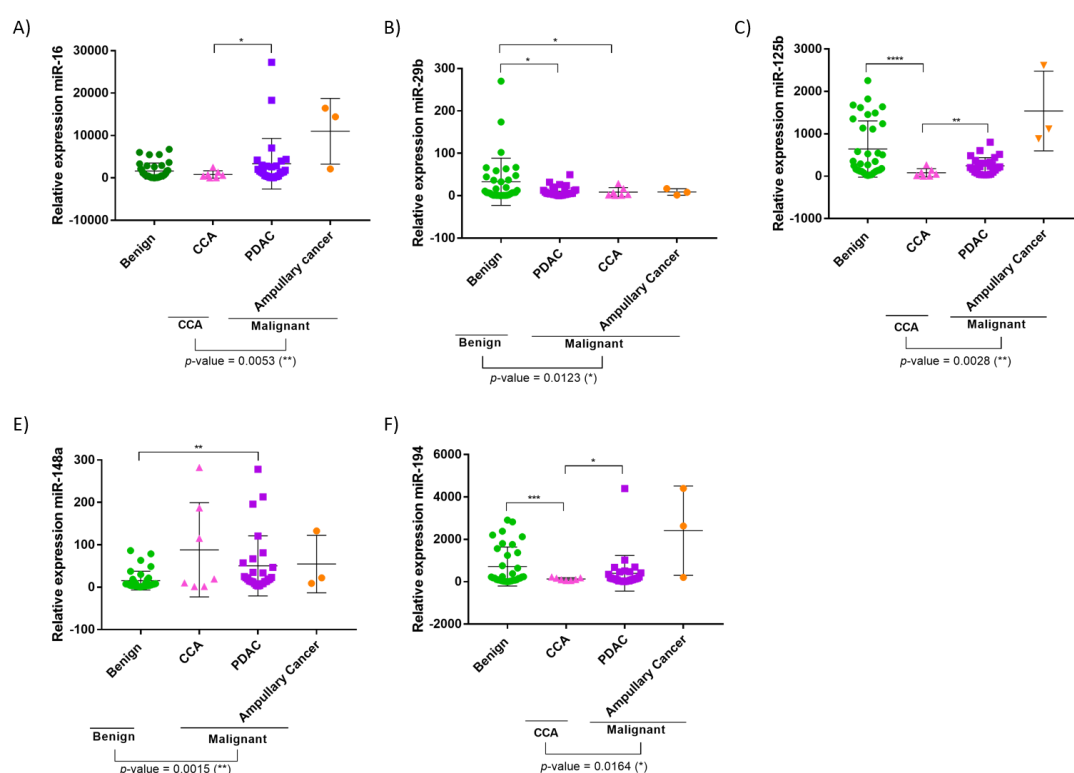


Figure 3.8. MicroRNA expression levels of different types of pancreatico-biliary tract cancers from the validation cohort.

A) miR-16, B) miR-125b, C) miR-148a and D) miR-194.

3.2.6 Performance of candidate miRNAs in the validation cohort

The performance of four miRNAs including miR-29b, miR-125b, miR-148a and miR-194 was assessed by ROC curve analysis and AUC measurement to determine the discriminatory power of these miRNAs in differentiating between benign and malignant disease and between pancreatic and BTCs.

3.2.6.1 Bile miRNAs discriminate benign lesions from malignant tumours

In **Figure 3.9**, miRNA-148a discriminated benign lesions from malignant disease with an AUC > 0.75 (AUC = 0.7552; CI: 0.64 – 0.87; *p*-value = 0.0002).

In contrast, both miR-125b and miR-194 showed an AUC lower than 0.65 (see Appendix Section B.2, **Figure B.2.2**) in differentiating benign controls from malignant disease.

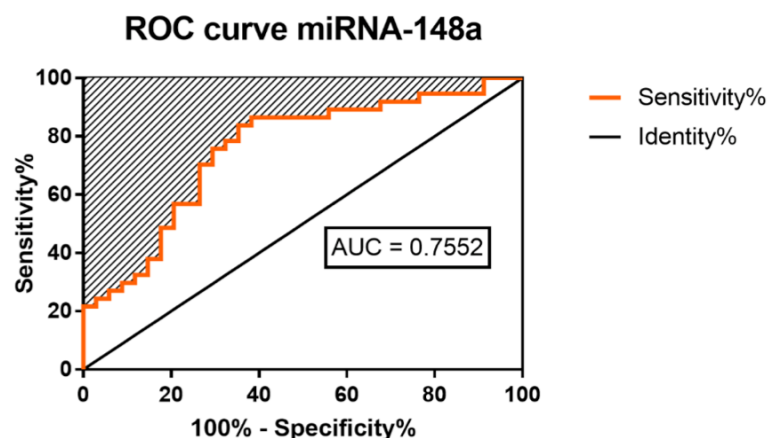


Figure 3.9 ROC curve analysis of miR-148a in discriminating benign from malignant lesions (PDAC, CCA, AC and IPMN carcinoma).

3.2.6.2 Bile miRNAs can differentiate benign lesions from pancreatic cancer and biliary-tract cancers

MiR-148 can distinguish PDAC from benign lesions with an AUC higher than 0.75 (see Appendix Section B.2, **Figure B.2.3**). MiRNA-125b distinguished CCA from

benign controls with an AUC > 0.80 (see Appendix Section B.2, **Figure B.2.4**). Conversely, miR-194 exhibited an AUC lower than 0.65 in discriminating CCA from benign disease (see Appendix Section B.2, **Fig. B2.4**). Similarly, miR-16 differentiated CCA from benign disease (see Appendix Section B.2, **Figure B.2.5**).

3.2.6.3 Bile miRNAs distinguish CCA from PDAC and from other malignant biliary-tract cancers

In **Figure 3.10**, miR-125b distinguished between CCA and PDAC with an AUC > 0.70 (AUC = 0.7937, CI: 0.61 – 0.98, p -value = 0.0181) and between CCA and pancreatico-biliary tract cancers (i.e. PDAC and AC) with an AUC higher than 0.80 (see Appendix Section B.2, **Figure B.2.6**). In contrast, miR-194 showed an AUC = 0.65 (**Fig. 3.10**) in discriminating CCA from PDAC (CI: 0.36 – 0.75; p -value = 0.6547) and an AUC < 0.60 in discriminating CCA from other pancreatico-biliary tract cancers (see Appendix Section B.2, **Fig. B.2.6**). Additionally, miR-16 differentiated CCA from pancreatico-biliary tract cancers (i.e. PDAC and AC) with an AUC > 0.70 (see Appendix Section B.2, **Figure B.2.7**). Moreover, this miRNA also distinguished between CCA and PDAC (AUC > 0.70).

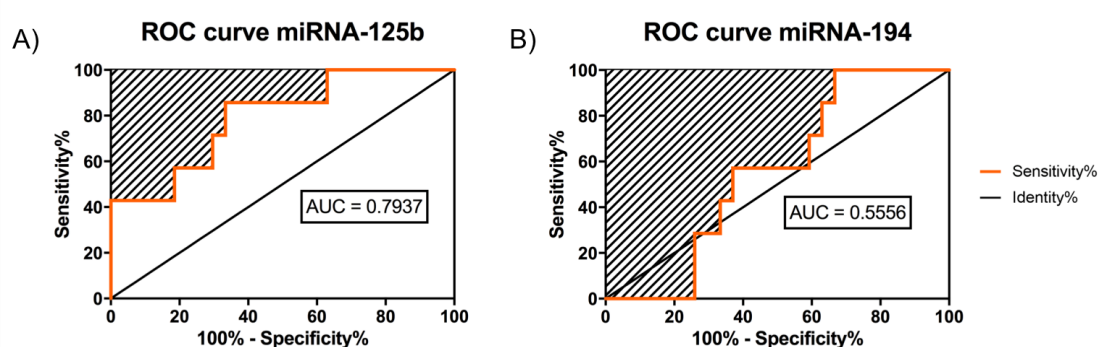


Figure 3.10 ROC curve analysis of miR-125b and miR-194 in discriminating CCA from PDAC

A) miR-125b and **B)** miR-194

3.2.7 Flow chart

A flow chart showing the expression of the most significantly de-regulated miRNAs was created (**Figure 3.11**). It shows miR-148a as the classifier between benign and malignant disease. Moreover, within malignant disease two branches divide two major malignant types of tumour: PDAC and CCA. From each group, there is a list of up- and down-regulated miRNAs found during the bioinformatics analysis when comparing CCA *versus* PDAC and *vice versa*. An initial validation of these findings was performed in the discovery cohort, where most of the miRNAs found up- or down-regulated in the bioinformatics analysis also showed the same expression after being validated, with the exception of three miRNAs from the CCA group (miR15a, miR-15b and miR-16). Contrary to the bioinformatic analysis, these miRNAs were not found to be up-regulated in CCA, but instead down-regulated after validating the miRNA microarray data, although they were not significantly differentially expressed. Subsequently, an alternative cohort of samples was collected (validation cohort) and a second validation was performed. In this final validation, not all the previously discovered miRNAs showed a significantly differential expression. In the PDAC group, there was a shift between the initial validation and the final validation: miR-29b did not exhibit a significantly increased expression when compared with CCA. On the other hand, in the CCA group, from a list of six miRNAs found to be up- and down-regulated, only two were confirmed to be down-regulated in CCA compared to PDAC (miR-125b and miR-194) in all the analysis. MiRNA-16 was initially up-regulated (bioinformatic analysis), but its expression decreased in the following analysis, showing a down-regulation in CCA when compared to PDAC. In the discovery cohort miR-16 (*) was not significantly up-regulated, whereas in the validation cohort it was.

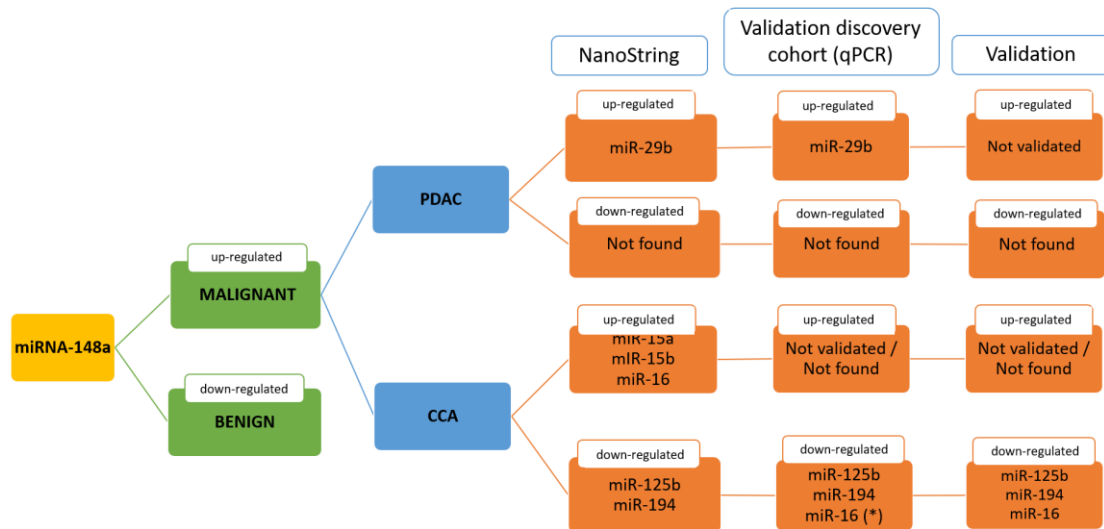


Figure 3.11. Flow chart showing de-regulated miRNAs across PDAC and CCA.

MicroRNA-148a acts as a classifier between benign and malignant disease. The bioinformatics analysis revealed some de-regulated miRNAs in PDAC and CCA, which were validated *a posteriori* in different cohort of samples. Two miRNAs (miR-125b and miR-194) are significantly down-regulated in all the analysis when comparing CCA to PDAC. (*) *in the discovery cohort, miR-16 was not significantly down-regulated in CCA compared to PDAC.*

3.3 Discussion

Currently, imaging techniques alone such as CT and MRI cannot detect pancreatic or BTCs at an early stage, nor differentiate between benign and malignant lesions. Despite that detection of pancreatic cancer at an early stage increases the likelihood of being resectable, in the majority of the cases the cancer present at an advanced stage when surgical resection is no longer possible. Furthermore, CCA and PDAC have overlapping immunohistochemical profiles and are very difficult to distinguish from a clinical and histopathological view. Moreover, blood-based diagnostic markers lack specificity and sensitivity (e.g. CA 19-9) (42). Therefore, sensitive and specific biomarkers to detect PDAC and BTCs at an early stage are necessary. Furthermore,

there are no biomarkers able to robustly stratify these tumours at the time of presentation.

Initial profiling studies focused on discovering a tumoural miRNA expression “signature” in various cancers, especially PDAC and BTCs (169-173, 175). These studies indicated that each tumour exhibited a distinct miRNA expression pattern, which enabled the discrimination between benign and malignant lesions. As a consequence, many researchers have aimed to discover novel diagnostic biomarkers for cancer in liquid biopsies (i.e. blood, saliva, urine) in order to allow more non-invasive methods for detection. Consequently, a large number of studies have focused on demonstrating the feasibility of a clinical useful blood-based miRNA biomarkers for distinguishing between cancer patients and healthy individuals with a high specificity and sensitivity. Findings from these studies indicated that panels of miRNAs could even be effective for discriminating malignant lesions from benign disease (183, 186, 187, 189, 244). However, more specific biomarkers for PDAC and BTCs are still needed. Amazingly, only a few studies have investigated the potential of bile-based miRNAs as useful biomarkers to detect and/or stratify PDAC and BTCs (190, 191).

For the first time, we have identified a miRNA signature in pancreatobiliary tract cancers. The study was divided into two cohorts (discovery and validation) and was composed of benign and malignant disease groups (see **Fig. 3.1**). Next, miRNA profiling of 800 human miRNAs was performed on the discovery cohort (n = 51).

Bioinformatic analyses showed that miR-148a was differentially up-regulated between malignant tumours (PDAC, CCA, AC and IPMN carcinoma) and benign controls (see Venn diagram, **Figure 3.2**), and between PDAC and benign lesions (**Figure 3.3**) with a near 2-fold change (see **Table 3.1** and **Table 3.2**). Moreover, the validation of these results by RT-qPCR confirmed that this miRNA was significantly up-regulated in malignant pancreatobiliary lesions and in IPMN carcinoma compared to benign disease (**Figure 3.5**), exhibiting a good discriminatory power (AUC = 0.7532).

Additionally, miR-148a was significantly up-regulated when compared to PDAC patients vs. benign controls (**Fig 3.5 and Figure B.1.2**).

Another miRNA that was found up-regulated after performing the bioinformatic analysis in PDAC compared to other malignant lesions was miR-29b. Results from the RT-qPCR performed in the discovery cohort (**Fig 3.5**) confirmed it was significantly up-regulated in PDAC when compared to BTCs and IPMN carcinoma. However, miR-29b exhibited a low discriminatory power ($AUC < 0.70$) when comparing PDAC vs. BTCs and IPMN carcinoma (see Appendices, **Figure B.1.7**). Furthermore, bioinformatic analysis showed this miRNA was down-regulated between CCA and PDAC (**Table 3.6**).

Of the miRNAs up-regulated in PDAC compared to benign lesions or to other malignant lesions (CCA, AC, IPMN), but particularly to CCA, miR-148a and miR-29b were chosen for further investigation in an independent cohort of samples (validation cohort). Surprisingly, the results from the validation cohort exposed that miR-29b was not significantly up-regulated when comparing PDAC to BTCs; in fact, it was significantly down-regulated when comparing PDAC *versus* benign controls and between CCA and benign lesions (see Appendices, **Figure B.2.1**). These results showed a change in the expression of miR-29b: from being up-regulated in PDAC as compared to other malignant lesions (**Figure 3.3 and Fig 3.5**), in both bioinformatic analysis and the initial confirmation of these results in a RT-qPCR performed in the discovery cohort, to be down-regulated in an alternative cohort of samples (validation cohort) - where it exhibited a decreased expression and it is unable to significantly discriminate PDAC from CCA or other malignant lesions (**Figure B.2.1**). Therefore, this miRNA was not further validated and additionally analyses were not carried out using this miRNA.

Interestingly, miRNA-148a was significantly up-regulated in malignant disease compared to benign lesions, showing a good discriminatory power ($AUC > 0.75$) in the

validation cohort. In this study, miR-148a has been successfully validated as being significantly up-regulated in malignant lesions compared to benign lesions when performing bioinformatic analyses and when further validating these results in two independent cohorts. Therefore, this miRNA could potentially serve as a classifier between benign and malignant disease.

Conversely, earlier studies described that miR-148a was down-regulated in PDAC compared to normal pancreas (169-171, 173) and its expression was enriched in normal pancreas (170). Furthermore, Bloomston and Schultz showed that miR-148a was down-regulated in PDAC compared to chronic pancreatitis (169, 172). According to Schultz, expression levels of miR-148a were one of the most significantly decreased in PDAC compared to chronic pancreatitis and normal pancreas (172). Hanoun *et al.* indicated that down-regulation of miR-148a is an early marker of PDAC, and microscopic PanIN lesions also have decreased expression of this miRNA (245). Furthermore, miR-148a was included in a 5-miRNA panel, which in combination with standard cytology was able to improve PDAC detection up to 91% (246). Taken together, these studies have demonstrated that miR-148a is significantly down-regulated in PDAC tissue compared to normal pancreas and chronic pancreatitis. These studies performed miRNA expression profiling in tissues (i.e. FFPE and EUS-FNA biopsies), whereas in the present study profiling was performed using bile samples. A few studies have shown that cancer cells secrete miRNAs via EVs into the circulation enabling cellular communication with the microenvironment (198, 239). Thus, one reasonable explanation for the difference in expression between the bile fluid and tissues is that malignant cells in the pancreas and/or the biliary tree actively release this miR-148a into the bile and it is therefore detected at higher levels compared with patients with benign disease. Perhaps this is a mechanism by which cancer cells get rid of this documented tumour suppressor miRNA (170, 245, 247-250)

in order to ensure their survival (i.e. low expression inside the cell, and higher in the extracellular space).

Moreover, several studies have demonstrated that miR-148 is down-regulated in other types of cancer, including gastric, colorectal, melanoma and hepatoblastoma (247, 251, 252). Furthermore, Zheng *et al.* suggested that miR-148a acts as a tumour suppressor in metastatic gastric cancer and its decreased expression contributes to lymph node metastasis and tumour progression (248). However, in osteosarcoma the expression levels of miR-148a were increased (253). Also, Murata *et al.* suggested that up-regulation of miR-148a could promote cell growth in prostate cancer cells (254). Expression of this miRNA is therefore clearly tumour specific.

In contrast, plasma levels of miR-148a were up-regulated in multiple myeloma patients compared with healthy controls, and its increased expression was associated with a shorter relapse-free survival time (255). Nonetheless, serum levels of miR-148a were significantly low in non-small cell lung cancer (NSCLC) patients as compared to healthy individuals (256). Yang *et al.* suggested that miR-148a has a tumour suppressive role in NSCLC and its low expression is associated with large size tumours and lymph node metastasis (256).

In summary, several studies have been able to show that miR-148a is dysregulated in cancer and its expression is dependent on cell context.

On the other hand, several microRNAs were also observed to be de-regulated in CCA compared to other types of cancers and benign lesions. The bioinformatic analysis revealed five of them to be up-regulated, including miR-15a, miR-15b, miR-16, miR-25 and miR-93, whilst miR-125 and miR-194 were observed to be down-regulated (see **Table 3.5**, **Table 3.6** and **Figure 3.4**). However, none of the five up-regulated miRNAs showed a significant difference when comparing bile samples from CCA and PDAC patients in the discovery and validation cohorts. This is most likely to be due to the

normalization techniques used during bioinformatic analyses and also the increased sensitivity of TaqMan assays. Nonetheless, the levels of expression of miR-16 were decreased, rather than increased as shown in previous analyses, between CCA and PDAC, as well as when comparing CCA to other malignant tumours and to benign lesions (see **Figure 3.5** and **Figure B.1.1**). Following the validation of the bioinformatic analysis, miR-125b and miR-194 were shown to be significantly decreased in CCA in comparison to PDAC, other malignant lesions (PDAC, CCA and IPMN carcinoma) and benign lesions (see **Fig. 3.5**). Moreover, miR-194 showed an excellent discriminatory power in differentiating CCA from benign controls (see **Figure B.1.3**). Both miRNAs (miR-125b and miR-194) also exhibited good to moderate discriminatory power in distinguishing CCA from PDAC (**Figure 3.7**), and in differentiating CCA from other pancreatobiliary malignancies and IPMN carcinoma (**Figure B.1.6**).

To further validate these results, an independent cohort of samples was used. Subsequently, miR-125b and miR-194, which were found to be down-regulated in CCA compared to PDAC, and to other pancreatobiliary cancers and benign lesions, were further validated in this cohort. Likewise, miR-16 was selected to confirm whether its' down-regulation could be further validated in an independent cohort. As observed in the previous results from this study, miR-125b and miR-194 were significantly down-regulated in CCA compared to PDAC, as well as when comparing CCA to benign lesions, and CCA to other malignant tumours (**Figure 3.8**). Furthermore, miR-125b was able to differentiate CCA from PDAC and showed moderate discriminatory power (see **Figure 3.10**). In contrast, the discriminatory power of miR-194 in differentiating CCA from PDAC was lower in the validation cohort compared to the results from the discovery cohort (AUC = 0.7884 (**Figure 3.7**), AUC = 0.5556 (**Figure 3.10**), respectively). Moreover, miR-194 was unable to distinguish CCA subjects from controls or from other pancreatic and biliary-tract cancers with sufficient discriminatory power (see **Figure B.2.4** and **Figure B.2.6**) compared to the discovery cohort.

Deregulation of miRNAs has been reported in multiple human cancer types. For example, Visone *et al.* has shown that miR-125b was down-regulated in thyroid carcinoma samples compared to normal thyroid tissue. Additionally, expression of miR-125b was lower in mouse thyroid cancer tissues compared to normal tissues. Therefore, miR-125b down-regulation may be implicated in thyroid carcinogenesis (257). Likewise, Nam *et al.* have identified several differentially expressed miRNAs in serous ovarian cancers compared to normal ovarian tissues. Among these, miR-125b was found to be down-regulated in ovarian carcinoma (258). Furthermore, Kappelmann and colleagues found that miR-125b levels were decreased in malignant melanoma tissue samples and cell lines compared to primary human melanocytes (259). These data suggest that miR-125b could be a potential tumour suppressor in malignant melanoma, serous ovarian carcinoma and thyroid cancer. Conversely, Lee and colleagues showed that inhibition of miR-125b decreased cell growth in prostate cancer cells (260).

Shi and co-workers have demonstrated that increased expression of miR-125b triggers prostate cancer growth in xenograft tumours in intact and castrated nude mice (261). Thus, this miRNA has oncogenic features in prostate cancer, and its down-regulation results in suppression of prostate cancer growth. Furthermore, inhibition of miR-125b showed an increase in the sensitivity of prostate cancer cells to different therapeutic treatments leading to cell death (261). Ottaviani *et al.* showed that miR-125b is induced by TGF- β signalling pathway and regulates PDAC progression by promoting stemness, epithelial-mesenchymal transition (EMT) and tumourigenesis (262). They also demonstrated that inhibition of miR-125b sensitises PDAC cells to gemcitabine (GEM) triggering apoptosis. Similarly, Wang *et al.* found that serum levels of miR-125b were associated with chemotherapeutic resistance in breast cancer (263). Indeed, higher levels of circulating miR-125b were observed in non-responsive breast cancer (263) patients. Furthermore, reducing expression of miR-125b sensitised breast cancer cells

to chemotherapy (263). Moreover, expression levels of miR-125b were associated with tumour size, lymph node metastasis and disease stage. Advanced breast cancer patients exhibited a higher expression of miR-125b than early stage patients and healthy controls (263). Taken together, miR-125b has an oncogenic and/or tumour suppressor role depending on the type of human cancer.

Recently, Zhang *et al.* demonstrated that levels of miR-194 were significantly increased in PDAC tissue compared to adjacent non-tumorous tissue. Additionally, serum levels of miR-194 were higher in PDAC patients compared to healthy controls (264). Hence, there was a positive correlation between the levels of miR-194 in tissue and serum. However, the discriminatory power to differentiate PDAC patients from healthy individuals was markedly low for this miRNA; therefore, it may not be a suitable serum diagnostic marker for PDAC. Moreover, Bernuzzi and colleagues also showed that serum levels of miR-125b and miR-194 were increased in CCA compared with healthy control subjects (265). Furthermore, two different studies also demonstrated that miR-194 was overexpressed in human breast (266) and colon cancer cell lines (267) promoting cell proliferation and tumour angiogenesis, respectively.

Schultz *et al.* demonstrated that miR-194 was down-regulated in PDAC and AC compared with chronic pancreatitis. Moreover, miR-194 was significantly down-regulated in PDAC compared to AC (172). Likewise, Khella (268) and Meng (269) suggested that miR-194 could act as a tumour suppressor in renal cell and hepatocellular carcinoma, respectively. They found that overexpression of miR-194 reversed cell migration and invasion.

In the present study, miR-148a was significantly up-regulated in bile from patients with malignant lesions compared to benign controls, whereas miR-125b and miR-194 were significantly down-regulated in CCA in comparison to PDAC. All three miRNAs showed moderate discriminatory power, in both discovery and validation cohorts, in differentiating, on one side benign lesions from malignant disease (miR-148a), and on

the other side CCA from PDAC (miR-125b and miR-194). To show an overall view of the good performance of these three miRNAs, a combined cohort composed of the discovery cohort and the validation cohort was created. Each cancer type, except IPMN lesions, was included in this combined cohort (see **Appendices Section B.3**). Within this combined cohort, miR-148a was significantly up-regulated in malignant disease compared to benign lesions, and was able to differentiate benign from malignant lesions with good discriminatory power (**Figure B.3.1**). Furthermore, miR-125b and miR-194 were significantly down-regulated in CCA compared to PDAC (**Figure B.3.2**) and differentiated with a good discriminatory power between CCA and PDAC patients (**Figure B.3.3**). Additionally, these two miRNAs differentiated CCA from malignant disease and benign lesions with a good discriminatory power (**Figure B.3.4 and Figure B.3.5**). Therefore, it is suggested that miR-148a could serve as a classifier of malignancy, whilst miR-125b and miR-194 might discriminate CCA from PDAC, being unique bile-based biomarkers for CCA.

In summary, previous studies focused on profiling the expression of miRNAs in various types of cancers, in which tissue samples, cell lines and bodily fluids were used, showed a dysregulation in the expression levels of miR-125b, miR-148a and miR-194, which directly and/or reversely correlate with the findings in this thesis. In particular, the expression of miR-125b, miR-148a and miR-194 in pancreatic cancer tissue samples and cell lines is reversely correlated with the expression in bile. Taken together, this suggests that all the aforementioned miRNAs display a different expression in human cancers depending on the type of tumour.

CHAPTER 4. Pancreatic cell medium contains exosomal miRNAs

4.1. Background

Exosomes are small extracellular vesicles (30 to 150 nm in size) of endocytic origin, which contain proteins, lipids and nucleic acids (e.g. DNA, RNA and miRNAs) characteristic of their cellular origin (270). Exosomes can be secreted to the extracellular microenvironment by a variety of cells including stem cells (271), neurons (272, 273) and cancer cells (239), where they play a vital role in cell-cell communication transferring information through their cargo (209). Furthermore, they are found in many bodily fluids such as (e.g. blood, saliva, urine, breast milk and synovial fluid) and are implicated in cancer development, angiogenesis, pre-metastatic niche formation and metastasis (229-231, 235-237).

Isolation of extracellular vesicles (EV) from biological fluids such as saliva, urine, plasma, and cell culture medium is performed following standard methods including differential ultracentrifugation, size exclusion chromatography, ultrafiltration, density gradient separation, polymer-based reagents and immunoaffinity capture. Each methodology has its advantages and disadvantages, and the choice of a particular technique is based on the cost, time, equipment and downstream analysis. For instance, differential ultracentrifugation is the gold standard method and uses high speed centrifugation steps to obtain EV. However, it requires a large amount of starting material and most of the time is difficult to collect. Thus, an alternative to isolate exosomes from small volumes of samples is to use chemical reagents provided in commercial kits specific for exosome purification.

4.2 Results section

4.2.1 Pancreatic cell media contains exosomes

4.2.1.1 Pancreatic cell lines

A total number of six pancreatic cell lines were used to purify exosomes, which include two normal pancreatic ductal epithelium cell lines (HPNE and H6c7) and four PDAC cell lines (TKCC-02, TKCC-10, PANC-1 and MiaPaCa-2). All six pancreatic cell lines were grown with their corresponding cell culture growth media (see [section 2.1.5](#)) supplemented with exosome-depleted FCS.

4.2.1.2 Isolation of exosomes from pancreatic cell culture medium

Exosomes were isolated from cell culture supernatants from a total of six pancreatic cell lines using a commercial kit (Exo-Quick TC). Pancreatic cell lines were grown in exosome-depleted medium for 24-72 hours and then their cell media was collected. Exosomes have several markers on their surface membrane, including tetraspanins CD9, CD63 and CD81. A transmission electron microscope (TEM; JEOL 1200 EX) was used to assess the size of the EV followed by immunogold labelling with CD63 antibody in order to evaluate the exosomes seen. In **Figure 4.1** TEM images show exosomes with gold-labelled anti-CD63 attached to all pancreatic cell lines and negative controls without labelling. TEM analysis indicated that the isolated EV are exosomes (40-100 nm in diameter) and have CD63 surface marker.

To further confirm the isolation of exosomes was successful, detection of other exosomal surface markers (CD9 and CD81), as well as CD63, was determined by ELISA. The total number of exosomes present in each sample was also quantified using the ExoELISA kit specific for CD9, CD63 and CD81. The standard curves for each protein (CD9, CD63 and CD81) are shown in the Appendix Section B.4 (**Figure B.4.1**).

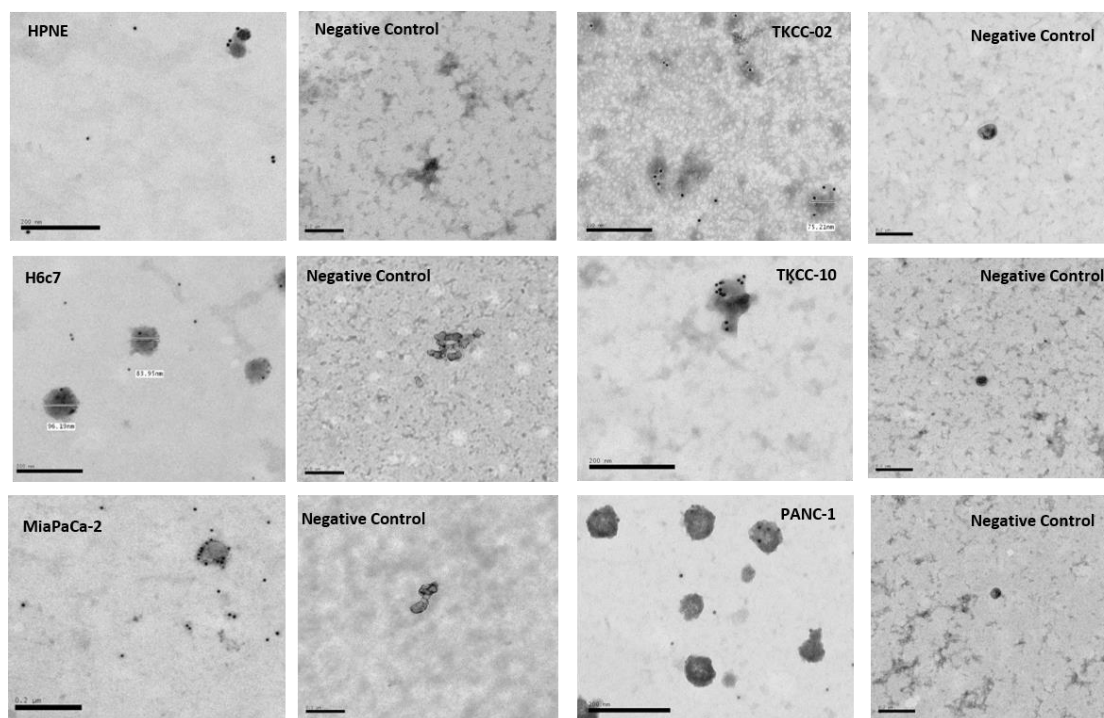


Figure 4.1. Transmission electron microscopy.

Exosomes from six pancreatic cell lines were visualised under TEM and immunogold labelling of CD63 was performed. The black dots represent the immunogold staining with CD63 antibody. The negative controls are exosomes without immunogold labelling for CD63.

The ExoELISA protein standards are calibrated to NanoSight, and therefore the number of exosome readings (particles/ml) can be obtained after isolating exosomes from biofluids. Exosomes were quantified from four pancreatic cell lines, including H6c7, TKCC-02, TKCC-10 and MiaPaCa-2. The number of exosomes detected in each of these pancreatic cell lines is summarised in **Table 4.1**.

Table 4.1. Number of exosomes in pancreatic cell lines

Pancreatic cell line	Number of exosomes	Detection exosomal marker
H6c7	5.49×10^7	CD63
TKCC-02	6.35×10^6	CD63
TKCC-10	2.54×10^9	CD81
MiaPaCa-2	1.36×10^9	CD9

4.2.2 Identification of exosomal microRNAs in pancreatic cell medium

4.2.2.1 Exosomal RNA isolation

Exosome pellets from all six pancreatic cell lines were obtained using ExoQuick-TC kit and were re-suspended in 500 µl of PBS. Then, TRIzol LS Reagent was used to extract RNA from exosome pellets following manufacturer's instructions, with a few modifications of the protocol (see [section 2.2.3.1](#)).

The quality and quantity of all exosomal RNA samples was assessed with a microfluidics-based platform (Agilent Bioanalyzer). However, the RNA concentration was lower than 1 ng/µl and the RNA Integrity Number (RIN) ranged between 1.5 – 2.5.

4.2.2.2 Exosomal miRNA expression in pancreatic cell lines

To determine the exosomal microRNA expression levels in all six pancreatic cell lines, a reverse transcription quantitative PCR (RT-qPCR) was performed. The expression of three miRNAs including miR-125b, miR-148a and miR-194, which were discovered in a miRNA profiling performed on human bile samples, was measured using TaqMan assays. Despite the low RIN, we obtained reasonable C_T values. The miRNA expression levels were normalised using small nucleolar U6.

Furthermore, the expression of the aforementioned miRNAs was also measured in the pellet and supernatant of these six pancreatic cell lines. Nevertheless, the miRNA expression in the supernatant of MiaPaCa-2 was only performed for two miRNAs (miR-148a and miR-194) as there was not sufficient supernatant material to measure an additional miRNA. Therefore, the miRNA expression levels of miR-125b in MiaPaCa-2 cell line were only assessed in the cell pellet and in exosomes.

4.2.2.2.1 miR-125b

The cell pellet of H6c7, TKCC-10 and PANC-1 exhibited significantly decreased levels of miR-125b compared to the supernatant (p -value = 0.0020, p -value = 0.0173, p -value = 0.0036, respectively), whereas the expression of this miRNA was significantly higher in the supernatant of the aforementioned pancreatic cell lines compared to their exosomes (p -value = 0.0023, p -value = 0.0207, p -value = 0.0021, respectively). Furthermore, this miRNA showed a tendency towards a high expression in the supernatant of TKCC-02 compared to the cell pellet (p -value = 0.0725) and the exosomes (p -value = 0.0878). Moreover, miR-125b was significantly high expressed in exosomes compared to the cell pellet of H6c7 and TKCC-02 (p -value = 0.0372 and p -value = 0.0474, respectively). (**Figure 4.2**). In contrast, the expression of this miR-125b in HPNE was not statistically significant when comparing the supernatant, the cell pellet and the exosomes (cell pellet vs supernatant, p -value = 0.203; cell pellet vs exosomes, p -value = 0.2584; supernatant vs exosomes, p -value = 0.1842). In MiaPaCa-2, the expression of miR-125b was only measured in the cell pellet and in the exosomes (**Fig 4.2**). However, despite observing increased expression in exosomes compared to the cell pellet, it was not statistically significant (p -value = 0.2145).

4.2.2.2.2 miR-148a

The expression of miR-148a was significantly lower in the cell pellet of three PDAC cell lines compared to the supernatant (TKCC-10, p -value = 0.0058; PANC-1, p -value = 0.0200; MiaPaCa-2, p -value = 0.0196, respectively) as well as a significantly lower expression in the cell pellet of HPNE compared to the supernatant (p -value = 0.0260). Conversely, miR-148a exhibited significantly higher expression in the cell pellet of H6c7 and MiaPaCa-2 (p -value = 0.0209 and p -value = 0.0019). In contrast, the exosomal expression of miR-148 was higher in four pancreatic cell lines: two normal pancreatic cell lines (HPNE and H6c7) and two PDAC cell lines, including the primary

cell cultures TKCC-02 and TKCC-10, when comparing supernatant vs. exosomes. Whereas PANC-1 and MiaPaCa-2 exhibited a higher expression of miR-148a in the supernatant compared to the exosomes (**Fig 4.2**). However the expression of miR-148a was not statistically significant when comparing the supernatant and the exosomes, except in TKCC-02 (p -value = 0.0163). Furthermore, decreased expression of miR-148a was observed in the cell pellet of all six pancreatic cell lines compared to exosomes. However, only H6c7, TKCC-02 and PANC-1 exhibited a statistical differential significance (p -value = 0.0269, p -value = 0.0231 and p -value = 0.0024, respectively).

4.2.2.2.3 miR-194

Overall, the expression of miR-194 was observed to be lower in the cell pellet compared to the supernatant in all six pancreatic cell lines. However, only HPNE and PANC-1 exhibited a statistically differential significance (p -value = 0.0303 and p -value < 0.0001, respectively). Moreover, TKCC-10 and MiaPaCa-2 showed a tendency towards a lower level of expression in the cell pellet compared to the supernatant (p -value = 0.0649 and p -value = 0.0620, respectively). Moreover, the aforementioned pancreatic cell lines also showed a lower expression of miR-194 in the cell pellet compared to exosomes; although only TKCC-02 exhibited a statistical differential significance (p -value = 0.0016). The expression of miR-194 was higher in exosomes compared to the supernatant of pancreatic normal cell lines (HPNE and H6c7), whilst in the four PDAC cell lines the expression of this miRNA was lower in the exosomes compared to the supernatant. However, only TKCC-02 and PANC-1 showed a statistical differential significance (p -value < 0.001).

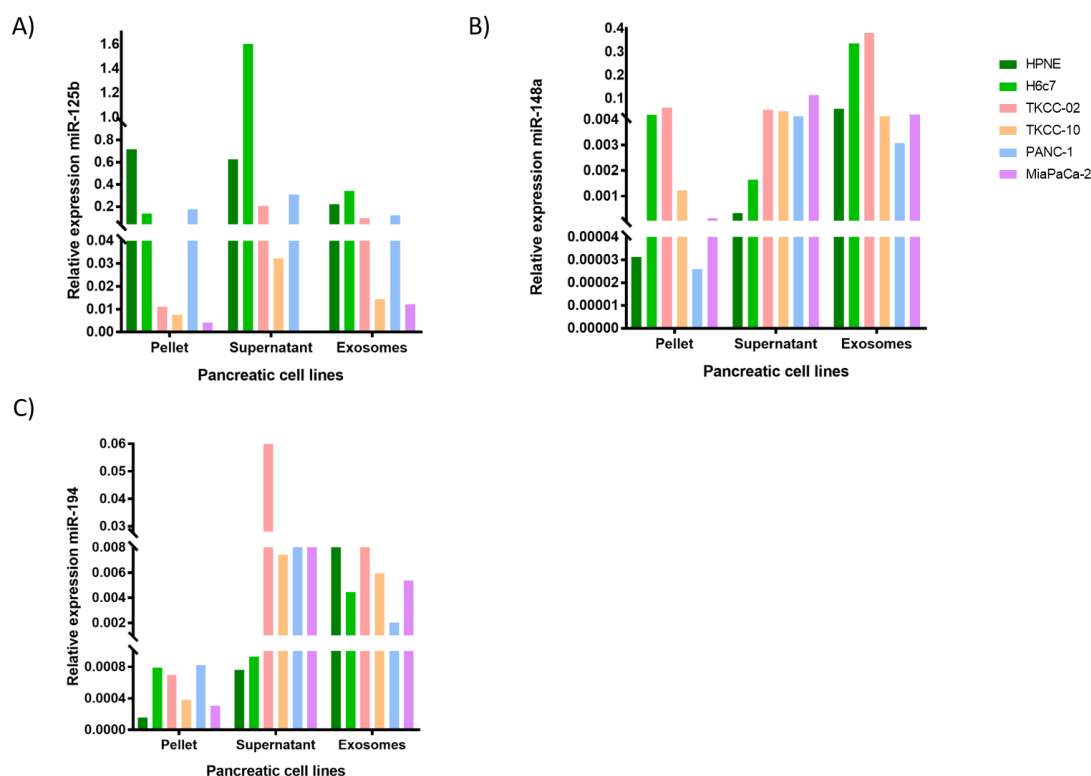


Figure 4.2. Expression levels of miRNAs in cell pellet, supernatant and exosomes from normal and cancer pancreatic cell lines.

A) miRNA-125b; B) miRNA-148a and C) miRNA-194

** MiaPaCa-2 is only expressed in pellet and exosomes of miR-125b.*

4.3 Discussion

4.3.1 Purification and characterisation of exosomes in pancreatic cell lines

There are different methodologies to purify exosomes from cell culture medium and biofluids. Nevertheless, the purification of extracellular vesicles from either biological fluids such as saliva, blood, urine, or cell culture medium results in a heterogeneous population of vesicles. Moreover, these extracellular vesicles overlap in size range and have a similar morphology, which hinders a precise separation. However, they are

different in terms of origin, biogenesis mechanisms and composition. Thus, they can be distinguished based on their extravesicular membrane and lumen composition (205).

It is important to verify the existence of exosomes by characterising their size distribution and the presence of specific protein markers. Therefore, different techniques have to be performed in order to confirm the isolation of exosomes. In this project, the confirmation of exosome isolation was carried out using two different methods: 1) to determine the size of the EV, TEM was used and 2) immunogold staining of the exosomal surface marker CD63.

After reading the absorbance of the samples at 450 nm with the spectrophotometer, standard curves for protein standards CD9, CD3 and CD81 were generated and they were used to know the exact number of exosomes contained in the medium of six pancreatic cell lines. After the analysis, some pancreatic cell line samples showed a negative absorbance value meaning that the absorbance value was lower than the blank sample. Thus, it can be assumed that the number of exosomes in those particular samples was zero or below the range of the ELISA.

One possible reason for this might be the small volume of pancreatic cell medium used to isolate exosomes. According to Thery and colleagues exosomes are successfully detected from cell culture medium using higher amounts of volume (e.g. 70 ml) because the yield of the purification process increases with the starting volume (217). Additionally, these authors also recommend to proceed with exosome purification as soon as possible, to avoid potential exosome loss due to storing the supernatants at +4°C or -80°C for prolonged periods. For example, Tauro and co-workers used 750 ml of cell culture medium to purify exosomes by ultracentrifugation and they obtained a yield of 375 µg of protein from 6×10^8 cells (220).

In this project, exosome-depleted supernatants were kept at -80°C for a couple of months until they were used. Therefore, this may be one of the reasons behind the difficulty in detecting exosomes in some pancreatic cell lines. Although Théry and colleagues indicated that some investigators have successfully isolated exosomes from cell supernatants kept at -80°C (217). Furthermore, a second reason could be the type of isolation protocol chosen, which was by polymer-based reagent. This method enables an enrichment of EV in the sample, however, it compromises the purity of the material as other protein contaminants might be co-isolated together, resulting in a low purity of the exosome material.

Exosomes were only quantified in four pancreatic cell lines: TKCC-02 contained the lowest number of exosomes (6.35×10^6), whereas TKCC-10 had 2.54×10^9 exosomes, followed by MiaPaCa-2 with 1.36×10^9 exosomes. A total of 5.49×10^7 exosomes were present in H6c7 pancreatic cell line. Nonetheless, the fact that exosomal markers were not detected by ELISA in HPNE and PANC-1, and therefore, the concentrations of exosomes were not quantified, does not necessarily mean that exosomes are not present in these samples as they were observed in TEM images (**Figure 4.1**). Perhaps when performing the ExoELISA more standard dilutions should have been done to cover a larger range of concentrations of exosomes in order to detect the minimal number of exosomes present in the samples.

4.3.2 Exosomal microRNA expression in pancreatic cell lines

The exosomal expression of different miRNAs was assessed in six pancreatic cell lines. The expression of miR-125b was significantly higher in the supernatant of three pancreatic cancer cell lines (H6c7, TKCC-10 and PANC-1) compared to either the cell pellet or the exosomes. Moreover, this miRNA showed a tendency towards an increased expression in the supernatant of TKCC-02 compared to the cell pellet and

exosomes, whereas the expression of miR-125b in HPNE was not statistically significant when comparing the supernatant to the cell pellet or the exosomes. The exosomal expression of miR-125b was significantly higher only in H6c7 and TKCC-02 when compared to the cell pellet (**Figure 4.2**).

Exosomal miR-148a was significantly over-expressed in the three pancreatic cell lines H6c7, TKCC-02 and PANC-1 compared to the cell pellet and significantly over-expressed in exosomes from TKCC-02 in comparison to the supernatant. The lowest level of expression of miR-148a was observed in the cell pellet of HPNE, TKCC-10, PANC-1 and MiaPaCa-2 in comparison to the cell supernatant, whereas H6c7 and TKCC-02 showed an overexpression of this miRNA in the cell pellet. Furthermore, three cell lines including H6c7, TKCC-02 and PANC-1 demonstrated a significantly higher expression of miR-148a in their exosomes compared to the cell pellet (**Fig 4.2**). Despite the fact that all four PDAC cell lines showed higher expression levels of miR-194 in exosomes compared to the supernatant, only TKCC-02 and PANC-1 cells exhibited a significantly over-expression. Likewise, this miRNA was significantly higher in exosomes from TKCC-02 compared to the cell pellet. Two cell lines (H6c7 and TKCC-10) showed a tendency towards an increase level of expression of miR-194 in exosomes compared to the cell pellet. Markedly decreased expression levels of miR-194 were observed in the cell pellet of all six pancreatic cell lines compared to the supernatant or the exosomes. However, only HPNE and PANC-1 showed a significant down-regulation in the expression of miR-194 in the cell pellet compared to the supernatant. Two PDAC cell lines (TKCC-10 and MiaPaCa-2) showed a tendency towards a lower level of expression of miR-194 in the cell pellet compared to the supernatant (**Fig 4.2**).

Taken together, the expression of miR-125b, miR-148a and miR-194 was lower in the cell pellet of four PDAC cell lines (TKCC-02, TKCC-10, PANC-1 and MiaPaCa-2), whilst the expression of these miRNAs was higher in the supernatant and exosomes.

These findings correlate with the hypothesis that cancer cells release these miRNAs as circulating-free miRNAs and/or within EV (exosomes), where they are detected at higher levels compared with tumour (i.e. cell pellet) or normal cells.

Several studies have revealed that tumour-derived exosomes contain miRNA signatures specific to different types of cancer. For instance, Corcoran *et al.* indicated that miR-148a expression was reduced in cells and exosomes of prostate cancer cells. This miRNA was significantly increased in prostate cancer tissues compared with matched benign tissues. Nevertheless, in prostate cancer patients this circulating-free urinary miR-148a was significantly reduced in comparison to those patients with benign prostatic hyperplasia (274).

Furthermore, Alegre *et al.* found that expression levels of miR-125b in exosomes were significantly reduced in patients with melanoma compared to healthy individuals and disease-free melanoma patients (275). Interestingly, circulating-free miR-125b has been found to be down-regulated in metastatic melanoma (259), thyroid (257) and ovarian cancers (258). Alegre and colleagues observed a directly association between the levels of miR-125b in exosomes and the melanoma stage disease: lower levels of exosomal miR-125b were correlated with advanced melanoma stage (275).

Recently, two studies performed an expression profiling of miRNAs derived from exosomes using serum (276) and plasma (249) samples from patients with early-stage PDAC to discriminate patients from healthy individuals. In both studies, only one exosomal miRNA was found up-regulated and its expression was higher in PDAC cases compared to healthy controls. Chen *et al.* also reported that in the supernatant of PDAC cells the expression of miR-23b was higher compared to normal pancreatic cells (276). Takahasi and colleagues showed a significant association between exosomal miR-451a levels and PDAC stage and tumour size (249). Additionally, high exosomal miR-451a was associated with recurrence and poor prognosis in PDAC patients after surgical resection. Likewise, Xu *et al.* performed a next generation small

RNA sequencing of miRNAs derived from exosomes in PDAC cell lines and observed a significantly higher expression levels of three miRNAs in exosomes derived from PDAC cells compared to normal pancreatic cells (h-TERT-HPNE cells) (277). Moreover, these 3 exosomal miRNAs isolated from plasma samples of PDAC patients (early stage) exhibited significant increased levels when compared to controls (277). Other studies have also reported similar findings using serum samples from patients with PDAC where the expression of exosomal miRNAs (miR-17, miR-21, miR-191 and miR-451) was increased compared to healthy controls (278, 279). Wang *et al.* reported that exosomes released from hypoxic PDAC cells containing miR-301a activated macrophages, which facilitated migration, invasion and epithelial-mesenchymal transition (EMT) of pancreatic cancer cells both *in vivo* and *ex vivo* (280). They demonstrated that miR-301a was highly expressed in hypoxic PDAC cells and enriched in their exosomes (280). Likewise, Takikawa and colleagues observed that pancreatic stellate cells also released exosomes containing a variety of miRNAs, and their interaction with PDAC cells (PANC-1 and SUIT-2) stimulated cell migration and proliferation (281). Other studies have suggested that exosomes are involved in PDAC chemo-resistance to gemcitabine (282-284).

Therefore, exosomes appear to be important components of the tumour microenvironment as they mediated the cross talk between PDAC cells and adjacent stroma cells by delivering their content, which ultimately influences tumour biology.

CHAPTER 5. CONCLUSIONS

5.1 Conclusions

Pancreatic ductal adenocarcinoma (PDAC) and biliary tract cancers (BTC) have few early symptoms, and these are also often unspecific. These tumours are characterised by painless jaundice due to biliary obstruction and are difficult to differentiate from each other because of the close anatomical proximity of the structures affected. A substantial improvement in non-invasive imaging techniques has enabled improved diagnosis and allowed better planning of therapies for patients with PDAC and BTC. However, biomarkers to improve diagnosis, and stratification of these tumours are lacking. Indeed, the only blood biomarker in clinical use for PDAC and BTC is CA 19-9. However, it is not an accurate screening blood test for early detection of PDAC, as it is not sensitive or specific enough. Taken together, further investigations are required to improve the detection of these tumours and discover molecular therapeutic strategies.

In the last two decades, miRNAs have emerged as potential diagnostic and therapeutic targets for human cancers. They can act as oncogenes or tumour suppressors in a cellular dependent manner contributing to cancer development. Therefore, the discovery of novel miRNA biomarkers associated with PDAC or BTCs in the bile fluid would be a considerable discovery, due to the close proximity of this biological fluid to the malignant lesion.

Previous studies in blood and tissues have demonstrated the existence of diagnostic and prognostic miRNA signatures for PDAC. In this thesis, human bile has been profiled to identify a miRNA signature for PDAC and BTCs in order to improve detection in cases of diagnostic dilemma. Due to the viscosity of the human bile, the methodology followed to isolate RNA had to be optimised by implementing a series of additional steps to obtain a good quality and quantity.

We have shown that miRNAs can be measured in bile specimens and can differentiate between benign and malignant pancreato-biliary disease. Moreover, bioinformatics analyses revealed a specific miRNA profile across all cancer subtypes: PDAC, CCA, AC and IPMN carcinoma. We identified three bile-based miRNAs, miR-125b, miR-148a and miR-194, able to differentiate benign from malignant lesions, and also PDAC from CCA. MiRNA-148a was up-regulated in malignant pancreato-biliary tract cancers compared to benign lesions. On the other hand, miR-125b and miR-194 were down-regulated in CCA compared to PDAC and other malignant tumours, as well as to benign lesions.

Unexpectedly, a few studies focused on the discovery of miRNAs in bile fluid as markers for diagnosis in PDAC (191) and CCA (190) did not showed these three miRNAs as significantly different. One of the reasons could be the misrepresentation of BTC patients. For instance, one study measured the differential expression of selected miRNAs, which was based on previous studies from the literature, in plasma and bile from patients with a benign condition, chronic pancreatitis and PDAC (191). A different study focused their investigation on the evaluation of miRNA patterns across patients with PSC and CCA in bile and serum (190). In this thesis, the main objective was to profile miRNAs from bile samples to be able to discriminate PDAC and BTCs from benign disease. Hence, it is possible that these three miRNAs discovered here (miR-125b, miR-148a and miR-194) are more specific to this type of malignancies related to the biliary tree system and the pancreas given that Cote *et al.* and Voigtländer *et al.* compare PDAC and CCA, respectively, to benign conditions rather than to other biliary pathologies such as CCA, AC or IPMNs (190, 191). Moreover, another difference between the study carried out by Voigtländer and colleagues and the present one is the lack of validation of the results in an alternative cohort of samples to confirm there is a correlation between previous findings and an independent series of patients.

On the other hand, previous miRNA profiling studies in cancer reported a differential expression of miR-125b, miR-148a and miR-194, indicating that all three miRNAs display a different expression in human cancers depending on the tumour type (169, 251, 258, 259). Particularly, in PDAC studies miR-148a has been shown to be down-regulated (170), whilst miR-125b and miR-194 (CCA vs PDAC) were up-regulated (172, 262). Surprisingly, our findings indicate that the expression of these three miRNAs is reversed in the bile, suggesting that the tumour either releases specific miRNAs into the extracellular space (i.e. bile) as they may have a tumour suppressor role, such as miR-148a, or preserves them inside the cell in order to promote survival (i.e. miR-125b and miR-194).

Furthermore, we have demonstrated that pancreatic cell lines contain exosomes, and that exosomal miRNAs can be measured. This was done to determine whether candidate miRNAs discovered in PDAC bile samples were also expressed in PDAC exosomes. Indeed, we demonstrate that these miRNAs are down-regulated in the cell pellet of almost all PDAC cell lines compared with the matched supernatant and isolated exosomes.

Therefore, it appears that PDAC cells release specific miRNAs, either circulating-free or enriched within exosomes, into the extracellular space and these may therefore appear in the bile at higher levels. These miRNAs may have tumour suppressor functions and therefore the cancer cells are trying to “excrete” them in order to ensure survival.

MiRNAs appear to be robust molecules able to serve as potential biomarkers in the bile to detect and stratify pancreatobiliary tumours. Furthermore, exosomes are EVs involved in intercellular communication and can modulate the behaviour of neighbouring cells, as well as the tumour microenvironment, inducing the formation of pre-metastatic niches. Exosomes contain miRNAs that are delivered to recipient tumour cells potentially stimulating migration and proliferation. The bile-based miRNAs

that we have identified appear enriched in cancer cell exosomes and their functions need to be further elucidated.

In summary, bile-based miRNAs may be clinically useful biomarkers to help detect pancreatobiliary cancers, stratify patients, inform treatment decisions and predict survival, therefore allowing a more “personalised” treatment strategy, and better outcomes.

5.2. Future directions

In light of the results presented in this thesis, it would be interesting to investigate a few aspects further.

Next, expression of the candidate bile miRNAs will be correlated with clinicopathological factors and survival outcomes for the patients with pancreatobiliary tumours, in order to see whether these biomarkers can indeed stratify patients and prognosticate.

Candidate miRNAs identified in this retrospective study will be validated in patients presenting with suspected pancreatobiliary disease and/or obstructive jaundice. During endoscopic retrograde cholangio-pancreatography (ERCP), bile will be aspirated and RNA isolation will be performed. The ability of the candidate miRNAs discovered in the retrospective study will be assessed to detect malignancy at the time of ERCP and compare the sensitivity, specificity and AUC to standard clinical tests. In parallel, blood from matched bile samples will be collected and processed to obtain plasma and isolate RNA. Then, miRNA levels will be measured and correlated with levels in matched bile samples. Moreover, to investigate the source of bile miRNAs and to determine whether their expression is associated with tumoural levels, a small fresh frozen core biopsy or section from the formalin-fixed paraffin-embedded (FFPE)

tissue blocks will be collected from patients undergoing any surgical intervention to perform a miRNA analysis (e.g. *in situ* hybridisation) to compare tissue levels to those in bile and blood.

In this thesis, we have demonstrated that pancreatic cell lines contain exosomes; however, this has not been tested in cholangiocarcinoma cell lines. Therefore, we will determine whether our bile-miRNA candidates are also expressed in exosomes from cholangiocarcinoma cell lines and normal cholangiocytes.

Early studies have suggested that serum and plasma exosomal miRNAs might be sensitive and specific diagnostic markers in PDAC (238, 278). Recently, it has been shown that biliary exosomes can be isolated, and their miRNA cargo may have potential as biomarkers in BTCs (198, 285). The methodology to isolate circulating exosomes from bile will have to be optimised. After isolating exosomes from benign, PDAC and BTC bile samples, RNA extraction will be performed and small RNA sequencing will be carried out in order to identify differentially expressed miRNAs. Moreover, their ability to discriminate between benign and malignant lesions will be assessed. Correlation between biliary miRNAs and biliary-exosomes miRNAs in these samples will be also assessed to elucidate their source in bile and their potential as clinically useful miRNA biomarkers in patients with pancreatobiliary disease.

APPENDICES

A. Clinical data

Table B.1. Clinicopathological data of the discovery cohort

Diagnosis		Benign (n = 15)	PDAC (n = 28)	CCA (n = 6)	AC (n = 3)	IPMN (n = 6)
Age (years)		38 - 77	49 - 11	41 - 81	62 - 78	55 - 72
Gender	F	8	11	2	-	3
	M	7	17	4	3	3

Table B.2. TNM staging of the discovery cohort

Diagnosis	TNM staging						
	I	IIa	IIb	IIIa	IIIb	IVa	IVb
PDAC (n = 28)	-	8	14	-	2	-	4
CCA (n = 6)	1	-	2	-	2	-	1
AC (n = 3)	1	-	2	-	-	-	-
IPMN (n = 6)	-	-	-	1	5	-	-

Table B.3. Clinicopathological data of the validation cohort

Diagnosis		Benign (n = 34)*	PDAC (n = 27)	CCA (n = 7)	AC (n = 3)	CP (n = 4)
Age (years)		29 - 74	46 - 80	49 - 80	53 - 68	33 - 69
Gender	F	19	11	3	-	2
	M	14	16	4	3	2

(*) missing: 1 benign

Table B.4. TNM staging of the validation cohort

Diagnosis	TNM staging						
	I	IIa	IIb	IIIa	IIIb	IVa	IVb
PDAC (n = 34)	1	1	13	3	5	-	4
CCA (n = 7)*	-	-	4	-	1	-	-
AC (n = 3)	-	-	3	-	-	-	-

(*) missing: 2 CCA

B.1 Expression and performance of miRNAs in the discovery cohort

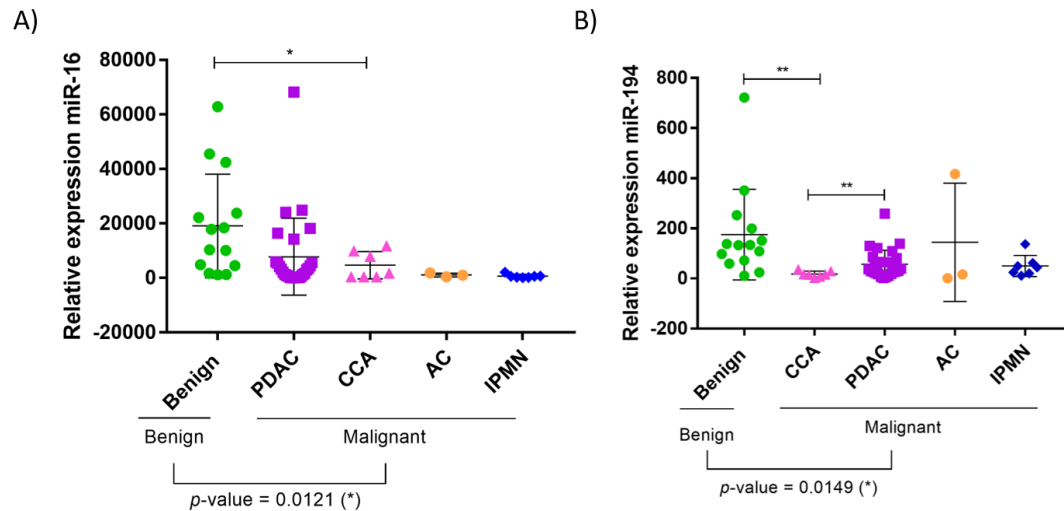


Figure B.1.1 Relative expression levels of miR-16 and miR-194 across different types of pancreatic and bile duct malignancies (discovery cohort).

A) miR-16 is down-regulated in CCA compared to benign lesions ($p\text{-value} = 0.0087$, mean = 4706 ± 5026 (SD); mean = 19090 ± 19028). MiRNA-16 is up-regulated in benign lesions as compared to malignant lesions (miR-16: $p\text{-value} = 0.0121$, 19090 ± 19028 vs 5724 ± 11523).

B) miR-194 is up-regulated in benign lesions as compared to malignant lesions (miR-194: $p\text{-value} = 0.0149$, 175.50 ± 181.20 vs 55.64 ± 74.44).

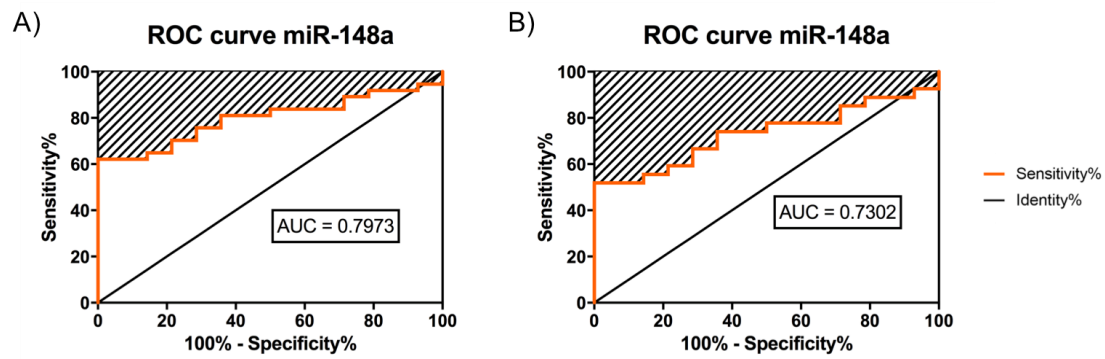


Figure B.1.2. ROC curve analysis of miR-148a in discriminating benign from pancreatico-biliary tract cancers (PDAC, CCA and AC).

A) miR-148a differentiates between benign lesions and pancreatico-biliary tract cancers (i.e. PDAC, CCA and AC) with an AUC = 0.7973 (CI: 0.69 - 0.92; p -value = 0.0012). **B)** miR-148a distinguishes benign from PDAC with a discriminatory power of 73.02% (CI: 0.58 - 0.88; p -value = 0.0168).

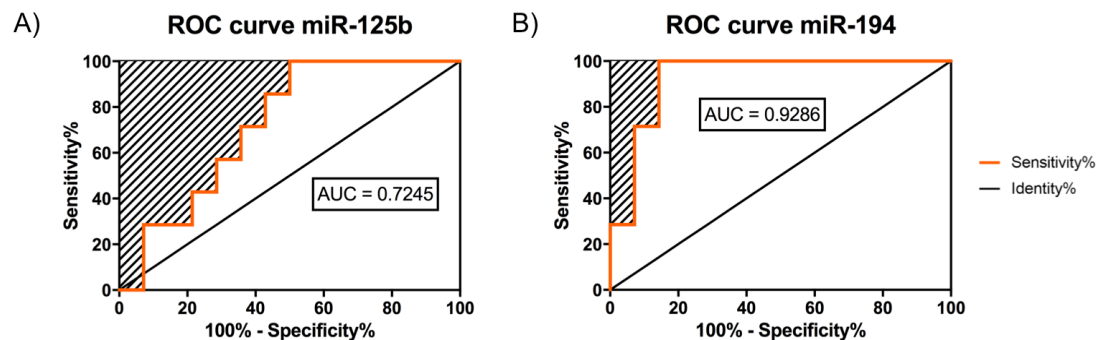


Figure B.1.3. ROC curve analysis of miR-125b and miR-194 in discriminating CCA from benign lesions.

A) miR-125: (AUC = 0.7245, CI: 0.5091 – 0.9339, p -value = 0.1007), and **B)** miR-194 (AUC = 0.9286, CI: 0.82 – 1.04; p -value = 0.0017).

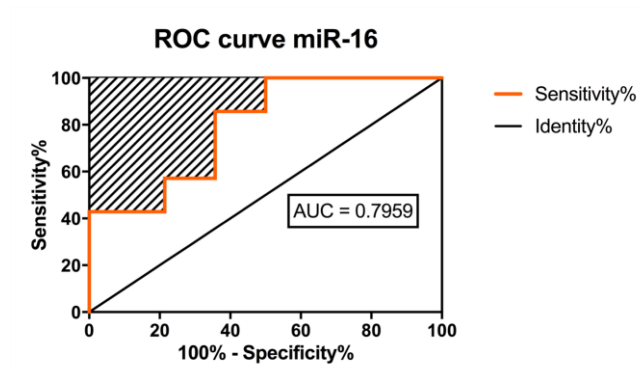


Figure B.1.4. ROC curve analysis of miR-16 in discriminating benign lesions from CCA.

MiRNA-16 exhibits an AUC = 0.7959 (CI: 0.5990 – 0.9928, p -value = 0.0305).

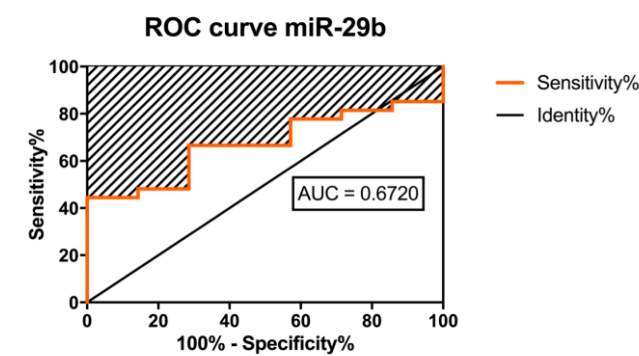


Figure B.1.5. ROC curve analysis of miR-29b in discriminating PDAC from CCA.

MiR-29b shows an AUC of 0.6720 (CI: 0.49 - 0.86, p -value = 0.1663) in distinguishing PDAC from CCA.

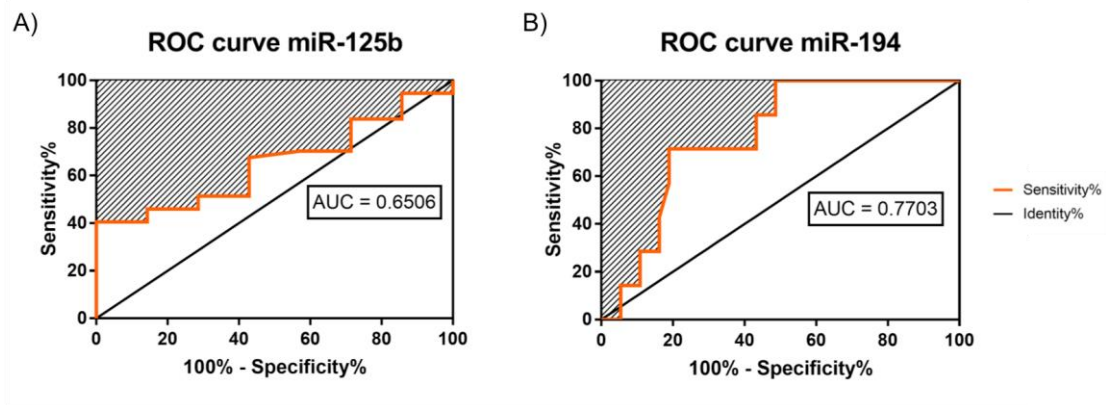


Figure B.1.6. ROC curve analysis of miR-125b and miR-194 in discriminating CCA from other malignant pancreatico-biliary tumours.

A) miR-125b distinguishes CCA from PDAC, AC and IPMN with a discriminatory power of 65.05% (CI: 0.50 – 0.83, p -value = 0.2108). **B)** miR-194 differentiates CCA from pancreatico-biliary tract cancers (i.e. PDAC and AC) and IPMN lesions with a 77.03% discriminatory power (CI: 0.62 -0.92; p -value = 0.0247).

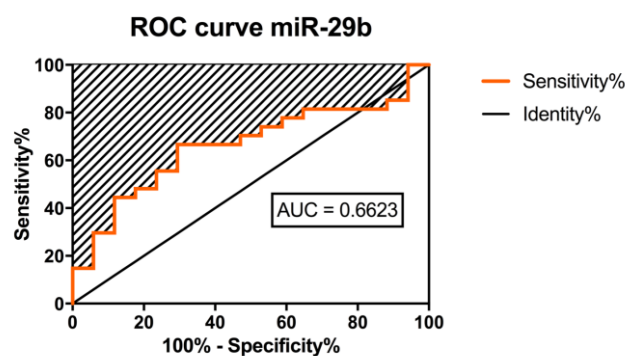


Figure B.1.7. ROC curve analysis of miR-29b discriminating PDAC from other cancers of the bile duct.

MiR-29b exhibits and AUC = 0.6623 (CI: 0.50 - 0.83; p -value = 0.0725).

B.2. Expression and performance of miRNAs in the validation cohort

cohort

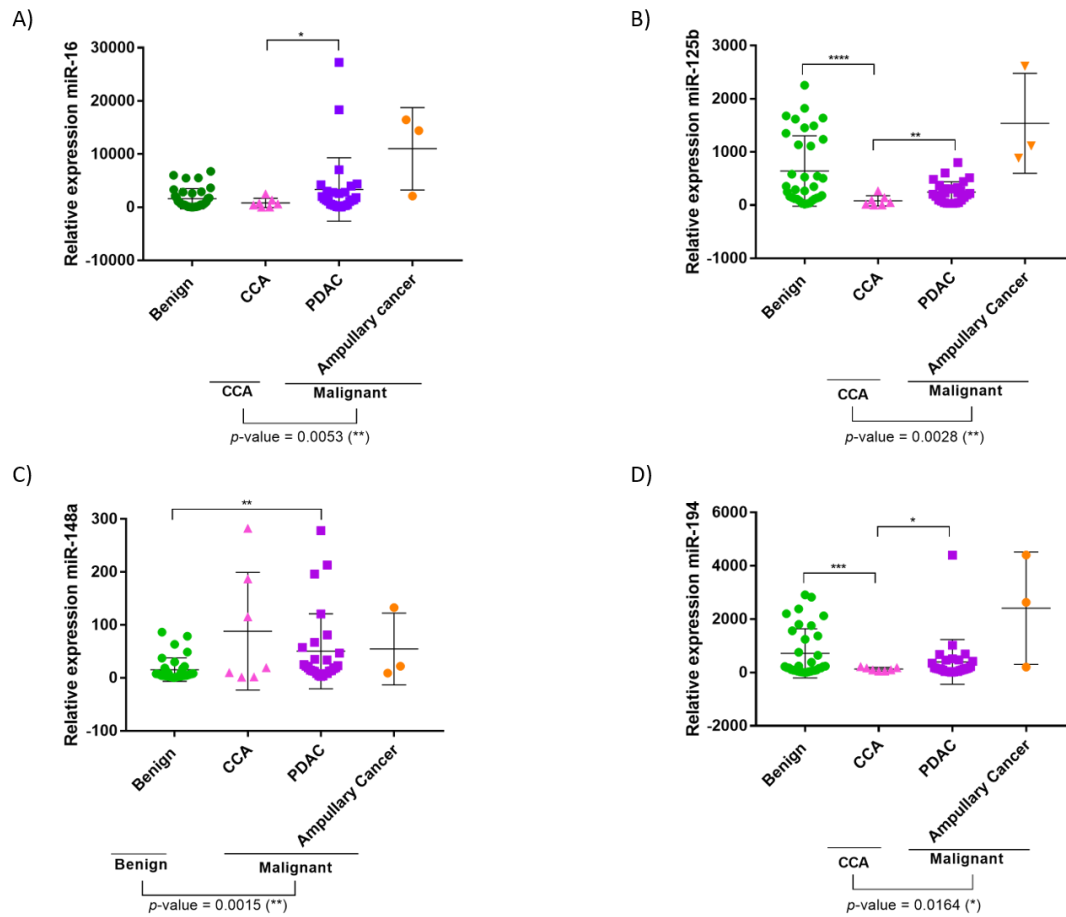


Figure B.2.1 Relative miRNA expression levels across different tumour types (validation cohort).

A) miR-16 is down-regulated in CCA compared to pancreatico-biliary tract cancers ($p\text{-value} = 0.0053$, mean = 819.30 ± 880.40 (SD), mean = 4127 ± 6440), and it is also down-regulated in CCA in comparison to benign lesions ($p\text{-value} = 0.0208$, 819.30 ± 880.40 vs 1621 ± 1907). **B)** miR-125b is down-regulated in CCA compared to benign lesions ($p\text{-value} < 0.0001$, 82.06 ± 97.38 vs 642.90 ± 662.90). **C)** miR-148a differentiates PDAC from benign disease ($p\text{-value} = 0.0098$; 50.42 ± 70.76 vs 15.57 ± 22.14). **D)** miR-194 is down-regulated in CCA in comparison to benign disease ($p\text{-value} = 0.0004$, 127.30 ± 65.87 vs 714.10 ± 919.30).

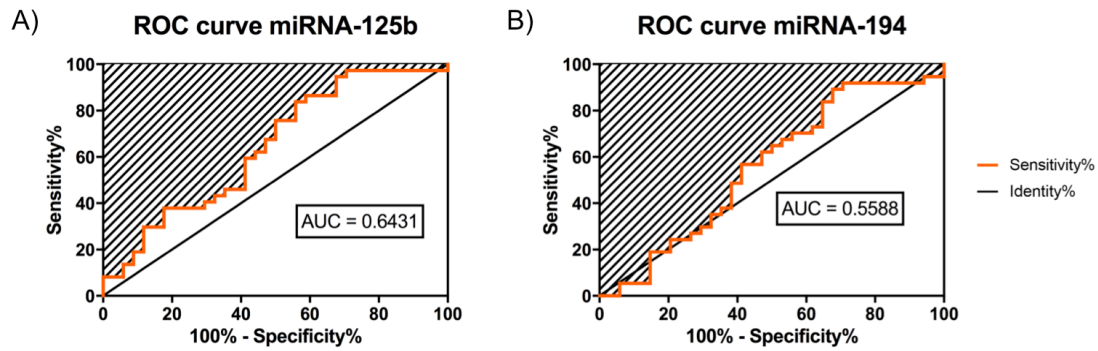


Figure B.2.2. ROC curve analysis of miR-125b and miR-194 in discriminating benign lesions from malignant disease.

A) miR-125b shows an AUC = 0.6431 (CI: 0.51 – 0.77, p -value = 0.0383) and **B)** miR-194 exhibits an AUC = 0.5588 (CI: 0.42 – 0.70, p -value = 0.3943).

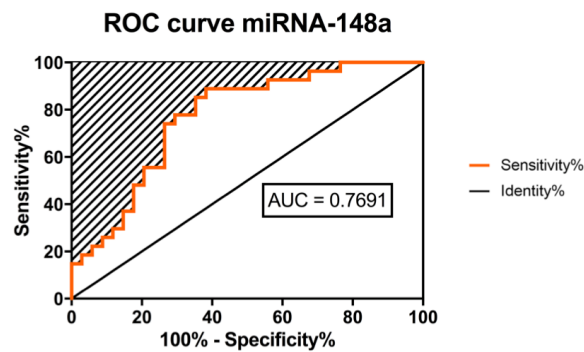


Figure B.2.3. ROC curve analysis of miR-148a in discriminating benign from PDAC.

MiRNA-148a shows an AUC = 0.7691 (CI: 0.65 – 0.89, p -value = 0.0003).

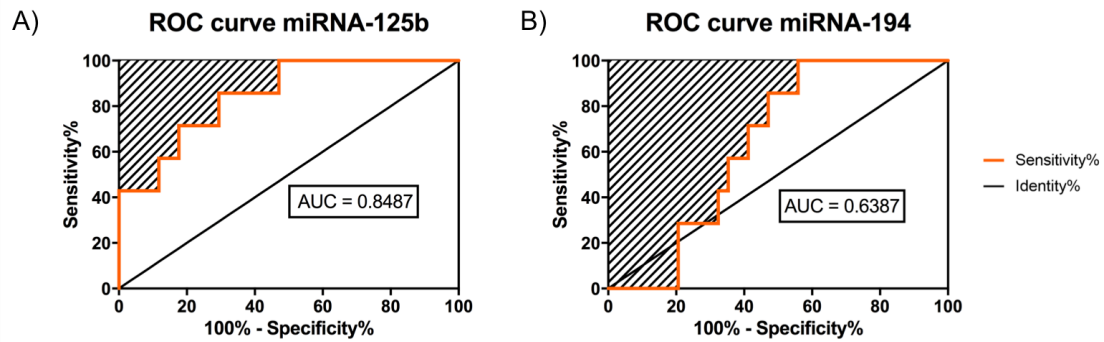


Figure B.2.4. ROC curve analysis of miR-125b and miR-194 in discriminating CCA from benign lesions

A) miR-125b shows an AUC = 0.8487 (CI: 0.71 – 0.99; p -value = 0.0040), and **b)** miR-194 exposes an AUC = 0.6387 (CI: 0.47 – 0.80; p -value = 0.2529).

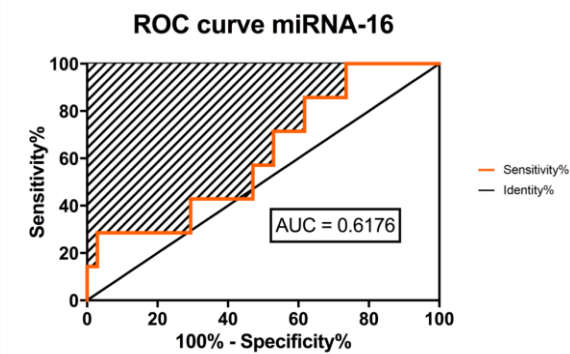


Figure B.2.5. ROC curve analysis of miR-16 in discriminating CCA from benign lesions.

MiRNA-16 exhibits an AUC = 0.6176 (CI: 0.40 – 0.84; p -value = 0.3320).

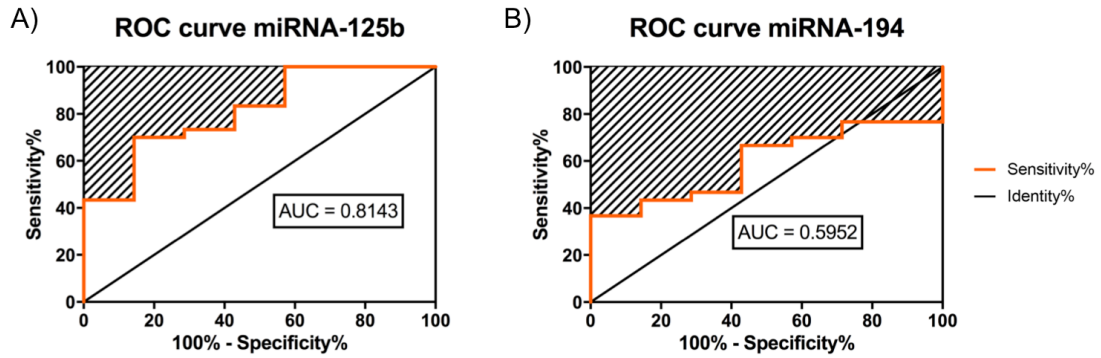


Figure B.2.6. ROC curve analysis of miR-125b and miR-194 in discriminating CCA from other malignant lesions.

A) miR-125b (AUC = 0.8143; CI: 0.65 – 0.98; p -value = 0.0105), and **B)** miR-194 (AUC = 0.5952, CI: 0.41– 0.78; p -value = 0.4380).

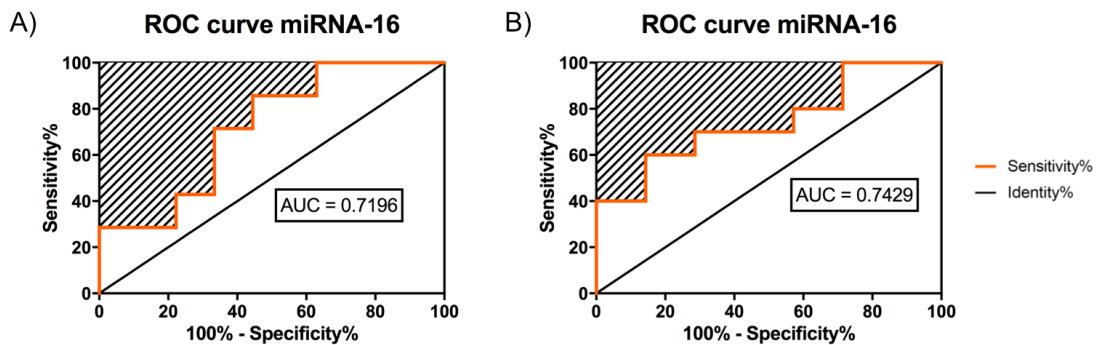


Figure B.2.7 ROC analysis of miR-16 in discriminating CCA from PDAC and CCA from other malignant lesions.

A) miR-16 differentiates CCA from PDAC (AUC = 0.7196, CI: 0.52 – 0.92, p -value = 0.071). **B)** miR-16 distinguishes CCA from other malignant lesions (i.e. PDAC and AC) with an AUC = 0.7429 (CI: 0.56 – 0.93, p -value = 0.0480).

B.3. Expression and performance of miRNA-125b, miR-148a and miRNA-194 in pancreatobiliary tract cancers after combining discovery and validation cohorts

A combined cohort composed of the discovery cohort and the validation cohort was created. Each cancer type from the discovery cohort and the validation cohort was included in this combined cohort, except IPMN lesions.

Table B.3.1. Number of subjects in each cohort classified by type of lesion.

	Cohort		
Type of lesion	Discovery (n)	Validation (n)	Combined (n)
Benign	14	34	48
PDAC	27	27	54
CCA	7	7	14
AC	3	3	6

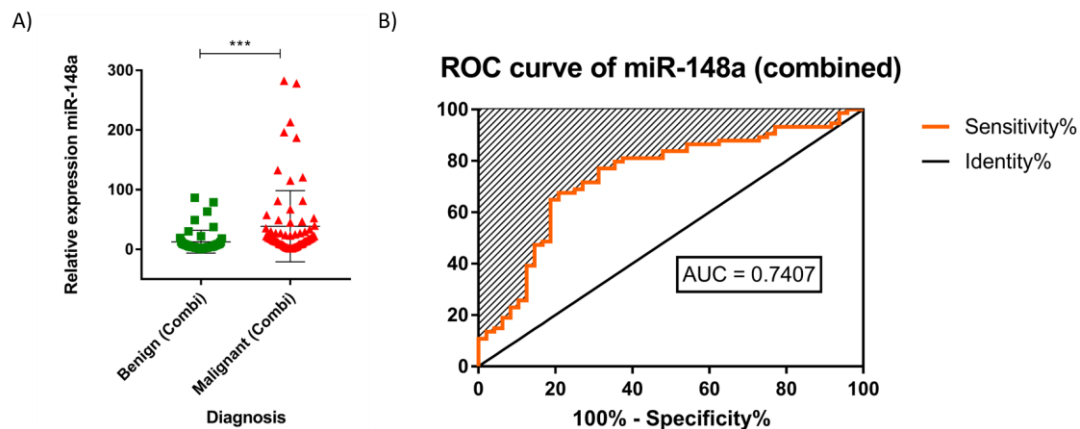


Figure B.3.1. Relative miRNA expression and ROC curve analysis of miR-148a in benign and malignant lesions (combined cohort).

A) MiRNA-148a significantly differentiates benign from malignant lesions (p -value = 0.0004; mean = 12.68 ± 19.17 (SD), mean = 38.56 ± 59.64). **B)** MiRNA-148a differentiates benign from malignant lesions with a good discriminatory power (AUC = 0.7407; CI = 0.65 – 0.83; p -value < 0.0001).

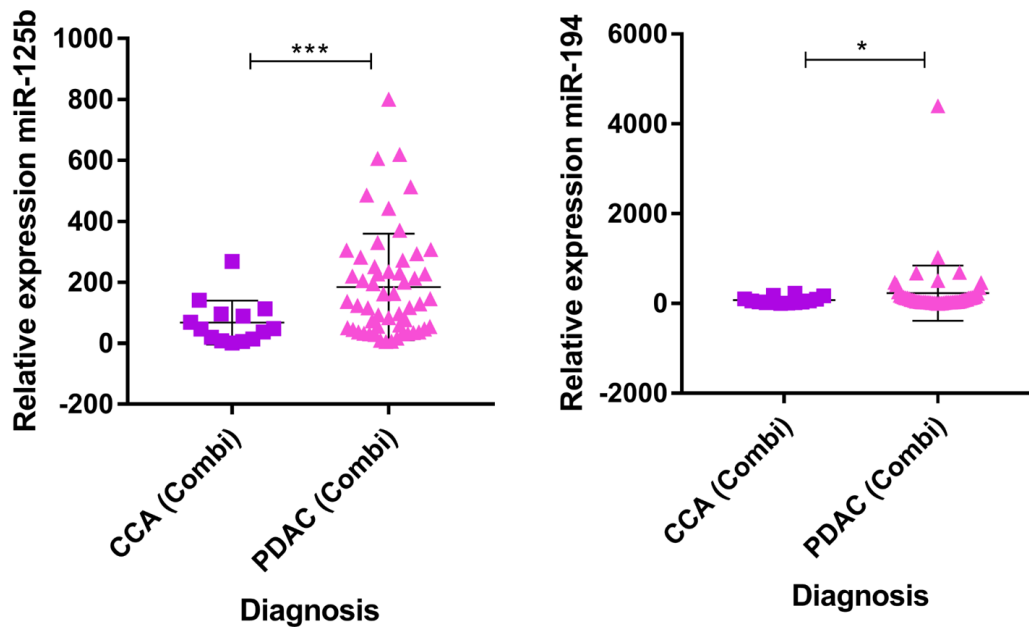


Figure B.3.2. Relative miRNA expression of miR-125b and miR-194 in CCA and PDAC (combined cohort)

A) miRNA-125b significantly differentiates CCA from PDAC (p -value = 0.0002; mean = 68.46 ± 72.32 (SD), mean = 184.90 ± 175.10) and **B)** miR-194 distinguishes CCA from PDAC with a p -value of 0.0382 (72.63 ± 72.68 vs 227.30 ± 613.50).

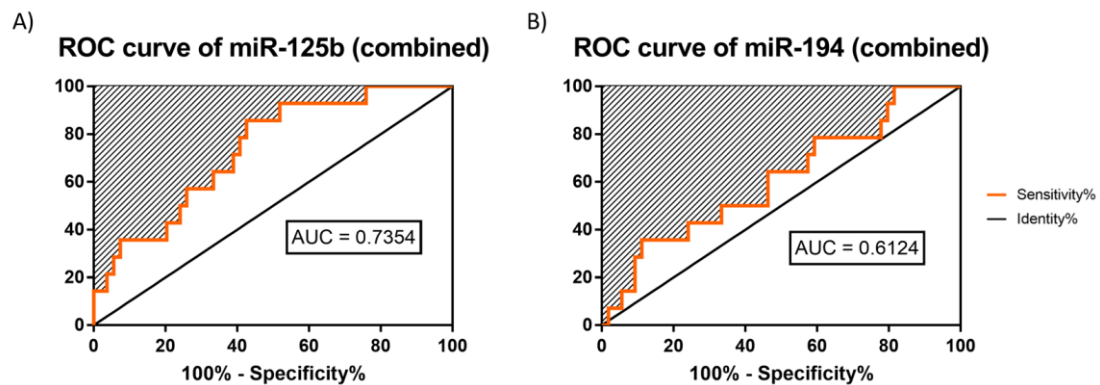


Figure B.3.3. ROC analysis of miR-125b and miR-194 in discriminating CCA from PDAC in a combined cohort.

A) miRNA-125b differentiates CCA from PDAC with an AUC = 0.7354 (CI: 0.60 – 0.87, p -value = 0.0069) and **B)** miR-194 distinguishes CCA from PDAC (AUC = 0.6124; CI: 0.45 – 0.78, p -value of 0.1973).

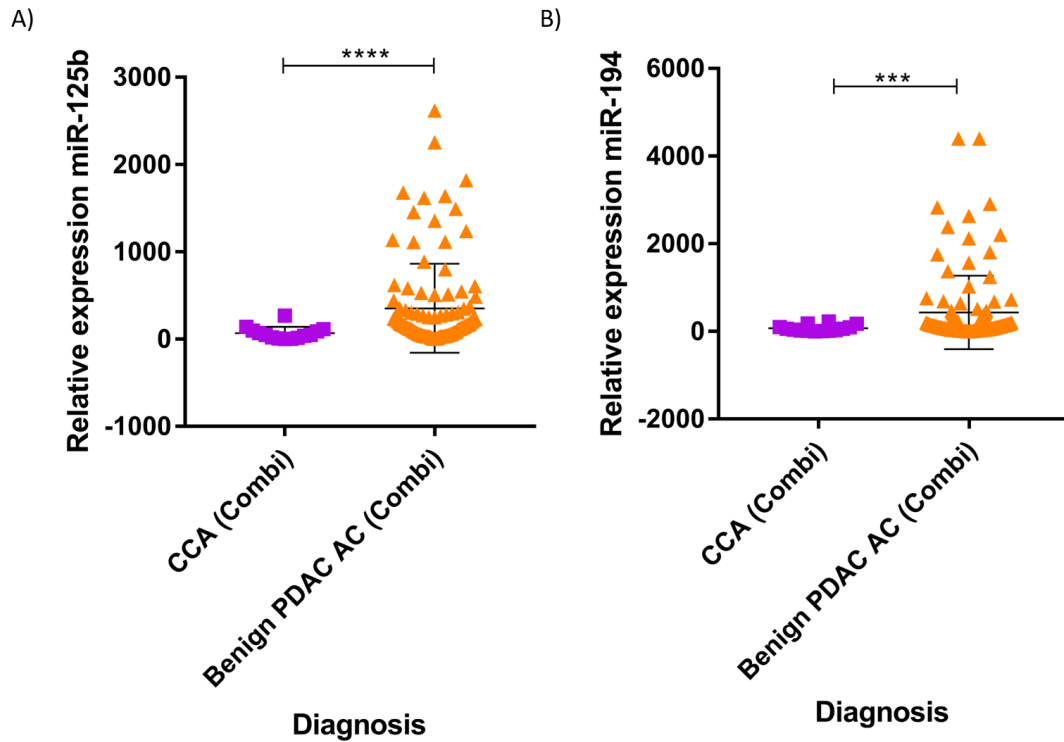


Figure B.3.4. Relative miRNA expression of miR-125b and miR-194 in CCA and other malignant lesions and benign lesions (combined cohort).

A) miRNA-125b significantly discriminates CCA from other malignant (i.e. PDAC and AC) and benign lesions (p -value < 0.0001 ; mean = 68.46 ± 72.32 (SD), mean = 353.00 ± 508.60) and **B)** miR-194 significantly differentiates CCA from other malignant (PDAC and AC) and benign lesions (p -value < 0.0001 ; 72.63 ± 72.68 vs 432.20 ± 838.10).

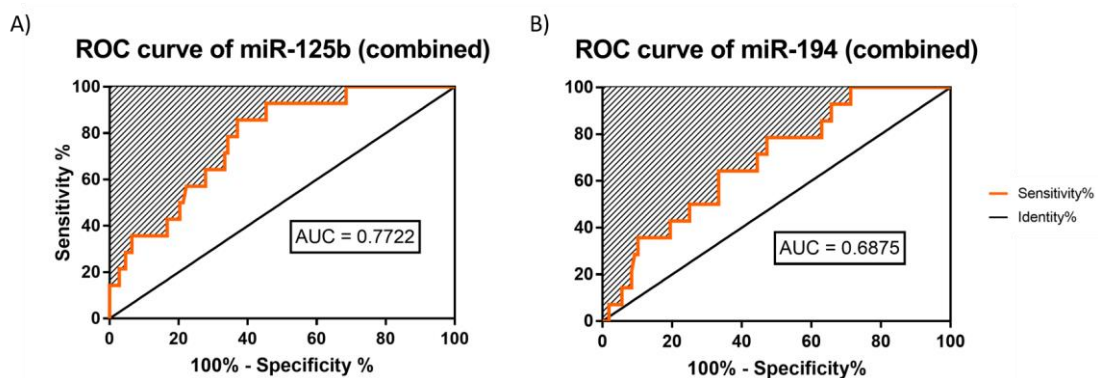


Figure B.3.5. ROC analysis of miR-125b and miR-194 in discriminating CCA other malignant tumours (PDAC and AC) and benign lesions (combined cohort)

A) miRNA-125b differentiates CCA from PDAC with an AUC = 0.7722 (CI: 0.66 – 0.89, p -value = 0.0009) and **B)** miR-194 distinguishes CCA from PDAC (AUC = 0.6875; CI: 0.55 – 0.82, p -value of 0.0228).

B.4. Standard Curves

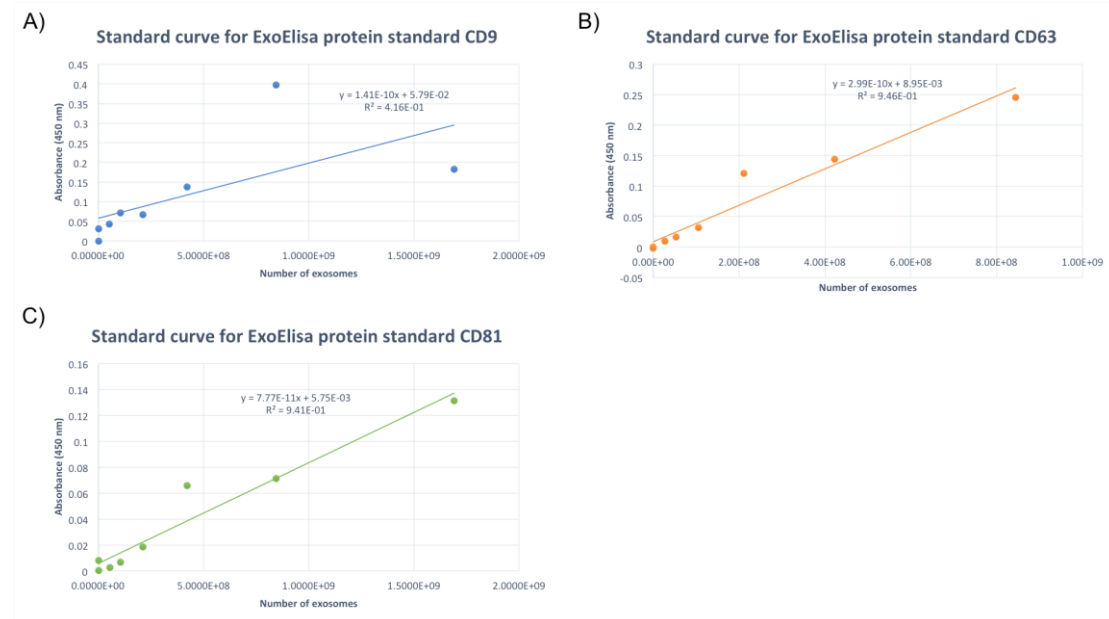


Figure B.4.1. Standard curves for protein standards of CD63, CD9 and CD81

A) Standard curve for protein standard CD9, **B)** Standard curve for protein standard CD63, and **C)** Standard curve for protein standard CD81.

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Reference

1. Garcea G, Ong SL, Rajesh A, Neal CP, Pollard CA, Berry DP, et al. Cystic lesions of the pancreas. A diagnostic and management dilemma. *Pancreatology*. 2008;8(3):236-51.
2. Basturk O, Coban I, Adsay NV. Pancreatic cysts: pathologic classification, differential diagnosis, and clinical implications. *Archives of pathology & laboratory medicine*. 2009;133(3):423-38.
3. D'Angelica M, Brennan MF, Suriawinata AA, Klimstra D, Conlon KC. Intraductal papillary mucinous neoplasms of the pancreas: an analysis of clinicopathologic features and outcome. *Ann Surg*. 2004;239(3):400-8.
4. Tanaka M, Chari S, Adsay V, Fernandez-del Castillo C, Falconi M, Shimizu M, et al. International consensus guidelines for management of intraductal papillary mucinous neoplasms and mucinous cystic neoplasms of the pancreas. *Pancreatology*. 2006;6(1-2):17-32.
5. Crippa S, Fernandez-Del Castillo C, Salvia R, Finkelstein D, Bassi C, Dominguez I, et al. Mucin-producing neoplasms of the pancreas: an analysis of distinguishing clinical and epidemiologic characteristics. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2010;8(2):213-9.
6. Mondiere JT. Diseases of the pancreas. *Arch gen de Paris*. 1836:236.
7. Ferlay J, Steliarova-Foucher E, Lortet-Tieulent J, Rosso S, Coebergh JW, Comber H, et al. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer*. 2013;49(6):1374-403.
8. Zhu A, Xia J, Zuo J, Jin S, Zhou H, Yao L, et al. MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in gastric cancer. *Medical oncology*. 2012;29(4):2701-9.
9. Rahib L, Smith BD, Aizenberg R, Rosenzweig AB, Fleshman JM, Matrisian LM. Projecting cancer incidence and deaths to 2030: the unexpected burden of thyroid, liver, and pancreas cancers in the United States. *Cancer Res*. 2014;74(11):2913-21.
10. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *International journal of cancer Journal international du cancer*. 2015;136(5):E359-86.
11. Yadav D, Lowenfels AB. The epidemiology of pancreatitis and pancreatic cancer. *Gastroenterology*. 2013;144(6):1252-61.
12. Takhar AS, Palaniappan P, Dhingra R, Lobo DN. Recent developments in diagnosis of pancreatic cancer. *BMJ*. 2004;329(7467):668-73.
13. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. *Lancet*. 2011;378(9791):607-20.
14. Gullo L. Diabetes and the risk of pancreatic cancer. *Ann Oncol*. 1999;10 Suppl 4:79-81.
15. Vrieling A, Bueno-de-Mesquita HB, Boshuizen HC, Michaud DS, Severinsen MT, Overvad K, et al. Cigarette smoking, environmental tobacco smoke exposure and pancreatic cancer risk in the European Prospective Investigation into Cancer and Nutrition. *International journal of cancer Journal international du cancer*. 2010;126(10):2394-403.
16. Raimondi S, Lowenfels AB, Morselli-Labate AM, Maisonneuve P, Pezzilli R. Pancreatic cancer in chronic pancreatitis; aetiology, incidence, and early detection. *Best practice & research Clinical gastroenterology*. 2010;24(3):349-58.
17. Guerra C, Collado M, Navas C, Schuhmacher AJ, Hernandez-Porras I, Canamero M, et al. Pancreatitis-induced inflammation contributes to pancreatic cancer by inhibiting oncogene-induced senescence. *Cancer Cell*. 2011;19(6):728-39.

18. Petersen GM, de Andrade M, Goggins M, Hruban RH, Bondy M, Korczak JF, et al. Pancreatic cancer genetic epidemiology consortium. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* 2006;15(4):704-10.
19. Shi C, Klein AP, Goggins M, Maitra A, Canto M, Ali S, et al. Increased Prevalence of Precursor Lesions in Familial Pancreatic Cancer Patients. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2009;15(24):7737-43.
20. Li D. Diabetes and pancreatic cancer. *Mol Carcinog.* 2012;51(1):64-74.
21. Brat DJ, Lillemoe KD, Yeo CJ, Warfield PB, Hruban RH. Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas. *The American journal of surgical pathology.* 1998;22(2):163-9.
22. Hruban RH, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN, et al. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. *The American journal of surgical pathology.* 2001;25(5):579-86.
23. Hruban RH, Takaori K, Klimstra DS, Adsay NV, Albores-Saavedra J, Biankin AV, et al. An illustrated consensus on the classification of pancreatic intraepithelial neoplasia and intraductal papillary mucinous neoplasms. *The American journal of surgical pathology.* 2004;28(8):977-87.
24. Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2000;6(8):2969-72.
25. Murphy SJ, Hart SN, Lima JF, Kipp BR, Klebig M, Winters JL, et al. Genetic alterations associated with progression from pancreatic intraepithelial neoplasia to invasive pancreatic tumor. *Gastroenterology.* 2013;145(5):1098-109 e1.
26. Iacobuzio-Donahue CA, Velculescu VE, Wolfgang CL, Hruban RH. Genetic basis of pancreas cancer development and progression: insights from whole-exome and whole-genome sequencing. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2012;18(16):4257-65.
27. Hruban RH, Wilentz RE, Maitra A. Identification and analysis of precursors to invasive pancreatic cancer. *Methods in molecular medicine.* 2005;103:1-13.
28. Klein AP, Brune KA, Petersen GM, Goggins M, Tersmette AC, Offerhaus GJ, et al. Prospective risk of pancreatic cancer in familial pancreatic cancer kindreds. *Cancer Res.* 2004;64(7):2634-8.
29. Rachakonda PS, Bauer AS, Xie H, Campa D, Rizzato C, Canzian F, et al. Somatic mutations in exocrine pancreatic tumors: association with patient survival. *PloS one.* 2013;8(4):e60870.
30. Hruban RH, Goggins M, Kern SE. Molecular genetics and related developments in pancreatic cancer. *Current opinion in gastroenterology.* 1999;15(5):404-9.
31. Maitra A, Kern SE, Hruban RH. Molecular pathogenesis of pancreatic cancer. *Best practice & research Clinical gastroenterology.* 2006;20(2):211-26.
32. Hidalgo M. Pancreatic cancer. *N Engl J Med.* 2010;362(17):1605-17.
33. Yachida S, White CM, Naito Y, Zhong Y, Brosnan JA, Macgregor-Das AM, et al. Clinical significance of the genetic landscape of pancreatic cancer and implications for identification of potential long-term survivors. *Clinical cancer research : an official journal of the American Association for Cancer Research.* 2012;18(22):6339-47.
34. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, et al. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. *Nature.* 2012;491(7424):399-405.
35. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, et al. Genomic analyses identify molecular subtypes of pancreatic cancer. *Nature.* 2016;531(7592):47-52.

36. Collisson EA, Sadanandam A, Olson P, Gibb WJ, Truitt M, Gu S, et al. Subtypes of pancreatic ductal adenocarcinoma and their differing responses to therapy. *Nat Med.* 2011;17(4):500-3.
37. Lockhart AC, Rothenberg ML, Berlin JD. Treatment for pancreatic cancer: current therapy and continued progress. *Gastroenterology.* 2005;128(6):1642-54.
38. Ozaki H. Improvement of pancreatic cancer treatment from the Japanese experience in the 1980s. *International journal of pancreatology : official journal of the International Association of Pancreatology.* 1992;12(1):5-9.
39. Pang J, Minter R, Kwon R, Simeone D, Roh M. The Role of Cytology in the Preoperative Assessment and Management of Patients with Pancreaticobiliary Tract Neoplasms. *Journal of Gastrointestinal Surgery.* 2013;17(3):501-10.
40. Katz DS, Friedel DM, Kho D, Georgiou N, Hines JJ. Relative accuracy of CT and MRI for characterization of cystic pancreatic masses. *AJR Am J Roentgenol.* 2007;189(3):657-61.
41. Visser BC, Yeh BM, Qayyum A, Way LW, McCulloch CE, Coakley FV. Characterization of cystic pancreatic masses: relative accuracy of CT and MRI. *AJR Am J Roentgenol.* 2007;189(3):648-56.
42. Ballehaninna UK, Chamberlain RS. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. *Journal of gastrointestinal oncology.* 2012;3(2):105-19.
43. Whipple AO, Parsons WB, Mullins CR. Treatment of Carcinoma of the Ampulla of Vater. *Ann Surg.* 1935;102(4):763-79.
44. Semaan A, Maitra A. Pancreatic cancer in 2017: Rebooting pancreatic cancer knowledge and treatment options. *Nature reviews Gastroenterology & hepatology.* 2018;15(2):76-8.
45. Berlin JD, Catalano P, Thomas JP, Kugler JW, Haller DG, Benson AB, 3rd. Phase III study of gemcitabine in combination with fluorouracil versus gemcitabine alone in patients with advanced pancreatic carcinoma: Eastern Cooperative Oncology Group Trial E2297. *J Clin Oncol.* 2002;20(15):3270-5.
46. Colucci G, Labianca R, Di Costanzo F, Gebbia V, Carteni G, Massidda B, et al. Randomized phase III trial of gemcitabine plus cisplatin compared with single-agent gemcitabine as first-line treatment of patients with advanced pancreatic cancer: the GIP-1 study. *J Clin Oncol.* 2010;28(10):1645-51.
47. Cunningham D, Chau I, Stocken DD, Valle JW, Smith D, Steward W, et al. Phase III randomized comparison of gemcitabine versus gemcitabine plus capecitabine in patients with advanced pancreatic cancer. *J Clin Oncol.* 2009;27(33):5513-8.
48. Heinemann V, Quietzsch D, Gieseler F, Gonnermann M, Schonekas H, Rost A, et al. Randomized phase III trial of gemcitabine plus cisplatin compared with gemcitabine alone in advanced pancreatic cancer. *J Clin Oncol.* 2006;24(24):3946-52.
49. Herrmann R, Bodoky G, Ruhstaller T, Glimelius B, Bajetta E, Schuller J, et al. Gemcitabine plus capecitabine compared with gemcitabine alone in advanced pancreatic cancer: a randomized, multicenter, phase III trial of the Swiss Group for Clinical Cancer Research and the Central European Cooperative Oncology Group. *J Clin Oncol.* 2007;25(16):2212-7.
50. Louvet C, Labianca R, Hammel P, Lledo G, Zampino MG, Andre T, et al. Gemcitabine in combination with oxaliplatin compared with gemcitabine alone in locally advanced or metastatic pancreatic cancer: results of a GERCOR and GISCAD phase III trial. *J Clin Oncol.* 2005;23(15):3509-16.
51. Poplin E, Feng Y, Berlin J, Rothenberg ML, Hochster H, Mitchell E, et al. Phase III, randomized study of gemcitabine and oxaliplatin versus gemcitabine (fixed-dose rate infusion) compared with gemcitabine (30-minute infusion) in patients with pancreatic carcinoma E6201: a trial of the Eastern Cooperative Oncology Group. *J Clin Oncol.* 2009;27(23):3778-85.

52. Von Hoff DD, Ervin T, Arena FP, Chiorean EG, Infante J, Moore M, et al. Increased survival in pancreatic cancer with nab-paclitaxel plus gemcitabine. *N Engl J Med*. 2013;369(18):1691-703.
53. Conroy T, Desseigne F, Ychou M, Bouche O, Guimbaud R, Becouarn Y, et al. FOLFIRINOX versus gemcitabine for metastatic pancreatic cancer. *N Engl J Med*. 2011;364(19):1817-25.
54. Manji GA, Olive KP, Saenger YM, Oberstein P. Current and Emerging Therapies in Metastatic Pancreatic Cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2017;23(7):1670-8.
55. Wang-Gillam A, Li CP, Bodoky G, Dean A, Shan YS, Jameson G, et al. Nanoliposomal irinotecan with fluorouracil and folinic acid in metastatic pancreatic cancer after previous gemcitabine-based therapy (NAPOLI-1): a global, randomised, open-label, phase 3 trial. *Lancet*. 2016;387(10018):545-57.
56. Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. *N Engl J Med*. 2004;350(12):1200-10.
57. Oettle H, Neuhaus P. Adjuvant therapy in pancreatic cancer: a critical appraisal. *Drugs*. 2007;67(16):2293-310.
58. Regine WF, Winter KA, Abrams R, Safran H, Hoffman JP, Konski A, et al. Fluorouracil-based chemoradiation with either gemcitabine or fluorouracil chemotherapy after resection of pancreatic adenocarcinoma: 5-year analysis of the U.S. Intergroup/RTOG 9704 phase III trial. *Ann Surg Oncol*. 2011;18(5):1319-26.
59. Uesaka K, Boku N, Fukutomi A, Okamura Y, Konishi M, Matsumoto I, et al. Adjuvant chemotherapy of S-1 versus gemcitabine for resected pancreatic cancer: a phase 3, open-label, randomised, non-inferiority trial (JASPAC 01). *Lancet*. 2016;388(10041):248-57.
60. Neoptolemos JP, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, et al. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. *Lancet*. 2017;389(10073):1011-24.
61. Klaassen DJ, MacIntyre JM, Catton GE, Engstrom PF, Moertel CG. Treatment of locally unresectable cancer of the stomach and pancreas: a randomized comparison of 5-fluorouracil alone with radiation plus concurrent and maintenance 5-fluorouracil--an Eastern Cooperative Oncology Group study. *J Clin Oncol*. 1985;3(3):373-8.
62. Burris H, Storniolo AM. Assessing clinical benefit in the treatment of pancreas cancer: gemcitabine compared to 5-fluorouracil. *Eur J Cancer*. 1997;33 Suppl 1:S18-22.
63. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, et al. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol*. 2007;25(15):1960-6.
64. Bruns CJ, Solorzano CC, Harbison MT, Ozawa S, Tsan R, Fan D, et al. Blockade of the epidermal growth factor receptor signaling by a novel tyrosine kinase inhibitor leads to apoptosis of endothelial cells and therapy of human pancreatic carcinoma. *Cancer Res*. 2000;60(11):2926-35.
65. Ng SS, Tsao MS, Nicklee T, Hedley DW. Effects of the epidermal growth factor receptor inhibitor OSI-774, Tarceva, on downstream signaling pathways and apoptosis in human pancreatic adenocarcinoma. *Mol Cancer Ther*. 2002;1(10):777-83.
66. Li J, Kleeff J, Giese N, Buchler MW, Korc M, Friess H. Gefitinib ('Iressa', ZD1839), a selective epidermal growth factor receptor tyrosine kinase inhibitor, inhibits pancreatic cancer cell growth, invasion, and colony formation. *Int J Oncol*. 2004;25(1):203-10.
67. Durkin AJ, Osborne DA, Yeatman TJ, Rosemurgy AS, Armstrong C, Zervos EE. EGF receptor antagonism improves survival in a murine model of pancreatic adenocarcinoma. *J Surg Res*. 2006;135(1):195-201.

68. Dragovich T, Huberman M, Von Hoff DD, Rowinsky EK, Nadler P, Wood D, et al. Erlotinib plus gemcitabine in patients with unresectable pancreatic cancer and other solid tumors: phase IB trial. *Cancer chemotherapy and pharmacology*. 2007;60(2):295-303.
69. Hidalgo M, Siu LL, Nemunaitis J, Rizzo J, Hammond LA, Takimoto C, et al. Phase I and pharmacologic study of OSI-774, an epidermal growth factor receptor tyrosine kinase inhibitor, in patients with advanced solid malignancies. *J Clin Oncol*. 2001;19(13):3267-79.
70. Von Hoff DD, Ramanathan RK, Borad MJ, Laheru DA, Smith LS, Wood TE, et al. Gemcitabine plus nab-paclitaxel is an active regimen in patients with advanced pancreatic cancer: a phase I/II trial. *J Clin Oncol*. 2011;29(34):4548-54.
71. Goldstein D, El-Maraghi RH, Hammel P, Heinemann V, Kunzmann V, Sastre J, et al. nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. *J Natl Cancer Inst*. 2015;107(2).
72. Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, et al. Cisplatin plus gemcitabine versus gemcitabine for biliary tract cancer. *N Engl J Med*. 2010;362(14):1273-81.
73. Blechacz B, Komuta M, Roskams T, Gores GJ. Clinical diagnosis and staging of cholangiocarcinoma. *Nature reviews Gastroenterology & hepatology*. 2011;8(9):512-22.
74. Blechacz B. Cholangiocarcinoma: Current Knowledge and New Developments. *Gut and liver*. 2017;11(1):13-26.
75. Blechacz B, Gores GJ. Cholangiocarcinoma: advances in pathogenesis, diagnosis, and treatment. *Hepatology*. 2008;48(1):308-21.
76. Lazaridis KN, Gores GJ. Cholangiocarcinoma. *Gastroenterology*. 2005;128(6):1655-67.
77. Lim JH, Park CK. Pathology of cholangiocarcinoma. *Abdominal imaging*. 2004;29(5):540-7.
78. Razumilava N, Gores GJ. Classification, diagnosis, and management of cholangiocarcinoma. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2013;11(1):13-21 e1; quiz e3-4.
79. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. *Seminars in liver disease*. 2004;24(2):115-25.
80. Witjes CD, Karim-Kos HE, Visser O, de Vries E, JN IJ, de Man RA, et al. Intrahepatic cholangiocarcinoma in a low endemic area: rising incidence and improved survival. *HPB : the official journal of the International Hepato Pancreato Biliary Association*. 2012;14(11):777-81.
81. Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC cancer*. 2002;2:10.
82. Aljiffry M, Walsh MJ, Molinari M. Advances in diagnosis, treatment and palliation of cholangiocarcinoma: 1990-2009. *World J Gastroenterol*. 2009;15(34):4240-62.
83. Gores GJ. Cholangiocarcinoma: current concepts and insights. *Hepatology*. 2003;37(5):961-9.
84. Patel T. Cholangiocarcinoma--controversies and challenges. *Nature reviews Gastroenterology & hepatology*. 2011;8(4):189-200.
85. Annunziata S, Caldarella C, Pizzuto DA, Galiandro F, Sadeghi R, Giovanella L, et al. Diagnostic accuracy of fluorine-18-fluorodeoxyglucose positron emission tomography in the evaluation of the primary tumor in patients with cholangiocarcinoma: a meta-analysis. *BioMed research international*. 2014;2014:247693.
86. Charatcharoenwitthaya P, Enders FB, Halling KC, Lindor KD. Utility of serum tumor markers, imaging, and biliary cytology for detecting cholangiocarcinoma in primary sclerosing cholangitis. *Hepatology*. 2008;48(4):1106-17.

87. Rizvi S, Gores GJ. Pathogenesis, diagnosis, and management of cholangiocarcinoma. *Gastroenterology*. 2013;145(6):1215-29.
88. Heimbach JK, Sanchez W, Rosen CB, Gores GJ. Trans-peritoneal fine needle aspiration biopsy of hilar cholangiocarcinoma is associated with disease dissemination. *HPB : the official journal of the International Hepato Pancreato Biliary Association*. 2011;13(5):356-60.
89. Blechacz B, Gores GJ. Positron emission tomography scan for a hepatic mass. *Hepatology*. 2010;52(6):2186-91.
90. Masselli G, Manfredi R, Vecchioli A, Gualdi G. MR imaging and MR cholangiopancreatography in the preoperative evaluation of hilar cholangiocarcinoma: correlation with surgical and pathologic findings. *Eur Radiol*. 2008;18(10):2213-21.
91. Farges O, Fuks D, Le Treut YP, Azoulay D, Laurent A, Bachellier P, et al. AJCC 7th edition of TNM staging accurately discriminates outcomes of patients with resectable intrahepatic cholangiocarcinoma: By the AFC-IHCC-2009 study group. *Cancer*. 2011;117(10):2170-7.
92. Bismuth H, Corlette MB. Intrahepatic cholangioenteric anastomosis in carcinoma of the hilus of the liver. *Surgery, gynecology & obstetrics*. 1975;140(2):170-8.
93. Deoliveira ML, Schulick RD, Nimura Y, Rosen C, Gores G, Neuhaus P, et al. New staging system and a registry for perihilar cholangiocarcinoma. *Hepatology*. 2011;53(4):1363-71.
94. DeOliveira ML, Cunningham SC, Cameron JL, Kamangar F, Winter JM, Lillemoe KD, et al. Cholangiocarcinoma: thirty-one-year experience with 564 patients at a single institution. *Ann Surg*. 2007;245(5):755-62.
95. Andre T, Tournigand C, Rosmorduc O, Provent S, Maindrault-Goebel F, Avenin D, et al. Gemcitabine combined with oxaliplatin (GEMOX) in advanced biliary tract adenocarcinoma: a GERCOR study. *Ann Oncol*. 2004;15(9):1339-43.
96. Harder J, Riecken B, Kummer O, Lohrmann C, Otto F, Usadel H, et al. Outpatient chemotherapy with gemcitabine and oxaliplatin in patients with biliary tract cancer. *British journal of cancer*. 2006;95(7):848-52.
97. Lee J, Park SH, Chang HM, Kim JS, Choi HJ, Lee MA, et al. Gemcitabine and oxaliplatin with or without erlotinib in advanced biliary-tract cancer: a multicentre, open-label, randomised, phase 3 study. *Lancet Oncol*. 2012;13(2):181-8.
98. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, et al. Cancer statistics, 2008. *CA: a cancer journal for clinicians*. 2008;58(2):71-96.
99. Cattell RB, Pyrtle LJ. Premalignant lesions of the ampulla of Vater. *Surgery, gynecology & obstetrics*. 1950;90(1):21-30, illust.
100. Agoff SN, Crispin DA, Bronner MP, Dail DH, Hawes SE, Haggitt RC. Neoplasms of the ampulla of vater with concurrent pancreatic intraductal neoplasia: a histological and molecular study. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2001;14(3):139-46.
101. Beger HG, Treitschke F, Gansauge F, Harada N, Hiki N, MatTFeldt T. Tumor of the ampulla of Vater: experience with local or radical resection in 171 consecutively treated patients. *Archives of surgery*. 1999;134(5):526-32.
102. O'Connell JB, Maggard MA, Manunga J, Jr., Tomlinson JS, Reber HA, Ko CY, et al. Survival after resection of ampullary carcinoma: a national population-based study. *Ann Surg Oncol*. 2008;15(7):1820-7.
103. Overman MJ, Varadhachary GR, Kopetz S, Adinin R, Lin E, Morris JS, et al. Phase II study of capecitabine and oxaliplatin for advanced adenocarcinoma of the small bowel and ampulla of Vater. *J Clin Oncol*. 2009;27(16):2598-603.
104. Demeure MJ, Craig DW, Sinari S, Moses TM, Christoforides A, Dinh J, et al. Cancer of the ampulla of Vater: analysis of the whole genome sequence exposes a potential therapeutic vulnerability. *Genome Med*. 2012;4(7):56.

105. Chen WX, Xie QG, Zhang WF, Zhang X, Hu TT, Xu P, et al. Multiple imaging techniques in the diagnosis of ampullary carcinoma. *Hepatobiliary Pancreat Dis Int*. 2008;7(6):649-53.
106. Tsukada K, Takada T, Miyazaki M, Miyakawa S, Nagino M, Kondo S, et al. Diagnosis of biliary tract and ampullary carcinomas. *Journal of hepato-biliary-pancreatic surgery*. 2008;15(1):31-40.
107. Cereda S, Passoni P, Reni M, Vigano MG, Aldrighetti L, Nicoletti R, et al. The cisplatin, epirubicin, 5-fluorouracil, gemcitabine (PEFG) regimen in advanced biliary tract adenocarcinoma. *Cancer*. 2010;116(9):2208-14.
108. Sikora SS, Balachandran P, Dimri K, Rastogi N, Kumar A, Saxena R, et al. Adjuvant chemo-radiotherapy in ampullary cancers. *Eur J Surg Oncol*. 2005;31(2):158-63.
109. Klinkenbijn JH, Jeekel J, Sahmoud T, van Pel R, Couvreur ML, Veenhof CH, et al. Adjuvant radiotherapy and 5-fluorouracil after curative resection of cancer of the pancreas and periampullary region: phase III trial of the EORTC gastrointestinal tract cancer cooperative group. *Ann Surg*. 1999;230(6):776-82; discussion 82-4.
110. Primrose JN, Fox R, Palmer DH, Prasad R, Mirza D, Anthoney DA, et al. Adjuvant capecitabine for biliary tract cancer: The BILCAP randomized study. *Journal of Clinical Oncology*. 2017;35(15_suppl):4006-.
111. Boyer JL. Bile formation and secretion. *Compr Physiol*. 2013;3(3):1035-78.
112. Kristiansen TZ, Bunkenborg J, Gronborg M, Molina H, Thuluvath PJ, Argani P, et al. A proteomic analysis of human bile. *Molecular & cellular proteomics : MCP*. 2004;3(7):715-28.
113. Chapman MH, Tidswell R, Dooley JS, Sandanayake NS, Cerec V, Deheragoda M, et al. Whole genome RNA expression profiling of endoscopic biliary brushings provides data suitable for biomarker discovery in cholangiocarcinoma. *J Hepatol*. 2012;56(4):877-85.
114. Mattick JS, Makunin IV. Non-coding RNA. *Hum Mol Genet*. 2006;15 Spec No 1:R17-29.
115. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*. 2004;116(2):281-97.
116. Zaratiegui M, Irvine DV, Martienssen RA. Noncoding RNAs and gene silencing. *Cell*. 2007;128(4):763-76.
117. Tomari Y, Zamore PD. Perspective: machines for RNAi. *Genes Dev*. 2005;19(5):517-29.
118. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. *Cell*. 2003;115(2):199-208.
119. Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB. Prediction of mammalian microRNA targets. *Cell*. 2003;115(7):787-98.
120. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, et al. The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature*. 2000;403(6772):901-6.
121. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. *Nat Rev Mol Cell Biol*. 2005;6(5):376-85.
122. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*. 1993;75(5):843-54.
123. Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. *Nature*. 2000;403(6808):86-9.
124. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. *Science*. 2001;294(5543):853-8.
125. Ambros V. microRNAs: tiny regulators with great potential. *Cell*. 2001;107(7):823-6.

126. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. *Genes Dev.* 2002;16(13):1616-26.
127. Lee Y, Kim M, Han J, Yeom KH, Lee S, Baek SH, et al. MicroRNA genes are transcribed by RNA polymerase II. *EMBO J.* 2004;23(20):4051-60.
128. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol.* 2006;13(12):1097-101.
129. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The Microprocessor complex mediates the genesis of microRNAs. *Nature.* 2004;432(7014):235-40.
130. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al. The nuclear RNase III Drosha initiates microRNA processing. *Nature.* 2003;425(6956):415-9.
131. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the Microprocessor complex. *Nature.* 2004;432(7014):231-5.
132. Winter J, Jung S, Keller S, Gregory RI, Diederichs S. Many roads to maturity: microRNA biogenesis pathways and their regulation. *Nat Cell Biol.* 2009;11(3):228-34.
133. Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and its *D. melanogaster* homolog are required for miRNA biogenesis. *Curr Biol.* 2004;14(23):2162-7.
134. Han J, Lee Y, Yeom KH, Kim YK, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. *Genes & development.* 2004;18(24):3016-27.
135. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol.* 2009;10(2):126-39.
136. Lingel A, Simon B, Izaurralde E, Sattler M. Nucleic acid 3'-end recognition by the Argonaute2 PAZ domain. *Nat Struct Mol Biol.* 2004;11(6):576-7.
137. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature.* 2005;436(7051):740-4.
138. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol.* 2007;23:175-205.
139. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell.* 2005;123(4):631-40.
140. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. *Science.* 2002;297(5589):2056-60.
141. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet.* 2004;5(7):522-31.
142. Papp I, Mette MF, Aufsatz W, Daxinger L, Schauer SE, Ray A, et al. Evidence for nuclear processing of plant micro RNA and short interfering RNA precursors. *Plant Physiol.* 2003;132(3):1382-90.
143. Rhoades MW, Reinhart BJ, Lim LP, Burge CB, Bartel B, Bartel DP. Prediction of plant microRNA targets. *Cell.* 2002;110(4):513-20.
144. Lewis BP, Burge CB, Bartel DP. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell.* 2005;120(1):15-20.
145. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell.* 2009;136(2):215-33.
146. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res.* 2009;19(1):92-105.
147. Hausser J, Zavolan M. Identification and consequences of miRNA-target interactions--beyond repression of gene expression. *Nat Rev Genet.* 2014;15(9):599-612.
148. Rizzo-Campos AL, Riquelme I, Brebi-Mieville P. Tools for Sequence-Based miRNA Target Prediction: What to Choose? *International journal of molecular sciences.* 2016;17(12).

149. Selbach M, Schwanhauser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N. Widespread changes in protein synthesis induced by microRNAs. *Nature*. 2008;455(7209):58-63.
150. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. *Nature*. 2008;455(7209):64-71.
151. Karginov FV, Conaco C, Xuan Z, Schmidt BH, Parker JS, Mandel G, et al. A biochemical approach to identifying microRNA targets. *Proc Natl Acad Sci U S A*. 2007;104(49):19291-6.
152. Chi SW, Zang JB, Mele A, Darnell RB. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. *Nature*. 2009;460(7254):479-86.
153. Hafner M, Landthaler M, Burger L, Khorshid M, Hausser J, Berninger P, et al. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. *Cell*. 2010;141(1):129-41.
154. Kishore S, Jaskiewicz L, Burger L, Hausser J, Khorshid M, Zavolan M. A quantitative analysis of CLIP methods for identifying binding sites of RNA-binding proteins. *Nat Methods*. 2011;8(7):559-64.
155. Zhang C, Darnell RB. Mapping in vivo protein-RNA interactions at single-nucleotide resolution from HITS-CLIP data. *Nat Biotechnol*. 2011;29(7):607-14.
156. Konig J, Zarnack K, Rot G, Curk T, Kayikci M, Zupan B, et al. iCLIP reveals the function of hnRNP particles in splicing at individual nucleotide resolution. *Nat Struct Mol Biol*. 2010;17(7):909-15.
157. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. *Cell*. 2013;153(3):654-65.
158. Kuhn DE, Martin MM, Feldman DS, Terry AV, Jr., Nuovo GJ, Elton TS. Experimental validation of miRNA targets. *Methods*. 2008;44(1):47-54.
159. Griffiths-Jones S. The microRNA Registry. *Nucleic acids research*. 2004;32(Database issue):D109-11.
160. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. *Nucleic acids research*. 2008;36(Database issue):D154-8.
161. Gaidatzis D, van Nimwegen E, Hausser J, Zavolan M. Inference of miRNA targets using evolutionary conservation and pathway analysis. *BMC Bioinformatics*. 2007;8:69.
162. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. *Nucleic acids research*. 2011;39(Database issue):D152-7.
163. Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, et al. Combinatorial microRNA target predictions. *Nat Genet*. 2005;37(5):495-500.
164. Enright AJ, John B, Gaul U, Tuschl T, Sander C, Marks DS. MicroRNA targets in *Drosophila*. *Genome biology*. 2003;5(1):R1.
165. Vejnar CE, Blum M, Zdobnov EM. miRmap web: Comprehensive microRNA target prediction online. *Nucleic acids research*. 2013;41(Web Server issue):W165-8.
166. Kiriakidou M, Nelson PT, Kouranov A, Fitziev P, Bouyioukos C, Mourelatos Z, et al. A combined computational-experimental approach predicts human microRNA targets. *Genes Dev*. 2004;18(10):1165-78.
167. Lu J, Getz G, Miska EA, Alvarez-Saavedra E, Lamb J, Peck D, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-8.
168. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. *Proc Natl Acad Sci U S A*. 2006;103(7):2257-61.
169. Bloomston M, Frankel WL, Petrocca F, Volinia S, Alder H, Hagan JP, et al. MicroRNA expression patterns to differentiate pancreatic adenocarcinoma from normal pancreas and chronic pancreatitis. *JAMA : the journal of the American Medical Association*. 2007;297(17):1901-8.

170. Szafranska AE, Davison TS, John J, Cannon T, Sipos B, Maghnouj A, et al. MicroRNA expression alterations are linked to tumorigenesis and non-neoplastic processes in pancreatic ductal adenocarcinoma. *Oncogene*. 2007;26(30):4442-52.
171. Szafranska AE, Doleshal M, Edmunds HS, Gordon S, Luttges J, Munding JB, et al. Analysis of microRNAs in pancreatic fine-needle aspirates can classify benign and malignant tissues. *Clin Chem*. 2008;54(10):1716-24.
172. Schultz NA, Werner J, Willenbrock H, Roslind A, Giese N, Horn T, et al. MicroRNA expression profiles associated with pancreatic adenocarcinoma and ampullary adenocarcinoma. *Modern pathology : an official journal of the United States and Canadian Academy of Pathology, Inc*. 2012;25(12):1609-22.
173. Collins AL, Wojcik S, Liu J, Frankel WL, Alder H, Yu L, et al. A differential microRNA profile distinguishes cholangiocarcinoma from pancreatic adenocarcinoma. *Ann Surg Oncol*. 2014;21(1):133-8.
174. Lubezky N, Loewenstein S, Ben-Haim M, Brazowski E, Marmor S, Pasmanik-Chor M, et al. MicroRNA expression signatures in intraductal papillary mucinous neoplasm of the pancreas. *Surgery*. 2013;153(5):663-72.
175. Gress TM, Lausser L, Schirra LR, Ortmüller L, Diels R, Kong B, et al. Combined microRNA and mRNA microfluidic TaqMan array cards for the diagnosis of malignancy of multiple types of pancreato-biliary tumors in fine-needle aspiration material. *Oncotarget*. 2017;8(64):108223-37.
176. Ferlinz A, Miller C, Formosa R, Lee KY. Gene expression analysis of both mRNA and miRNA on the same TaqMan(R) Array Card: development of a pancreatic tumor tissue classification methodology. *Methods*. 2013;59(1):S11-5.
177. Matthaei H, Norris AL, Tsiatis AC, Olino K, Hong SM, dal Molin M, et al. Clinicopathological characteristics and molecular analyses of multifocal intraductal papillary mucinous neoplasms of the pancreas. *Annals of surgery*. 2012;255(2):326-33.
178. Nakahara O, Takamori H, Iwatsuki M, Baba Y, Sakamoto Y, Tanaka H, et al. Carcinogenesis of intraductal papillary mucinous neoplasm of the pancreas: loss of microRNA-101 promotes overexpression of histone methyltransferase EZH2. *Ann Surg Oncol*. 2012;19 Suppl 3:S565-71.
179. Ryu JK, Matthaei H, Dal Molin M, Hong SM, Canto MI, Schulick RD, et al. Elevated microRNA miR-21 levels in pancreatic cyst fluid are predictive of mucinous precursor lesions of ductal adenocarcinoma. *Pancreatology*. 2011;11(3):343-50.
180. du Rieu MC, Torrisani J, Selves J, Al Saati T, Souque A, Dufresne M, et al. MicroRNA-21 is induced early in pancreatic ductal adenocarcinoma precursor lesions. *Clin Chem*. 2010;56(4):603-12.
181. Yu J, Li A, Hong SM, Hruban RH, Goggins M. MicroRNA alterations of pancreatic intraepithelial neoplasias. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2012;18(4):981-92.
182. Xue Y, Abou Tayoun AN, Abo KM, Pipas JM, Gordon SR, Gardner TB, et al. MicroRNAs as diagnostic markers for pancreatic ductal adenocarcinoma and its precursor, pancreatic intraepithelial neoplasm. *Cancer Genet*. 2013;206(6):217-21.
183. Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova-Agadjanyan EL, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105(30):10513-8.
184. Roldo C, Missiaglia E, Hagan JP, Falconi M, Capelli P, Bersani S, et al. MicroRNA expression abnormalities in pancreatic endocrine and acinar tumors are associated with distinctive pathologic features and clinical behavior. *J Clin Oncol*. 2006;24(29):4677-84.
185. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, et al. Expression profiling identifies microRNA signature in pancreatic cancer. *International journal of cancer Journal international du cancer*. 2007;120(5):1046-54.

186. Bauer AS, Keller A, Costello E, Greenhalf W, Bier M, Borries A, et al. Diagnosis of pancreatic ductal adenocarcinoma and chronic pancreatitis by measurement of microRNA abundance in blood and tissue. *PLoS One*. 2012;7(4):e34151.
187. Schultz NA, Dehlendorff C, Jensen BV, Bjerregaard JK, Nielsen KR, Bojesen SE, et al. MicroRNA biomarkers in whole blood for detection of pancreatic cancer. *JAMA*. 2014;311(4):392-404.
188. Kojima M, Sudo H, Kawauchi J, Takizawa S, Kondou S, Nobumasa H, et al. MicroRNA markers for the diagnosis of pancreatic and biliary-tract cancers. *PloS one*. 2015;10(2):e0118220.
189. Puik JR, Meijer LL, Large TYL, Heger M, Dijk F, Funel N, et al. Abstract 493: Circulating biliary tract microRNA signature discriminates cholangiocarcinoma from pancreatic cancer. *Cancer Research*. 2018;78(13 Supplement):493-.
190. Voigtlander T, Gupta SK, Thum S, Fendrich J, Manns MP, Lankisch TO, et al. MicroRNAs in Serum and Bile of Patients with Primary Sclerosing Cholangitis and/or Cholangiocarcinoma. *PLoS One*. 2015;10(10):e0139305.
191. Cote GA, Gore AJ, McElyea SD, Heathers LE, Xu H, Sherman S, et al. A pilot study to develop a diagnostic test for pancreatic ductal adenocarcinoma based on differential expression of select miRNA in plasma and bile. *The American journal of gastroenterology*. 2014;109(12):1942-52.
192. Wang J, Chen J, Chang P, LeBlanc A, Li D, Abbruzzesse JL, et al. MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease. *Cancer Prev Res (Phila)*. 2009;2(9):807-13.
193. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PloS one*. 2012;7(3):e30679.
194. Park NJ, Zhou H, Elashoff D, Henson BS, Kastratovic DA, Abemayor E, et al. Salivary microRNA: discovery, characterization, and clinical utility for oral cancer detection. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2009;15(17):5473-7.
195. Hanke M, Hoefig K, Merz H, Feller AC, Kausch I, Jocham D, et al. A robust methodology to study urine microRNA as tumor marker: microRNA-126 and microRNA-182 are related to urinary bladder cancer. *Urologic oncology*. 2010;28(6):655-61.
196. Lehmann SM, Kruger C, Park B, Derkow K, Rosenberger K, Baumgart J, et al. An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nature neuroscience*. 2012;15(6):827-35.
197. Weber JA, Baxter DH, Zhang S, Huang DY, Huang KH, Lee MJ, et al. The microRNA spectrum in 12 body fluids. *Clin Chem*. 2010;56(11):1733-41.
198. Li L, Masica D, Ishida M, Tomuleasa C, Umegaki S, Kalloo AN, et al. Human bile contains microRNA-laden extracellular vesicles that can be used for cholangiocarcinoma diagnosis. *Hepatology*. 2014;60(3):896-907.
199. Trams EG, Lauter CJ, Salem N, Jr., Heine U. Exfoliation of membrane ectoenzymes in the form of micro-vesicles. *Biochim Biophys Acta*. 1981;645(1):63-70.
200. Pan BT, Teng K, Wu C, Adam M, Johnstone RM. Electron microscopic evidence for externalization of the transferrin receptor in vesicular form in sheep reticulocytes. *J Cell Biol*. 1985;101(3):942-8.
201. Harding C, Heuser J, Stahl P. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol*. 1983;97(2):329-39.
202. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *The Journal of biological chemistry*. 1987;262(19):9412-20.
203. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, et al. B lymphocytes secrete antigen-presenting vesicles. *J Exp Med*. 1996;183(3):1161-72.

204. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, et al. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med*. 1998;4(5):594-600.
205. van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. *Nat Rev Mol Cell Biol*. 2018;19(4):213-28.
206. Montecalvo A, Larregina AT, Shufesky WJ, Stolz DB, Sullivan ML, Karlsson JM, et al. Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood*. 2012;119(3):756-66.
207. Batagov AO, Kuznetsov VA, Kurochkin IV. Identification of nucleotide patterns enriched in secreted RNAs as putative cis-acting elements targeting them to exosome nano-vesicles. *BMC Genomics*. 2011;12 Suppl 3:S18.
208. Colombo M, Raposo G, Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255-89.
209. Thery C. Exosomes: secreted vesicles and intercellular communications. *F1000 Biol Rep*. 2011;3:15.
210. Colombo M, Moita C, van Niel G, Kowal J, Vigneron J, Benaroch P, et al. Analysis of ESCRT functions in exosome biogenesis, composition and secretion highlights the heterogeneity of extracellular vesicles. *J Cell Sci*. 2013;126(Pt 24):5553-65.
211. Henne WM, Stenmark H, Emr SD. Molecular mechanisms of the membrane sculpting ESCRT pathway. *Cold Spring Harb Perspect Biol*. 2013;5(9).
212. Hanson PI, Cashikar A. Multivesicular body morphogenesis. *Annu Rev Cell Dev Biol*. 2012;28:337-62.
213. Stuffers S, Sem Wegner C, Stenmark H, Brech A. Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic*. 2009;10(7):925-37.
214. Zylbersztejn K, Galli T. Vesicular traffic in cell navigation. *FEBS J*. 2011;278(23):4497-505.
215. Stenmark H. Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol*. 2009;10(8):513-25.
216. Halvaei S, Daryani S, Eslami SZ, Samadi T, Jafarbeik-Iravanian N, Bakhshayesh TO, et al. Exosomes in Cancer Liquid Biopsy: A Focus on Breast Cancer. *Molecular therapy Nucleic acids*. 2018;10:131-41.
217. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current protocols in cell biology / editorial board, Juan S Bonifacino [et al]*. 2006;Chapter 3:Unit 3 22.
218. Rani S, O'Brien K, Kelleher FC, Corcoran C, Germano S, Radomski MW, et al. Isolation of exosomes for subsequent mRNA, MicroRNA, and protein profiling. *Methods Mol Biol*. 2011;784:181-95.
219. Zaborowski MP, Balaj L, Breakefield XO, Lai CP. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Bioscience*. 2015;65(8):783-97.
220. Tauro BJ, Greening DW, Mathias RA, Ji H, Mathivanan S, Scott AM, et al. Comparison of ultracentrifugation, density gradient separation, and immunoaffinity capture methods for isolating human colon cancer cell line LIM1863-derived exosomes. *Methods*. 2012;56(2):293-304.
221. Greening DW, Xu R, Ji H, Tauro BJ, Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol*. 2015;1295:179-209.
222. Liga A, Vliegenthart AD, Oosthuyzen W, Dear JW, Kersaudy-Kerhoas M. Exosome isolation: a microfluidic road-map. *Lab Chip*. 2015;15(11):2388-94.
223. Rekker K, Saare M, Roost AM, Kubo AL, Zarovni N, Chiesi A, et al. Comparison of serum exosome isolation methods for microRNA profiling. *Clinical biochemistry*. 2014;47(1-2):135-8.

224. Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C. Exosomal-like vesicles are present in human blood plasma. *Int Immunol*. 2005;17(7):879-87.
225. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9(6):654-9.
226. Melo SA, Luecke LB, Kahlert C, Fernandez AF, Gammon ST, Kaye J, et al. Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*. 2015;523(7559):177-82.
227. Lai X, Wang M, McElyea SD, Sherman S, House M, Korc M. A microRNA signature in circulating exosomes is superior to exosomal glypican-1 levels for diagnosing pancreatic cancer. *Cancer letters*. 2017;393:86-93.
228. Frampton AE, Prado MM, Lopez-Jimenez E, Fajardo-Puerta AB, Jawad ZAR, Lawton P, et al. Glypican-1 is enriched in circulating-exosomes in pancreatic cancer and correlates with tumor burden. *Oncotarget*. 2018;9(27):19006-13.
229. Webber J, Steadman R, Mason MD, Tabi Z, Clayton A. Cancer exosomes trigger fibroblast to myofibroblast differentiation. *Cancer Res*. 2010;70(23):9621-30.
230. Peinado H, Aleckovic M, Lavotshkin S, Matei I, Costa-Silva B, Moreno-Bueno G, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18(6):883-91.
231. O'Brien K, Rani S, Corcoran C, Wallace R, Hughes L, Friel AM, et al. Exosomes from triple-negative breast cancer cells can transfer phenotypic traits representing their cells of origin to secondary cells. *Eur J Cancer*. 2013;49(8):1845-59.
232. Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, et al. Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers. *Nat Cell Biol*. 2008;10(12):1470-6.
233. Taylor DD, Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecol Oncol*. 2008;110(1):13-21.
234. Fabbri M, Paone A, Calore F, Galli R, Gaudio E, Santhanam R, et al. MicroRNAs bind to Toll-like receptors to induce prometastatic inflammatory response. *Proc Natl Acad Sci U S A*. 2012;109(31):E2110-6.
235. Ohyashiki JH, Umezaki T, Ohyashiki K. Exosomes promote bone marrow angiogenesis in hematologic neoplasia: the role of hypoxia. *Curr Opin Hematol*. 2016;23(3):268-73.
236. Zhang L, Zhang S, Yao J, Lowery FJ, Zhang Q, Huang WC, et al. Microenvironment-induced PTEN loss by exosomal microRNA primes brain metastasis outgrowth. *Nature*. 2015;527(7576):100-4.
237. Costa-Silva B, Aiello NM, Ocean AJ, Singh S, Zhang H, Thakur BK, et al. Pancreatic cancer exosomes initiate pre-metastatic niche formation in the liver. *Nat Cell Biol*. 2015;17(6):816-26.
238. Madhavan B, Yue S, Galli U, Rana S, Gross W, Muller M, et al. Combined evaluation of a panel of protein and miRNA serum-exosome biomarkers for pancreatic cancer diagnosis increases sensitivity and specificity. *Int J Cancer*. 2015;136(11):2616-27.
239. Richards KE, Zeleniak AE, Fishel ML, Wu J, Littlepage LE, Hill R. Cancer-associated fibroblast exosomes regulate survival and proliferation of pancreatic cancer cells. *Oncogene*. 2017;36(13):1770-8.
240. Yan I, Patel T. Isolation of extracellular vesicle RNA from bile. *Protocol Exchange*. 2015.
241. Schroeder A, Mueller O, Stocker S, Salowsky R, Leiber M, Gassmann M, et al. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Mol Biol*. 2006;7:3.
242. Bossler AVD, Viviana. Chapter 4 - Conventional and Real-Time Polymerase Chain Reaction 2009.

243. Ambros V. The functions of animal microRNAs. *Nature*. 2004;431(7006):350-5.
244. Zhang Y, Li M, Wang H, Fisher WE, Lin PH, Yao Q, et al. Profiling of 95 microRNAs in pancreatic cancer cell lines and surgical specimens by real-time PCR analysis. *World journal of surgery*. 2009;33(4):698-709.
245. Hanoun N, Delpu Y, Suriawinata AA, Bournet B, Bureau C, Selves J, et al. The silencing of microRNA 148a production by DNA hypermethylation is an early event in pancreatic carcinogenesis. *Clin Chem*. 2010;56(7):1107-18.
246. Brand RE, Adai AT, Centeno BA, Lee LS, Rateb G, Vignesh S, et al. A microRNA-based test improves endoscopic ultrasound-guided cytologic diagnosis of pancreatic cancer. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association*. 2014;12(10):1717-23.
247. Chen Y, Song Y, Wang Z, Yue Z, Xu H, Xing C, et al. Altered expression of MiR-148a and MiR-152 in gastrointestinal cancers and its clinical significance. *Journal of gastrointestinal surgery : official journal of the Society for Surgery of the Alimentary Tract*. 2010;14(7):1170-9.
248. Zheng B, Liang L, Wang C, Huang S, Cao X, Zha R, et al. MicroRNA-148a suppresses tumor cell invasion and metastasis by downregulating ROCK1 in gastric cancer. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2011;17(24):7574-83.
249. Takahashi K, Iinuma H, Wada K, Minezaki S, Kawamura S, Kainuma M, et al. Usefulness of exosome-encapsulated microRNA-451a as a minimally invasive biomarker for prediction of recurrence and prognosis in pancreatic ductal adenocarcinoma. *J Hepatobiliary Pancreat Sci*. 2018;25(2):155-61.
250. Xu Q, Liu LZ, Yin Y, He J, Li Q, Qian X, et al. Regulatory circuit of PKM2/NF-kappaB/miR-148a/152-modulated tumor angiogenesis and cancer progression. *Oncogene*. 2015;34(43):5482-93.
251. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, et al. A microRNA DNA methylation signature for human cancer metastasis. *Proc Natl Acad Sci U S A*. 2008;105(36):13556-61.
252. Magrelli A, Azzalin G, Salvatore M, Viganotti M, Tosto F, Colombo T, et al. Altered microRNA Expression Patterns in Hepatoblastoma Patients. *Transl Oncol*. 2009;2(3):157-63.
253. Zhang H, Wang Y, Xu T, Li C, Wu J, He Q, et al. Increased expression of microRNA-148a in osteosarcoma promotes cancer cell growth by targeting PTEN. *Oncol Lett*. 2016;12(5):3208-14.
254. Murata T, Takayama K, Katayama S, Urano T, Horie-Inoue K, Ikeda K, et al. miR-148a is an androgen-responsive microRNA that promotes LNCaP prostate cell growth by repressing its target CAND1 expression. *Prostate Cancer Prostatic Dis*. 2010;13(4):356-61.
255. Huang JJ, Yu J, Li JY, Liu YT, Zhong RQ. Circulating microRNA expression is associated with genetic subtype and survival of multiple myeloma. *Medical oncology*. 2012;29(4):2402-8.
256. Yang JS, Li BJ, Lu HW, Chen Y, Lu C, Zhu RX, et al. Serum miR-152, miR-148a, miR-148b, and miR-21 as novel biomarkers in non-small cell lung cancer screening. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*. 2015;36(4):3035-42.
257. Visone R, Pallante P, Vecchione A, Cirombella R, Ferracin M, Ferraro A, et al. Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. *Oncogene*. 2007;26(54):7590-5.
258. Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, et al. MicroRNA expression profiles in serous ovarian carcinoma. *Clinical cancer research : an official journal of the American Association for Cancer Research*. 2008;14(9):2690-5.

259. Kappelmann M, Kuphal S, Meister G, Vardimon L, Bosserhoff AK. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression. *Oncogene*. 2013;32(24):2984-91.
260. Lee YS, Kim HK, Chung S, Kim KS, Dutta A. Depletion of human micro-RNA miR-125b reveals that it is critical for the proliferation of differentiated cells but not for the down-regulation of putative targets during differentiation. *The Journal of biological chemistry*. 2005;280(17):16635-41.
261. Shi XB, Xue L, Ma AH, Tepper CG, Kung HJ, White RW. miR-125b promotes growth of prostate cancer xenograft tumor through targeting pro-apoptotic genes. *Prostate*. 2011;71(5):538-49.
262. Ottaviani S, Stebbing J, Frampton AE, Zagorac S, Krell J, de Giorgio A, et al. TGF-beta induces miR-100 and miR-125b but blocks let-7a through LIN28B controlling PDAC progression. *Nat Commun*. 2018;9(1):1845.
263. Wang H, Tan G, Dong L, Cheng L, Li K, Wang Z, et al. Circulating MiR-125b as a marker predicting chemoresistance in breast cancer. *PloS one*. 2012;7(4):e34210.
264. Zhang J, Zhao CY, Zhang SH, Yu DH, Chen Y, Liu QH, et al. Upregulation of miR-194 contributes to tumor growth and progression in pancreatic ductal adenocarcinoma. *Oncol Rep*. 2014;31(3):1157-64.
265. Bernuzzi F, Marabita F, Lleo A, Carbone M, Mirolo M, Marzioni M, et al. Serum microRNAs as novel biomarkers for primary sclerosing cholangitis and cholangiocarcinoma. *Clin Exp Immunol*. 2016;185(1):61-71.
266. Iizuka D, Imaoka T, Nishimura M, Kawai H, Suzuki F, Shimada Y. Aberrant microRNA expression in radiation-induced rat mammary cancer: the potential role of miR-194 overexpression in cancer cell proliferation. *Radiat Res*. 2013;179(2):151-9.
267. Sundaram P, Hultine S, Smith LM, Dews M, Fox JL, Biyashev D, et al. p53-responsive miR-194 inhibits thrombospondin-1 and promotes angiogenesis in colon cancers. *Cancer Res*. 2011;71(24):7490-501.
268. Khella HW, Bakhet M, Allo G, Jewett MA, Girgis AH, Latif A, et al. miR-192, miR-194 and miR-215: a convergent microRNA network suppressing tumor progression in renal cell carcinoma. *Carcinogenesis*. 2013;34(10):2231-9.
269. Meng Z, Fu X, Chen X, Zeng S, Tian Y, Jove R, et al. miR-194 is a marker of hepatic epithelial cells and suppresses metastasis of liver cancer cells in mice. *Hepatology*. 2010;52(6):2148-57.
270. Thery C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat Rev Immunol*. 2002;2(8):569-79.
271. Quesenberry PJ, Aliotta JM. The paradoxical dynamism of marrow stem cells: considerations of stem cells, niches, and microvesicles. *Stem cell reviews*. 2008;4(3):137-47.
272. Lachenal G, Pernet-Gallay K, Chivet M, Hemming FJ, Belly A, Bodon G, et al. Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Molecular and cellular neurosciences*. 2011;46(2):409-18.
273. Kramer-Albers EM, Bretz N, Tenzer S, Winterstein C, Mobius W, Berger H, et al. Oligodendrocytes secrete exosomes containing major myelin and stress-protective proteins: Trophic support for axons? *Proteomics Clinical applications*. 2007;1(11):1446-61.
274. Corcoran C, Rani S, O'Driscoll L. miR-34a is an intracellular and exosomal predictive biomarker for response to docetaxel with clinical relevance to prostate cancer progression. *Prostate*. 2014;74(13):1320-34.
275. Alegre E, Sanmamed MF, Rodriguez C, Carranza O, Martin-Algarra S, Gonzalez A. Study of circulating microRNA-125b levels in serum exosomes in advanced melanoma. *Archives of pathology & laboratory medicine*. 2014;138(6):828-32.
276. Chen D, Wu X, Xia M, Wu F, Ding J, Jiao Y, et al. Upregulated exosomal miR23b3p plays regulatory roles in the progression of pancreatic cancer. *Oncol Rep*. 2017;38(4):2182-8.

277. Xu YF, Hannafon BN, Zhao YD, Postier RG, Ding WQ. Plasma exosome miR-196a and miR-1246 are potential indicators of localized pancreatic cancer. *Oncotarget*. 2017;8(44):77028-40.
278. Que R, Ding G, Chen J, Cao L. Analysis of serum exosomal microRNAs and clinicopathologic features of patients with pancreatic adenocarcinoma. *World J Surg Oncol*. 2013;11:219.
279. Goto T, Fujiya M, Konishi H, Sasajima J, Fujibayashi S, Hayashi A, et al. An elevated expression of serum exosomal microRNA-191, -21, -451a of pancreatic neoplasm is considered to be efficient diagnostic marker. *BMC cancer*. 2018;18(1):116.
280. Wang X, Luo G, Zhang K, Cao J, Huang C, Jiang T, et al. Hypoxic Tumor-Derived Exosomal miR-301a Mediates M2 Macrophage Polarization via PTEN/PI3Kgamma to Promote Pancreatic Cancer Metastasis. *Cancer Res*. 2018;78(16):4586-98.
281. Takikawa T, Masamune A, Yoshida N, Hamada S, Kogure T, Shimosegawa T. Exosomes Derived From Pancreatic Stellate Cells: MicroRNA Signature and Effects on Pancreatic Cancer Cells. *Pancreas*. 2017;46(1):19-27.
282. Patel GK, Khan MA, Bhardwaj A, Srivastava SK, Zubair H, Patton MC, et al. Exosomes confer chemoresistance to pancreatic cancer cells by promoting ROS detoxification and miR-155-mediated suppression of key gemcitabine-metabolising enzyme, DCK. *British journal of cancer*. 2017;116(5):609-19.
283. Han S, Gonzalo DH, Feely M, Rinaldi C, Belsare S, Zhai H, et al. Stroma-derived extracellular vesicles deliver tumor-suppressive miRNAs to pancreatic cancer cells. *Oncotarget*. 2018;9(5):5764-77.
284. Mikamori M, Yamada D, Eguchi H, Hasegawa S, Kishimoto T, Tomimaru Y, et al. MicroRNA-155 Controls Exosome Synthesis and Promotes Gemcitabine Resistance in Pancreatic Ductal Adenocarcinoma. *Scientific reports*. 2017;7:42339.
285. Severino V, Dumonceau JM, Delhaye M, Moll S, Annessi-Ramseyer I, Robin X, et al. Extracellular Vesicles in Bile as Markers of Malignant Biliary Stenoses. *Gastroenterology*. 2017;153(2):495-504 e8.

