Estimate of Mismatch Repair Deficient Pancreatic Cancer in Patients Enrolled in the Quebec Pancreas Cancer Study

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) has a 5-year survival rate of only 8%. Despite decades of research, treatment options for PDAC are largely ineffective. Identification of PDAC subtypes for precision oncology treatment strategies may lead to improved patient outcomes. Immunotherapy is emerging as a highly effective therapy in select subclasses of cancer. In general, PDAC is considered "immune-cold" without treatment responses to immune-mediated therapies. However, tumors demonstrating DNA mismatch repair deficiency (MMR-d) and/or associated microsatellite instability (MSI) have been shown to respond to immunotherapies. The prevalence of MMR-d in PDAC has not been well characterized and testing for MMR-d in PDAC is not routinely performed. While some studies estimate the frequency of MMR-d in PDAC to be as high as 22%, others have shown a frequency of only 0.3%. Considering these estimates and adhoc testing at the MUHC, we hypothesized that the incidence of MMR-d in PDAC is closer to 1%, as large-scale studies implementing sequencing technology have estimated. In the present study, the prevalence of MMR-d in PDAC was estimated by retrospectively evaluating PDAC cases enrolled in Quebec Pancreas Cancer Study. Tumor microarrays (TMA) were constructed from formalin-fixed paraffin-embedded (FFPE) tissue samples from 97 patients with pathologically confirmed PDAC. Immunohistochemistry staining of TMAs for MLH1, MSH2, MSH6 and PMS2 revealed 4 patients with absent staining in at least one MMR protein representing 4.4% of cases (95% CI 0.2%-8.7%). However, following confirmation using whole sections, only 1 patient was confirmed to be MMR deficient with remaining three cases demonstrating heterogeneous staining with intact nuclear staining in the majority of ductal cells and smaller regions displaying complete absence of staining. Despite the unusual staining pattern, these cases would be considered MMR proficient. Thus, the current

study suggests a MMR-d prevalence of 1.1% (95% CI 0-3.3%) in PDAC. The heterogeneous staining pattern observed may explain the high estimates of MMR-d prevalence in previous studies utilizing IHC staining on TMAs without a secondary confirmation method. It is unclear whether this heterogeneity reflects a technical limitation of IHC or tissue heterogeneity with a subset of tumor cells exhibiting MMR-d and others with intact mismatch repair. Due to the technical limitations of IHC and MSI, recent studies have implemented gene panel testing or whole genome sequencing in order to estimate the prevalence of MMR-d in PAC. These studies obtained similar results to ours with an estimated prevalence of MMR-d of approximately 1%. Despite the low prevalence of MMR-d in PDAC, clinical reflex testing may still be warranted since there are clinical implications in identifying such patients, including precision oncology treatment opportunities with immunotherapy and the identification of at-risk relatives for prevention and early detection of Lynch-associated malignancies.

Résumé

L'adénocarcinome du pancréas (PAC) a un taux de survie à 5 ans de seulement 8%. Malgré des décennies de recherche, les options de traitement pour la PAC sont limitées. À l'époque de la médecine génomique, l'identification des sous-types génomiques pouvant être utiles pour individualiser le traitement et améliorer les taux de survie pour les patients suscite un intérêt croissant. L'utilisation de l'immunothérapie est une nouvelle approche particulièrement efficace pour le traitement de certains types de cancers. En général, les tumeurs du pancréas sont considérées résistantes aux traitements à médiation immunitaire. Cependant, les tumeurs pancréatiques ayant un déficit en réparation de l'ADN, appelé déficit de réparation des mésappariements (MMR-d), et/ou l'instabilité des microsatellites (MSI) semblent être susceptible à certaines immunothérapies. Les tumeurs du pancréas sont rarement testées pour la déficience de MMR, ce qui fait que les tests ne sont ni systématiques, ni normalisés. Par conséquent, le taux de MMR-d dans les cancers du pancréas n'est pas bien caractérisé. Alors que certaines études estiment que la fréquence du MMR-d dans le PAC est aussi élevée que 22%, d'autres n'ont démontré que 0,3%. En prenant en compte les données publiées et les tests de façon ad hoc au CUSM, nous avons émis une hypothèse que l'incidence du MMR-d dans le PAC se rapprochait de 1%, comme l'avaient estimé des études à grande échelle mettant en œuvre une technologie de séquençage. Cette étude a completé une estimation du taux de prévalence de MMR-d en étudiant, de manière rétrospective, des cas de PDAC inscrit à l'Étude Québécoise sur le Cancer du Pancréas. Des microréseaux de tissues tumoraux (TMA) ont été construits à partir d'échantillons de tissus fixés au formol et inclus dans de la paraffine (FFPE) chez 97 patients atteints le PAC. L'immunohistochimie (IHC) des TMA pour les protéines MLH1, MSH2, MSH6 et PMS2 a révélé 4 patients avec une absence de coloration pour au moins une protéine testée représentant

4,4% des cas (IC à 95% 0,2% à 8,7%). Cependant, après confirmation sur des sections entières, un seul patient a été confirmé MMR-d, avec les trois cas restants présentant une coloration hétérogène avec une coloration nucléaire intacte dans la majorité des cellules canalaires et des régions plus petites présentant une absence complète de coloration. Malgré le caractère hétérogène, ces cas seraient considérés comme compétents en MMR et par conséquent, nous estimons que la prévalence du MMR-d dans la PAC est d'environ 1,1% (IC à 95% de 0 à 3,3%). Le schéma de coloration hétérogène observé peut expliquer les estimations élevées de la prévalence du MMR-d dans des études antérieures utilisant l'IHC sur des TMA sans méthode de confirmation secondaire. Il n'est pas clair si cette hétérogénéité reflète une limitation technique de l'IHC ou une hétérogénéité de la tumeur avec un sous-ensemble de cellules tumorales présentant une déficience en MMR et d'autres avec un system de réparation intacte. En raison des limitations techniques d'IHC et de MSI, des études récentes ont mis en œuvre des tests comprenant un panneau de gènes ou un séquençage du génome entier afin d'estimer la prévalence du MMR-d dans le PAC. Ces études ont obtenu des résultats similaires aux nôtres avec une prévalence estimée du MMR-d d'environ 1%. Malgré la faible prévalence du MMR-d dans le PAC, un test de réflexe clinique peut toujours être justifié, car l'identification de tels patients a plusieurs implications importantes. Ceux-ci incluent la possibilité d'un traitement par immunothérapie, l'identification de membres de la famille à risque et le dépistage subséquent de ces patients pour la prévention et la détection précoce des tumeurs malignes associées au syndrome de Lynch.

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Contributions

I designed and performed all experiments presented in this dissertation, including all associated data analyses. I prepared the dissertation.

Dr. George Zogopoulos provided mentorship and guidance in experimental design and the preparation of this dissertation.

Ms. Adeline Cuggia and **Dr. Yifan Wang** assisted with the collection of patient clinical data.

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List of Tables and Figures

List of Abbreviations

Abbreviation	Definition
5-FU	5-fluorouracil
AJCC	American Joint Committee on Cancer
AP	Apurinic/apyrimidinic
BER	Base excision repair
BMI	Body mass index
CA19-9	Cancer antigen 19-9
CMMRD	Constitutional mismatch repair deficiency
CRC	Colorectal cancer
DSBR	Double-strand break-repair
EC	Endometrial carcinoma
EMT	Epithelial to mesenchymal transition
FAMMM	Familial atypical multiple mole melanoma
FAP	Familial adenomatous polyposis
FC	French Canadian
FFPE	Formalin-fixed paraffin-embedded
FNA	Fine needle aspiration
FPC	Familial pancreatic cancer
GI	Gastrointestinal
H&E	Hematoxylin & eosin
HBOC	Hereditary breast & ovarian cancer
HNPCC	Hereditary non-polyposis colorectal cancer
HP	Hereditary pancreatitis
HR	Homologous recombination
IHC	Immunohistochemistry
IPMN	Intraductal papillary mucinous neoplasm
LFS	Li-fraumeni syndrome
LS	Lynch syndrome
MCN	Mucinous cystic neoplasm
MDM2	Mouse double minute 2 homolog
MMR	Mismatch repair
MMR-d	Mismatch repair deficiency
MSI	Microsatellite instability
MSI-H	Microsatellite instability high
MSI-L	Microsatellite instability low
MSK-IMPACT	Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets

MSS	Microsatellite stable
MUHC	McGill University Health Center
NCCN	National Comprehensive Cancer Network
NER	Nucleotide excision repair
NGS	Next-generation sequencing
NHEJ	Non-homologous end-joining
OR	Odds ratio
OS	Overall survival
PAC	Pancreatic adenocarcinoma
PanIN	Pancreatic intraepithelial neoplasia
PC	Pancreatic cancer
PD-1	Programmed cell death protein 1
PDAC	Pancreatic ductal adenocarcinoma
PJS	Peutz-Jeghers syndrome
PNET	Pancreatic neuroendocrine tumor
QPCS	Quebec Pancreas Cancer Study
RR	Relative risk
SIR	Standardized incidence ratio
SSBR	Single-strand break-repair
TMA	Tumor Microarray/Tissue Microarray
WGS	Whole genome sequencing

Chapter I: Literature Review

I.1 Overview of Pancreatic Cancer

I.1.1 Anatomy and Physiology of the Pancreas

The pancreas is an elongated, tapered organ located deep in the retroperitoneal portion of the abdomen, behind the stomach.[1] It is divided into five main regions: the head, neck, body, tail and uncinate process.[1] The head and uncinate process of the pancreas are surrounded by a C-shaped loop of duodenum.[2] The neck is a small portion of the pancreas lying directly anterior to the portal vein confluence and the superior mesenteric artery.[1] The body sits posterior to the stomach and the tail extends to the hilum of the spleen.[1] Exocrine secretions flow through the pancreatic duct and drain into the duodenum at the ampulla.[1]

Functionally, the pancreas is divided into the exocrine portion, comprising 95% of the of the pancreas, and the endocrine portion, comprising 1-2% of the pancreas.[3] The exocrine pancreas contains two main cell types: acinar cells and duct cells.[3] Acinar cells are rich in rough endoplasmic reticulum allowing them to produce, store and secrete digestive enzymes into the duodenum for the digestion of sugars, proteins and fats.[3] Duct cells, on the other hand, are rich in mitochondria, allowing sufficient ATP production for the transport of water and ions such as NaHCO3.[3] The exocrine pancreas also contains two other cell types: centroacinar cells, which act as pancreatic stem cells, and stellate cells which direct proper formation of epithelial structures.[3] The endocrine portion is composed islets of Langerhans which contain 4 main cell types: alpha, beta, delta and F cells which secrete the sugar-regulating hormones glucagon, insulin, somatostatin and pancreatic polypeptide, respectively.[2]

1.1.2 Histology of Solid Pancreatic Neoplasms

Pancreatic neoplasms can be broadly divided into two categories: those arising from the endocrine portion of the pancreas; and those arising from the exocrine pancreas.[4] Pancreatic neuroendocrine tumors (PNET), as its name suggests, arise from the islet cells of the endocrine pancreas.[5] These tumors are rare, accounting for less than 3% of all primary neoplasms of the pancreas.[5] PNETs are soft, well-demarcated and richly vascular solid neoplasms.[4] Microscopically, the neoplastic cells form trabeculae with granular cytoplasm and characteristic "salt and pepper" chromatin.[6] PNETs are generally less aggressive than tumors of the exocrine pancreas with a 5-year survival rate of 45%.[4] PNETS are further divided into non-functional tumors and functional tumors depending whether or not they secrete peptide hormones, such as insulin.[5]

Pancreatic ductal adenocarcinomas (PDAC) arise from the ductal cells of the exocrine pancreas and account for 95% of solid pancreatic tumors.[7] Grossly, PDACs are white to yellow, firm, ill-defined solid masses.[6] Microscopically, the tumor is composed of unevenly arranged infiltrating ductal and glandular structures typically surrounded by abundant and dense stromal tissue.[8] This is caused by an intense desmoplastic reaction, a process in which fibroblasts, inflammatory cells, endothelial cells and a complex extracellular matrix pool near the site of the tumor causing increased interstitial fluid pressure.[4] This elevated pressure impedes the perfusion of the tumor, diminishing the ability of therapeutic agents to reach neoplastic cells.[4] Consequently, many systemic therapies have limited efficacy in treating PDAC.[4] PDAC tumors also commonly exhibit perineural and lymphovascular invasion allowing projections of neoplastic cells to extend far beyond the main tumor area and metastasize.[4,8] As a result, PDAC is an aggressive cancer and is often diagnosed late, when surgical resection is no longer

possible.[4] Rare variants of pancreatic adenocarcinoma include adenosquamous carcinoma, colloid carcinoma, medullary carcinoma, signet-ring carcinoma, hepatoid carcinoma and undifferentiated or anaplastic carcinoma.[9] Finally, other solid neoplasms of the pancreas are extremely rare and include acinar carcinomas and pancreatoblastoma.[4] Because PDAC accounts for the vast majority of pancreatic neoplasms, it is often simply referred to as pancreatic cancer and will be the focus of this dissertation.

1.1.3 PDAC Precursor Lesions

The transformation from a normal epithelial cell to a cancer cell is caused by a series of genetic mutations which lead to the rapid proliferation and abnormal growth of cells, termed hyperplasia and dysplasia respectively.[8] The accumulation of further genetic mutations leads to the development of small, non-invasive tumors otherwise known as carcinoma *in situ*.[8] Finally, the acquisition of invasive properties allows for cancer progression and metastasis. [8]

In the progression model of PDAC development, these precursors are termed pancreatic intraepithelial neoplasia (PanIN) **(Figure 1).** PanINs are lesions composed of columnar or cuboidal cells with varying amounts of dysplasia and mucin production.[10] The PanIN model of PDAC development classifies lesions into several grades depending on their architecture, cytology and associated genetic driver events.[11,12]

PanIN-1 lesions are low-grade with minimal atypical features.[13] They are sub-classified as PanIN-1A when they form flat lesions and PanIN-1B when they form papillary lesions.[12] PanIN-1 lesions are associated with activating *KRAS* mutations, which are found in over 90% of cases, and telomere dysfunction.[13,14] *KRAS* is a proto-oncogene which encodes a GTPase involved in cell proliferation, differentiation, survival and migration.[13] Under normal

physiological conditions, *KRAS* is predominantly in its inactive, GDP-bound state.[13] However, during the development of many cancers, including PDAC, mutations develop in *KRAS* which lock it in its active, GTP-bound state.[13] As a result, downstream pathways become constitutively activated and drive the early stages of tumor initiation, including the development of PanIN precursor lesions.[13]

PanIN-2 lesions are intermediate grade and show moderate atypical cytological and architectural features including nuclear pleomorphisms, crowding and hyperchromasia.[10] PanIN-2 lesions often present with a papillary morphology and are associated with the loss *CDKN2A* tumor suppressor gene.[10] *CDKN2A* encodes a cyclin-dependent kinase inhibitor, p16/INK4A, which restricts entry into the S-phase of the cell cycle.[13] In addition, *CDKN2A* utilizes an alternate reading frame in order to encode a second tumor suppressor, p14ARF, which inhibits proteolysis of p53 by MDM2, an E3 ubiquitin-protein ligase.[13] For this reason, 95% of PDAC tumors exhibit functional loss of CDKN2A in order to bypass the senescence response following KRAS activation and allow for disease progression.[13]

Finally, high-grade PanIN-3 are usually papillary and are characterized by the "budding-off" of small clusters of epithelial cells into the lumen, loss of polarity, nuclear crowding, enlarged nuclei and hyperchromatin.[12] These lesions are considered carcinoma *in situ*.[10] PanIN-3 lesions are associated with the loss of the tumor suppressors *p53* and *SMAD4*.[13] In response to cellular stress or DNA damage, p53 acts as a transcription factor, inducing the expression of genes involved in cell cycle arrest and apoptosis. Mutations in *p53* impede its ability to bind DNA thereby preventing this response.[13] Loss of *p53* is thought to be required for the survival and continued proliferation of tumorigenic cells.[13] Furthermore, *p53* mutations may also be involved in PDAC metastasis by inhibiting its ability to counteract cell migration and epithelial-

to-mesenchymal transition (EMT).[13] One of the final steps in tumor initiation, occurring in approximately 55% of PDACs, is the loss of *SMAD4*.[14] SMAD4 is a coactivator of the TGF-B signalling pathway, a key inducer of EMT.[13] Mutations in *SMAD4* contribute the invasive and metastatic nature of PDAC.[13]

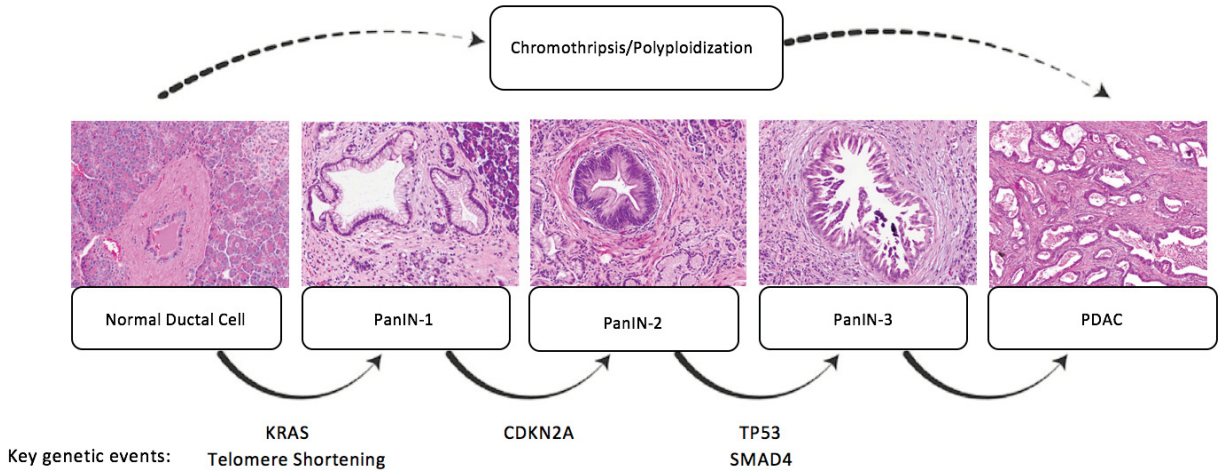


Figure 1. Models of PDAC development and associated histology. PDAC may arise progressively as hypothesized by the PanIN model (bottom), or instantaneously through catastrophic genetic events such as chromothripsis or polyploidization (top). Figure adapted from Loda *et al.* (2016)[8], approval obtained 03/10/19.

The PanIN model of pancreatic cancer development has been challenged as PanIN precursor lesions are not always present in pancreatic tumor specimens.[15] A study by Notta *et al.* found that in up to one-third of cases, the genetic driver events associated with the development of PDAC occur simultaneously rather than progressively as hypothesized by the PanIN model.[15] It is thought that this is due to large-scale catastrophic genetic events such as DNA shearing, known as chromothripsis, or genetic transformations associated with polyploidization.[15] As a

result, the development of invasive clones would occur almost instantaneously and metastasis would occur shortly after.[15] This alternative model of pancreatic cancer development has been termed the accelerated model **(Figure 1)**.[15]

Two other precursor lesions contribute to the development of PDAC: intraductal papillary mucinous neoplasms (IPMNs) and mucinous cystic neoplasms (MCNs).[10] These non-invasive cystic neoplasms are characterized by the production of vast amount of mucus.[4] IPMNs result in the cystic dilation of the large pancreatic ducts while MCNs typically arise in the tail of the pancreas and do not communicate with the ductal system.[4] Although these lesions typically present with low-grade dysplasia, they can progress to high-grade dysplasia and eventually develop into invasive carcinoma.[10] IPMNs tend to exhibit similar genetic changes to those seen in PanINs including mutations in KRAS, CDKN2A, SMAD4 and TP53.[10] However, a common and specific feature of IPMNs is the presence of GNAS mutations, either alone or in combination with those in KRAS.[10] On the contrary, MCNs do not exhibit mutations in GNAS but rather are associated with mutations in KRAS, TP53 and RNF43.[10] IPMNs can arise from the epithelium of the pancreatic duct (main duct type), its side branches (branch duct type), or both (mixed type).[16] This distinction is important as several studies have shown that side-branch IPMNs are less aggressive and less likely to progress into invasive cancer.[7] In fact, approximately 60-70% of main duct IPMNs contain malignant cells, compared to only 15-20% in isolated side-branch lesions.[16] Individuals with main duct IPMNs typically undergo prophylactic surgical resection, whereas side-branch IPMNs undergo surveillance with surgical resection if high-risk clinical features develop.[4,17] Mucinous cystic neoplasms (MCNs) are cystic, mucin-secreting tumors with ovarian-like stroma predominantly affecting middle-aged women.[7,16] Due to their malignant potential MCNs are treated with surgical resection.[17]

I.1.4 Clinical Overview of PDAC

Epidemiology

Pancreatic adenocarcinoma has one of the worst 5-year overall survival rates of any malignancy at only 8%.[18] In Canada, 5500 new cases of pancreatic cancer are diagnosed each year with 4800 succumbing to the disease.[18] Although the incidence of pancreatic cancer is relatively low at 1 in 73 (1.3%), it accounts for 6% of all cancer-related deaths due to its high mortality.[18] Pancreatic cancer affects men and women at roughly equal frequencies, with the highest incidence rates in African-American men and the lowest in Asian populations.[8] Unfortunately, unlike many cancer types, the incidence of pancreatic cancer is on the rise and decades of research have done little to improve patient outcomes.[18,19] In fact, the number of pancreatic cancer-related deaths is expected to increase by up to 50% by 2030.[20] It has been postulated that this may be a result of our aging population, as well as better detection methods.[20] As a result, although lung cancer is expected to remain the top killer, pancreatic cancer is projected to surpass both colon and breast cancer to become the second deadliest malignancy.[20]

Risk Factors

Like many cancers, age is a major determinant of pancreatic cancer development. Patients are generally diagnosed above the age of 50, with the peak incidence occurring in the seventh-to-eight decade of life.[21] The most common preventable risk factor for PDAC is cigarette smoking which increases the risk 2.2-fold compared to non-smokers and is responsible for approximately 25% of all pancreatic cancers.[4] While it is well established that new-onset type 2 diabetes can be an early sign of pancreatic cancer, long-standing (>10 years) type 2 diabetes mellitus can also contribute to pancreatic cancer development, conferring a 1.51-fold increased

risk.[4] More recently, obesity has been accepted as a clear risk factor for pancreatic cancer. A large meta-analysis reported a 10% increased risk for every 5-unit increase in body mass index (BMI), with an even more dramatic relative risk of 1.55 in obese patients (BMI > 35).[22] Other risk factors include heavy alcohol consumption (OR 1.46), chronic pancreatitis (RR 2.71), helicobacter pylori infection (OR 2.1), low physical activity and certain dietary factors including high intake of saturated fats, low intake of fruits and vegetables and consumption of processed and red meats.[4,8,21] In addition to environmental factors, there are also many inherited genetic conditions which pose an important risk for the development of pancreatic cancer. These conditions are outlined in **section 1.2.1.**

Symptoms & Diagnosis

Unfortunately, the majority of pancreatic cancers present with few, non-specific symptoms. For this reason, approximately 80% of diagnoses are made late in the disease course, after it has already spread, precluding curative-intent surgical resection.[23] Common symptoms include epigastric pain which often radiates to the back, unexplained weight loss, clay-colored stools and nausea.[4] Patients may also experience new onset diabetes and rarely migratory thrombophlebitis, an inflammation of the vein wall.[4] In addition, tumors at the head of the pancreas may obstruct the bile duct resulting in jaundice, sometimes aiding early diagnosis.[24]

When pancreatic cancer is suspected, a combination of computed tomography (CT), magnetic resonance imaging (MRI) and positron emission tomography (PET) is used for imaging of the pancreas, surrounding lymph nodes and any suspected metastases.[4] The results of these scans are key in evaluating metastatic spread as well as the involvement of important structures such as major vessels, and consequently, the resectability of the lesion.[21] In order to confirm a

diagnosis of pancreatic cancer, a pathological assessment is required, which is usually obtained through an endoscopic ultrasound with fine-needle aspiration (FNA) or core-needle biopsy.[21] Although a pancreatic cancer tumor biomarker, cancer antigen 19-9 (CA19-9) is useful in monitoring disease progression and response to treatment, its sensitivity and specificity is limited and cannot be reliably used as a definitive diagnostic tool.[21]

Once a diagnosis of pancreatic cancer is made, accurate clinical staging is required to establish prognosis and the most effective treatment plan. The American Joint Committee on Cancer (AJCC) staging system, based on TMN classification, is the most widely used to stage pancreatic cancer.[25] Based on this staging, cases are categorized into localized (stage I/II), locally advanced (stage III) or metastatic (stage IV).[25]

Treatment

Surgical resection is the only treatment for pancreatic cancer which offers the possibility of a cure. However, only 20% of patients are diagnosed with resectable disease which includes stage I, II and a subset of intermediate stage II-III cancers termed borderline resectable.[4] Patients with resectable tumors undergo a pancreaticoduodenectomy (Whipple's procedure) or distal/subtotal pancreatectomy with splenectomy for tumors located in the head and tail of the pancreas, respectively.[4] Tumors located in the neck or body of the pancreas are considered more difficult to treat surgically and although rare, may require a total pancreatectomy rather than one of the aforementioned procedures.[4] Following surgery, adjuvant chemotherapy is indicated in patients who are clinically fit. However, the oncologic survival benefit of adjuvant therapy is lost if the patients are unable to receive therapy within three months of surgery due to a complicated surgical recovery.[204] The current mainstays of adjuvant therapy are FOLFIRINOX (a cocktail

regimen of 5-fluorouracil (5-FU), leucovorin, irinotecan and oxaliplatin) or gemcitabine with or without concurrent capecitabine.[26] Although adjuvant chemotherapy has shown to increase survival compared to surgery alone, the median overall survival (OS) of resected patients remains only 54.4 months.[4,28]

Unfortunately, the remaining 80% of patients are diagnosed with locally advanced (unresectable) tumors or metastatic disease. Treatment options for these patients are limited and only moderately effective. Historically, the main chemotherapy regimen for the treatment of advanced PDAC was gemcitabine.[27] In 2010, FOLFIRINOX was shown to be superior with a 1-year OS of 48% and a median OS of 11.1 months compared to 20% and 6.8 months for gemcitabine and therefore became the standard of care.[28] However, it did cause significantly more adverse events and thus gemcitabine is still used in patients that cannot tolerate treatment with FOLFIRINOX.[28] More recently, gemcitabine in combination with protein-bound paclitaxel (nab-paclitaxel) may further improve survival compared to gemcitabine alone.[29-31] Despite improvements in chemotherapy regimens for advanced PAC, the five-year overall survival of these patients is only 8%.[18]

In the era of genomic-driven medicine, interest has grown in the use of precision oncology strategies.[32] For example, the use of platinum-based drugs, such as oxaliplatin and cisplatin, has shown increased efficacy in patients with defects in the homology-dependent repair pathway (*BRCA1*, *BRCA2* and *PALB2*) and is thus considered a targeted therapy for the DNA repair deficiency in this cancer subtype.[33] These strong DNA damaging agents, in combination with defective DNA repair lead to cellular death and therefore tumor regression.[33] There is also strong evidence for the use of immunotherapy for the treatment of pancreatic cancers exhibiting mismatch repair deficiency (MMR-d), described in **section 1.3.3.**

I.2 Genetics of Pancreatic Adenocarcinoma

I.2.1 Genetic Predisposition to PDAC

Hereditary Pancreatic Adenocarcinoma

Although most cases of pancreatic cancer are considered sporadic, arising via a combination of somatic genetic alterations and complex environmental factors, approximately 10% of pancreatic cancer cases have a hereditary basis.[34] Population-based studies have estimated the relative risk of individuals with a positive family history of pancreatic cancer at 2.49-3.2.[35-38] Familial pancreatic cancer (FPC) is defined as a kindred containing at least two first-degree relatives diagnosed with pancreatic cancer.[39] Individuals in FPC families are at an elevated risk for the development of pancreatic cancer (SIR 6.4 [95% CI, 1.8-16.4]). The risk further increases when three first-degree relatives are affected (SIR 32 [95%CI, 10.2-74.7]).[40] Although the genetic basis for the vast majority of FPC families have yet to be identified, several hereditary cancer predisposition syndromes are associated with an increased risk of pancreatic cancer, which are outlined below.[41] Together, these syndromes account for approximately 15% of FPC families and is termed hereditary pancreatic cancer.[42] A brief description of each genetic syndrome is outlined below and summarized in **Table 1.**

Peutz-Jeghers Syndrome

Peutz-Jeghers syndrome (PJS) is a rare, autosomal dominant condition caused by inherited germline mutations in the serine-threonine kinase 11 (*STK11*) gene.[43] *STK11* is a tumor suppressor gene involved in the regulation of the cell cycle, p53 mediated apoptosis and multiple signalling pathways.[44] Clinically, PJS is characterized by hamartomatous polyps in the gastrointestinal tract and mucocutaneous melanin pigmentation on the lips, fingers and toes.[45] In

addition, patients with PJS have a significantly increased risk for the development of various malignancies including gastrointestinal, breast, gynecological and pancreatic cancers.[46] The cumulative lifetime risk of any cancer type in patients with PJS is 76%-85%,[46,47] and for pancreatic cancer it is 26% representing a relative risk of up to 132.[43,48]

Li Fraumeni Syndrome

Li-Fraumeni Syndrome (LFS) is an autosomal dominant, hereditary, cancer predisposition syndrome usually caused by germline mutations in the *p53* tumor suppressor gene.[49] *p53* is a transcription factor which controls many essential cellular activities including DNA replication and repair, cell cycle arrest, apoptosis and senescence when cells are exposed to stress.[50] Because the p53 protein is constitutively expressed in most cell types and tissues, mutations in the *p53* gene predispose to a variety of cancer types including: acute leukemias and adrenocortical tumors in childhood; early-onset breast cancer, soft tissue sarcomas, osteosarcomas, colorectal and lung cancers in young adults; as well as prostate and pancreatic cancers in late adulthood.[50-52] The relative risk for pancreatic cancer in patients with LFS is estimated at 7.3.[50]

Hereditary Pancreatitis

First described by Comfort *et al.* in 1952, hereditary pancreatitis (HP) is a rare, inherited form of recurrent acute or chronic pancreatitis.[53] Although the precise prevalence of HP is still unknown, it is estimated to be 0.57 per 100 000.[53] HP is characterized by repeated episodes of abdominal pain, nausea and vomiting caused by inflammation of the pancreas, usually beginning with acute attacks in childhood and progressing into chronic disease by early adulthood.[54] Over

time, these episodes damage the pancreas which can lead to fibrosis, calcifications, biliary obstruction and diabetes mellitus due to pancreatic endocrine insufficiency.[55] Over 80% of hereditary pancreatitis cases are due to gain-of-function mutations in the protease serine 1 (*PRSS1*) gene, which follows an autosomal dominant form of inheritance.[56] *PRSS1* encodes trypsinogen which is released in the pancreas. Upon consumption of food, it is released from the pancreas into the duodenum where it is converted to the active digestive enzyme trypsin.[55] Mutations in *PRSS1* lead to the premature conversion of trypsinogen to trypsin leading to autodigestion of the pancreas causing inflammation and damage.[57] Loss-of-function mutations in the serine protease inhibitor, Kazal type 1 (*SPINK1*) gene cause an autosomal recessive form of HP.[58] SPINK1 encodes a trypsin inhibitor which is expressed in pancreatic acinar cells and protects from autodigestion.[58] Other genes associated with an increased risk of HP include *CFTR, CTRC, CTSB, CASR, CLDN-2* and *CPA1*.[55] Regardless of the specific genetic mutation involved, patients with HP have up to an 87-fold increased risk of developing pancreatic cancer and cumulative life-time risk of 40-55%.[59]

Familial Atypical Multiple Mole Melanoma

Approximately 5% to 12% of melanoma occurs in kindreds with a hereditary predisposition, a condition known as familial atypical multiple mole melanoma (FAMMM).[60] FAMMM is an autosomal dominant condition characterized by a high total body nevi count, often over 50, some of which are clinically atypical, as well as a family history of melanoma.[61] Approximately 20-40% of patients with FAMMM carry germline mutations in *CDKN2A*, a gene which, interestingly, is inactivated in 95% of sporadic pancreatic cancers.[61-63] *CDKN2A* encodes two proteins; $p16^{INK4a}$ and $p14^{ARF}$. Both proteins act as tumor suppressors with p16 controlling

the G1/S checkpoint by preventing the phosphorylation of the retinoblastoma protein and p14 controlling the G1 and G2 checkpoints by stabilizing p53.[64] Therefore, mutations in *CDKN2A* lead to uncontrolled cell proliferation and neoplastic formation.[64] *CDKN2A* mutations are associated with a cumulative lifetime risk of melanoma of 60-90% as well as a 13-22 fold increased risk of pancreatic cancer representing a lifetime risk of up to 25%.[34]

Hereditary Breast & Ovarian Cancer Syndrome

Hereditary breast and ovarian cancer (HBOC) syndrome is an autosomal dominant cancer predisposition syndrome primarily associated with inherited germline mutations in the *BRCA1* and *BRCA2* genes.[65] Both *BRCA1* and *BRCA2* function as tumor suppressor genes and are key players in double strand break repair by forming complexes with Rad51 and initiating homologous recombination.[66] When mutations in these genes occur, error-prone double strand breaks are not repaired leading to the development of chromosomal rearrangements and genomic instability.[67] Furthermore, *BRCA1* is involved in transcription regulation and is required for effective S-phase and G2/M-phase cell cycle checkpoints.[66] HBOC is suspected in individuals with a personal or family history of early-onset breast cancer (<50 years old), multiple primary breast and/or ovarian cancers in the same individual, male breast cancer or multiple relatives affected by breast, ovarian, prostate or pancreatic cancers.[65] In addition, HBOC should be suspected in any individual with breast or ovarian cancer and Ashkenazi Jewish ancestry as the *BRCA1* and *BRCA2* mutation prevalence in these individuals is 1 in 40, ten times more frequent than that of the general population.[68] Individuals with HBOC are at significantly increased risk for breast and ovarian cancers, as well as cancers of the colon, prostate and pancreas.[69] Individuals with *BRCA1* mutations have a lifetime risk of 65% and 39% for breast and ovarian

cancer respectively, as well as a 2.26-fold increased risk of pancreatic cancer.[68,70,71] Compared to individuals with *BRCA1* mutations, *BRCA2* mutation carriers are at lower risk for breast and ovarian cancers with lifetime risk estimates at 45% and 11% respectively. However, they are at greater risk for pancreatic cancer with a relative risk of 3.51.[68,70]

Hereditary Breast Cancer Syndrome

Approximately 5-10% of breast cancer cases are due to hereditary causes, however only 30% of these cases are explained by mutations in *BRCA1* and *BRCA2*.[72] Recently, *PALB2* and *ATM* have also been implicated in both breast and pancreatic cancer susceptibility in heterozygote mutation carriers.[72] Like *BRCA1* and *BRCA2*, both *PALB2* and *ATM* are critical in repairing double stranded breaks (DSB) through the homologous recombination pathway.[73] ATM is a kinase which recognizes DSB sites and subsequently recruits and phosphorylates proteins involved in the DNA damage response and cell cycle pathways, thereby activating them.[74] PALB2 is involved in localizing and loading BRCA2 and RAD51 onto DSB sites.[73] Furthermore, PALB2 is thought to play a role in later steps of homologous recombination (HR), by interacting with DNA polymerase nu (POLN) and sustaining its recruitment to stalled replication forks.[73]

Homozygous mutations in *PALB2* result in Fanconi anemia, an autosomal recessive disorder characterized by developmental abnormalities, bone marrow failure and an increased risk of malignancies.[75] On the other hand, mono-allelic loss-of-function mutations in *PALB2* are associated with a 33-58% lifetime risk of breast cancer development in female patients, depending on the number of relatives affected.[76] An exome sequencing study by Jones et al. in

2009 provided the first evidence for the role of *PALB2* in pancreatic cancer susceptibility.[77] Since then, *PALB2* mutations have been identified in 3-4% of familial pancreatic cancer cases.[78]

Homozygous mutations in the *ATM* gene result in a condition known as ataxia telangiectasia (AT), a rare neurodegenerative disease characterized by progressive cerebellar ataxia, immunodeficiency and cancer predisposition.[79] Specifically, 30% of AT patients develop lymphoma and leukemia in childhood, often proving fatal.[80] Heterozygous mutation carriers have a 4.9-fold increased risk for developing breast cancer under the age of 50 and an overall relative risk of 2.23 compared to the general population.[81] The role of *ATM* mutations in pancreatic cancer was discovered through a whole-genome sequencing study of individuals with FPC.[42] Although one study estimated the relative risk of pancreatic cancer in *ATM* mutation carriers to be 2.41, it was not statistically significant due to the low prevalence of the disease.[81] Although more research is needed, ATM carriers are considered to be at elevated risk for the development of pancreatic cancer.[41]

Familial Adenomatous Polyposis

Familial Adenomatous Polyposis (FAP) is an autosomal dominant, inherited, colorectal cancer syndrome caused by germline mutations in the adenomatous polyposis coli (APC) gene.[82] The *APC* gene is a tumor suppressor involved in cell adhesion and migration, the Wnt signalling pathway and the control of the cell cycle.[83] Loss-of-function mutations in *APC* inhibit its ability to phosphorylate and degrade of B-catenin, leading to accumulation of B-catenin in the cytoplasm.[83] B-catenin then binds to the Tcf family of transcription factors which alters the expression of various genes which encode for proteins, such as the c-myc and cyclin D1, which are involved in cell proliferation, differentiation, migration and apoptosis.[83] Furthermore, APC

has a critical role in chromosomal stability by stabilizing microtubules as well as regulating the G0/G1-S phase cell cycle transition.[83] Clinically, FAP is characterized by the early development of hundreds of adenomas throughout the gastrointestinal tract, which left untreated, almost always result in the development of colorectal cancer by the age of 35-40.[83] In addition, patients with FAP are at an increased risk for extra-intestinal malignancies such as those of the thyroid gland, adrenal gland, biliary tract, as well as a 4.5-fold increased risk for the development of pancreatic cancer.[84]

Lynch Syndrome

Lynch syndrome (LS), previously referred to as hereditary non-polyposis colorectal cancer syndrome (HNPCC), was one of the first hereditary cancer predisposition syndromes to be described in 1913.[85] LS occurs due to inherited germline mutations in one of four key DNA mismatch repair (MMR) genes: *MLH1, MSH2, MSH6* or *PMS2* or by germline mutations in *EPCAM* which lead to epigenetic silencing of *MSH2*.[86] MMR proteins act in mismatch repair, a post-replicative proof-reading and editing process which ensures genome integrity.[87] LS-associated cancers occur when a somatic loss of the remaining wild-type MMR allele occurs.[88] Because MMR proteins play a key role in DNA repair, these cancers exhibit a molecular phenomenon known as microsatellite instability (MSI) which is explained further in **section 1.3.2.1**.[89] LS is relatively common with an incidence between 1:660 and 1:2000, accounting for up to 5% of incident colorectal cancer diagnoses.[86,90] Individuals with LS have up to an 80% lifetime risk of colon cancer and develop it earlier than the general population, at a mean age of 44.[91] Furthermore, women with LS have a 30-50% lifetime risk of endometrial cancer.[92] The

risk of pancreatic cancer in individuals with LS is estimated at 3.7%, an 8.6-fold increased risk compared to the general population.[93]

Table 1. Hereditary predisposition syndromes associated with PDAC

Predisposition Syndrome	Gene(s) Involved	Lifetime risk of PDAC	Relative Risk
Familial Pancreatic Cancer	Unknown	2 FDRs 8-12%[40] ≥ 3 FDRs 40%[40]	6.4[40] 32[40]
Peutz-Jeghers Syndrome	*STK11*	26%[43]	132[48]
Li Fraumeni Syndrome	*TP53*	Elevated	7.3[50]
Hereditary Pancreatitis	*PRSS1* *SPINK1*	40-55%[59]	87[59]
Familial Atypical Multiple Mole Melanoma	*CDKN2A*	25%[34]	13-22[34]
Hereditary Breast & Ovarian Cancer Syndrome	*BRCA1* *BRCA2*	*BRCA1* Elevated *BRCA2* Elevated	2.26[68,70] 3.51[68,70]
Hereditary Breast Cancer Syndrome	*PALB2* *ATM*	*PALB2* Elevated *ATM* Elevated	Elevated Elevated
Familial Adenomatous Polyposis	*APC*	Elevated	4.5[84]
Lynch Syndrome	*MLH1* *MSH2* *MSH6* *PMS2*	3.7%[93]	8.6[93]

1.2.2 Quebec Pancreas Cancer Study

PDAC has largely been underrepresented in research studies due to its rarity and the rapid progression and fatality of the disease.[94] The establishment of prospective pancreatic cancer registries provides an opportunity to overcome these challenges by obtaining high-quality epidemiological data and associated biospecimens at the time of diagnosis. In Canada, the first of such registries, the Ontario Pancreas Cancer Study, began enrolling patients in 2003 and has served as an instrumental resource for basic, translational and clinical research studies centred around achieving a greater understanding of the disease and novel ways of treating it.[95]

In 2012, a similar registry, the Quebec Pancreas Cancer Study (QPCS) was established at the McGill University Health Center (MUHC) to create a resource for research focused on genetic susceptibilities of pancreatic cancer as well as other epidemiological, biomarker and cancer biology studies.[94] The QPCS enrolls men and women over the age of 18 who have a diagnosis of pancreatic cancer or other periampullary tumors; as well as unaffected individuals with FPC or another hereditary cancer predisposition syndrome.[94] At the time of enrollment, study participants are interviewed by a genetic counsellor and a three (or more) generation family history is obtained. Participants are also asked to complete a personal history questionnaire for the acquisition of epidemiological data.[94] Written consent is obtained allowing access to their medical records for confirmation of their diagnosis and subsequent follow-up of clinical data and outcomes.[94] Finally, biopspecimens are collected including a germline sample in the form of blood or saliva, as well as tumor samples such as archived tissue samples, and when possible, surgical samples during resection or biopsy.[94]

This resource in Quebec is of particular importance as over one-third (37.4%) of patients and their relatives enrolled in the QPCS are of French-Canadian (FC) ancestry, a founder

population with recurrent germline mutations in genes associated with development of hereditary pancreatic cancer.[94] Founder populations occur when a new population is created from a small number of individuals. Breeding within the population leads to decreased genetic variation and an increased prevalence of any genetic mutations present in the original colonizers.[96] Between 1608 and 1759, approximately 8500 settlers emigrated from France and established the French-Canadian population of Quebec.[96] Following the British Conquest of 1759, French immigration ceased leading to isolation, and the subsequent growth of the population propagated mutations present in the original colony.[96] Recurrent FC founder mutations have been identified in several pancreatic cancer predisposition genes including *BRCA1, BRCA2, PALB2, MLH1, MSH2* and *MSH6*. [97-102]

I.3 DNA Mismatch Repair

I.3.1 DNA repair pathways

As the cells of our body divide, the genome is replicated, a complicated process that can give rise to errors.[103] Furthermore, our cells are constantly exposed to agents, both endogenous and environmental, which result in innumerable DNA damaging events.[103] These changes can be highly detrimental to essential cellular functions.[104] Therefore, several highly specialized DNA repair mechanisms have evolved to maintain genomic integrity.[104] Outlined below are the major single-stranded break (SSB) and double-stranded break (DSB) DNA repair pathways. Deficiencies in these DNA repair mechanisms underlie several medical disorders, including cancer susceptibility syndromes.[103]

1.3.1.1. Single-Stranded Break Repair (SSBR)

***Mismatch Repair (MMR*)**

Mismatch repair is a highly conserved DNA repair mechanism responsible for correcting single base-pair mismatches.[103] These lesions are most often caused by base misincorporation during DNA replication which have evaded the proofreading mechanism of DNA replication polymerases, namely polymerases ε and δ.[105] MMR is accomplished through the action of four major proteins; mutL homologue 1 (MLH1), mutS homologue 2 (MSH2), mutS homologue 6 (MSH6) and postmeiotic segregation increased 2 (PMS2), named based on their homology to the E. coli MMR genes.[87,105] In the canonical pathway, the mutSα complex, composed of a MSH2-MSH6 heterodimer, recognizes the mismatched base and binds resulting in an ATP-dependent conformational change. This leads to the recruitment of the mutLα complex, comprised of a MLH1-PMS2 heterodimer.[105] MutLα and a DNA clamp protein, PCNA, initiate a single-stranded cut serving as an entry point for exonuclease 1 (Exo1), which becomes activated following the assembly of the MutS-MutL complex.[103] Exo1 degrades the area surrounding the mismatch, which is then resynthesized by DNA polymerase δ.[103] Lastly, DNA ligase I seals the remaining nick. Alternative MMR complexes exist including MutSß, a MSH2-MSH3 heterodimer which recognizes larger insertion/deletion loops and MutLβ (MLH1/PMS1) and MutLγ (MLH1/MLH3) which play minor roles in MMR which have yet to be fully understood.[103] An overview of mismatch repair is shown in **Figure 2.**

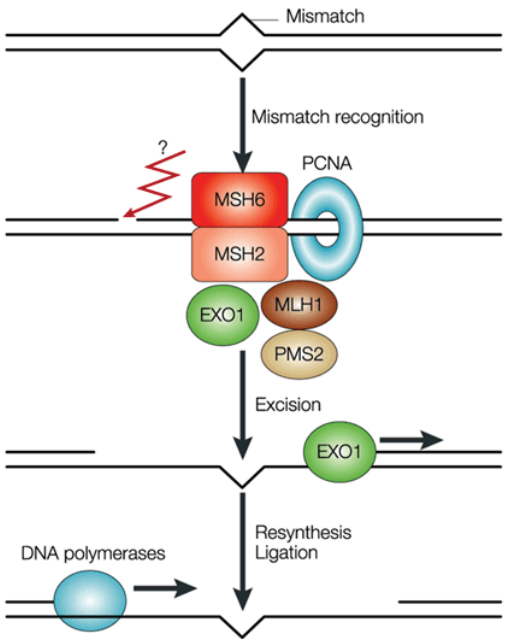


Figure 2: DNA mismatch repair system. Single-base pair mismatches are recognized by MutS, a MSH2-MSH6 heterodimer. MutL, a MLH1-PMS2 heterodimer, PCNA and Exo1 are subsequently recruited. Exo1 excises the region surrounding the mismatch allowing DNA polymerase and DNA ligase to repair the defect. Figure from Martin & Scharff (2002)[106], approval obtained 03/10/19.

Base Excision Repair

Base excision repair (BER) is another form of SSBR that corrects DNA base lesions that do not significantly alter the structure of the DNA helix such as oxidation, alkylation, deamination, and depurination/depyrimidination.[103] BER requires the function of four major proteins: a DNA glycosylase, an AP endonuclease or AP lyase, a DNA polymerase and a DNA ligase.[107] BER is initiated when the appropriate DNA glycosylase, such as uracil-DNA glycosylase (UNG) for uracil sites, recognizes the damaged base and cleaves an N-glycosidic

bond creating an apurinic or apyrimidinic site (AP site).[107] Next, AP endodeoxyribonuclease I (APEX1) cleaves the DNA backbone 5' to the AP site.[107] Finally, Polymerase ß will insert the correct nucleotide and DNA ligase III will seal the nick, thereby restoring the integrity of the DNA helix.[107]

Nucleotide Excision Repair

Nucleotide excision repair (NER) is responsible for removing bulky, helix distorting DNA lesions including pryrimidine dimers produced by exposure to UV radiation.[103] There are two NER pathways; global genome NER (GG-NER) which is active throughout the genome and transcription-couples NER (TC-NER) which repairs lesions on actively transcribed genes.[108] DNA lesions are recognized by an XPC-HR23B-CEN2 complex in GG-NER or RNAPII in the case of TC-NER.[109] Following recognition, both pathways converge with the recruitment of TFIIH to the repair site.[103,108] The TFIIH complex contains XPB and XPD which unwind the DNA creating a 30-nucleotide bubble, allowing XPA and RPA to enter, bind and stabilize the area. XPF and XPG bind and nick the 5' and 3' end of the bubble, respectively, leading to excision of the lesion-containing region.[103,108] Binding of PCNA and RFC allows DNA polymerase δ or ε to resynthesize the missing DNA, using the undamaged strand as a template.[103] Finally, DNA ligase repairs the nicks thereby completing the process.[103]

1.3.1.2 Double-Stranded Break-Repair (DSBR)

Non-homologous End-Joining (NHEJ)

Non-homologous end-joining is the simplest mechanism of DSBR, although it is non-conservative and may result in the loss of genomic material.[110] NHEJ is initiated when KU70/80

heterodimers bind to the ends of the double-strand break.[111] This protects against further degradation and triggers the recruitment of many proteins including the catalytic subunit of DNA-PK, DNA-PKcs, and the nuclease Artemis.[112] Single-stranded overhangs are either removed through the nuclease activity of the DNA-PKcs/Artemis complex or are used to resynthesize missing nucleotides through the action of the X family DNA polymerases, Pol μ and Pol λ.[103,112] Following processing of the DNA termini, ligation of the two ends is carried out by DNA ligase IV, XRCC4 and XLF. [103,112]

Homologous Recombination

Homologous recombination (HR) is the conservative, error-free form of DSBR restricted to the late-S and G2 phases of the cell cycle due to the need for a sister chromatid DNA template.[103] HR consists of three phases: presynapsis, synapsis and postsynapsis.[112] During presynapasis, the heterotrimeric MRN complex (Mre11-Rad50-Nbs1) and CtIP (RBBP8) process the DSB to produce single-stranded 3' overhangs.[103,111] Next, BRCA2, Rad51 and other paralogs coat the ssDNA tails, forming a presynaptic filament.[112] During synapsis, Rad51 searches for a homologous DNA sequence in the sister chromatid and then mediates strand invasion, wherein the damaged DNA strand invades the DNA template.[112] DNA polymerase η synthesizes the missing sequence from the 3' end of the invading strand.[112] Finally, during post-synapsis, ligation by DNA ligase I leads to the formation of a Holliday junction, a four-way intermediate structure which is subsequently resolved by BLM-mediated branch migration or through cleavage by a resolvase such as GEN1/Yen1, Slx1/Slx4 or Mus81/Eme1.[103,112]

I.3.2 Mismatch Repair Deficiency

1.3.2.1 Contribution of MMR-d to tumorigenesis

As previously discussed, the mismatch repair system is responsible for the repair of mismatched bases that occur during DNA replication and is therefore key in maintaining genomic stability.[105] Mutations in the MMR proteins, *MLH1, MSH2, MSH6* and *PMS2*, result in a functional deficiency of this process leading to the accumulation of mutations.[105] When these mutations occur in cancer-related genes, they cannot be repaired and therefore contribute to tumorigenesis.[113] Loss of expression of one or more MMR genes is associated with an increased predisposition to a number of cancer types including colorectal, endometrial, gastric ovarian, small bowel, hepatobiliary and brain.[114] MMR-deficient cancers represent a distinct subtype with unique clinical and pathological features, drug responses and patient outcomes. A brief overview of the predominant cancer types associated with MMR-d are outlined below and summarized in **Table 2.**

MMR-deficiency can be sporadic, caused by methylation, mutation or both, or hereditary, caused by heterozygous germline mutations in MMR genes which give rise to a condition known as Lynch Syndrome (LS), as previously described.[113] Biallelic germline mutations in the MMR genes result in an exceedingly rare condition called constitutional mismatch repair deficiency (CMMRD), a childhood cancer predisposition syndrome associated with the development of hematological, brain and intestinal tract malignancies, often during the first decade of life.[115,116] Other non-neoplastic features include “café au lait” spots, mild defects in immunoglobulin class switch recombination and congenital brain malformations.[115]

Colorectal Cancer

Classically, MMR-d is associated with predisposition to colorectal cancer (CRC).[105] Although it is now known that MMR-d contributes to a much broader tumor spectrum, the vast majority of studies of MMR-d have focused on colorectal tumors.[105] Approximately 20% of sporadic colonic adenocarcinomas and 5% of rectal carcinomas display MMR-d and associated MSI.[117] Hypermethylation of *MLH1* is the most common mechanism in the pathogenesis of MMR-d CRC and is associated with concomitant *BRAF* mutations.[118] A further 2-4% of cases are caused by patients with Lynch Syndrome.[88] Individuals with LS have a 52%-82% lifetime risk of developing colorectal cancer, with a lower mean age of onset than sporadic cases, at 44-61 years.[119] MMR-d colorectal tumors display distinct clinicopathologic features including a predilection for the proximal colon, poor differentiation, increased number of tumor-infiltrating lymphocytes and mucinous histology.[120,121] MSI is both a prognostic and predictive factor in patients diagnosed with CRC. Several studies have shown that patients with MSI-H tumors display lower recurrence rates and improved progression-free and overall survival rates compared to those with MSI-L or MSS tumors.[122-126] Furthermore, MSI negatively predicts response to adjuvant therapy with pyrimidine analogs such as gemcitabine and 5-fluorouracil.[122,123,126] In contrast, response to regimens including oxaliplatin (FOLFOX) is enhanced.[105]

Endometrial Cancer

Endometrial cancers represent the most common extra-colonic malignancy associated with defects in MMR.[88] Approximately 30% of endometrial carcinomas (EC) display MSI, of which 2-5% are caused by LS.[117,127] The cumulative lifetime risk for the development of

endometrial cancer in females with LS may even exceed the risk of CRC, with rates of up to 60%.[113,114,119] LS-associated endometrial cancers are characterized by young age on onset, lower uterine segment involvement and synchronous ovarian cancer.[88,128] As in CRC, MMR status is an independent prognostic factor of survival, with MMR-d patients demonstrating significantly improved overall survival rates compared to those with intact repair.[129,130] Only a handful of studies have assessed the predictive value of MMR-d in endometrial cancers with varying results.[105] More research is required to determine the effect of MMR status on treatment response.

Ovarian Cancer

Approximately 10% of incident ovarian cancers are caused by MMR-deficiency.[131] However, LS-associated ovarian cancer is rare. A recent study by *Latham et al.* did not identify any cases of LS in 343 ovarian tumors, 43 of which were MSI-H.[132] The prevalence of LS in ovarian cancer is estimated at approximately 1%.[133] Consistent with the association of mucinous histology in MMR-d CRC, non-serous ovarian cancers are overrepresented in MMR-d ovarian cancers.[134] The prognostic and predictive value of MMR-d in ovarian cancer remains unclear as very few studies have been published, and the results have not been consistent.[105]

Gastric Cancer

MSI-H tumors account for 10-20% of gastric cancers and are associated with the intestinal subtype, tendency to involve the distal antrum, early-stage disease and reduced lymph node involvement.[105,135] Patients with Lynch Syndrome are at a 6%-13% lifetime risk for the development of gastric cancer and represent an estimated 1% of cases.[119,132] Several studies have

demonstrated improved survival for patients with MMR-d gastric tumors compared to patients with proficiency in MMR, however MMR status does not seem to predict response to treatment.[135-138]

Urinary Tract Carcinomas

Approximately 5% of upper urothelial and bladder cancers are associated with MMR-deficiency.[205] Moreover, a large proportion (37.5%) of MMR-d urinary tract carcinomas are caused by LS-associated germline mutations.[132] MMR-d urothelial carcinomas present with unique histological features including low grade nuclear atypia, an inverted papilloma-like growth pattern and presence of villous-to-papillary structures.[205]

Pancreatic Cancer

The prevalence of MMR-d in pancreatic adenocarcinoma has been highly debated with some studies estimating high frequencies of up to 22%[139-143] and others as low as 0.3%.[132,144-149] An overview of the methods and results obtained from these studies is presented in **Table 4**. As opposed to other cancer types, it is well documented that the majority of MMR-d pancreatic cancers are associated with germline mutations.[139,146,150-152] For example, in a recent study by Hu *et al.,* 833 pancreatic adenocarcinomas were evaluated for mutations in 468 cancer-associated genes. MMR-d was found in 7 patients, all of which were found to have LS.[149] Patients with LS have a 4%-6% lifetime risk of developing pancreatic cancer.[88,93,153] MMR-d pancreatic cancers tend to be associated with improved survival and lack of response to pyrimide analogs, including gemcitabine and 5-FU, as seen in CRC.[139,140,142,154] Notably, the mutational load, number of neoantigens and presence of tumor-infiltrating lymphocytes is significantly higher in pancreatic

cancers with associated MMR-d providing a basis for treatment with immunotherapy in this subtype.[140,146,148]

Table 2. Cancer types associated with MMR deficiency

Cancer Type	% cases MMR-d/MSI-H	% cases with germline MMR mutations (LS)
Colorectal Cancer	20-25%[117]	2-4%[88]
Endometrial Cancer	30%[117]	2-5%[127]
Ovarian Cancer	10%[131]	~1%[133]
Gastric Cancer	10-20%[105]	~1%[132]
Urinary Tract Cancers	5%[132]	2%[132]
Pancreatic Cancer	0.3-22%[139-149]	0.6%[132]

1.3.2.2 Genetic Testing Guidelines

The first screening guidelines for LS, the Amsterdam I Criteria, were put forth in 1991 following a meeting of the International Collaborative Group on Hereditary Non-Polyposis Colon Cancer (ICG-HNPCC).[105] Often referred to as the "3-2-1 rule", the criteria required: a) at least three relatives with histologically confirmed colorectal cancer, one being a first-degree relative of the other two; b) two successive generations affected; and c) at least one relative diagnosed under the age of 50.[155] In 1998, these strict criteria were altered slightly to account for the vast tumor spectrum of Lynch Syndrome.[105] The Amsterdam II criteria stipulated that at least three relatives should have any histologically confirmed HNPCC-associated cancer (colorectal,

endometrial, small bowel, stomach, ovarian, pancreas, ureter, renal pelvis, biliary tract, brain), with the remaining two criteria unchanged.[155] Ultimately, the Amsterdam Criteria were found to have poor sensitivity and specificity in the identification of individuals with LS.[156]

Around the same time, the National Cancer Institute (NCI), released their own criteria, called the Bethesda Guidelines.[105] Patients who met one or more of the following criteria were considered likely to have Lynch syndrome: a) individuals who meet the Amsterdam criteria; b) individuals with two synchronous or metachronous HNPCC-related cancers; c) individuals with colorectal cancer and a first-degree relative with a HNPCC-related cancer, with one of the cancers diagnosed by age 45; d) individuals with colorectal cancer or endometrial cancer diagnosed by age 45; e) individuals with right-sided colorectal cancer with an undifferentiated pattern (solid/cribriform) on histopathology diagnosed by age 45; f) individuals with signet-ring-cell-type colorectal cancer diagnosed by age 45; and g) individuals with adenomas diagnosed by age 40.[157]

Finally, the most recent set of guidelines, the Revised Bethesda Guidelines, were published in 2004 and are the clinical standard used today **(Table 3)**.[105] Suspicion for LS is raised when patients meet one or more of the following criteria: a) individuals with CRC diagnosed by age 50; b) individuals with synchronous or metachronous CRC, or other HNPCC-associated tumors regardless of age; c) individuals with CRC and MSI-H histology diagnosed by age 60; d) individuals with CRC and more than 1 first degree relative with an HNPCC-associated tumor, with one cancer diagnosed by age 50; and e) individuals with CRC and more than 2 first degree relatives or second degree relatives with an HNPCC-associated tumor, regardless of age.[158]

Table 3. The Revised Bethesda Guidelines for MSI testing

Revised Bethesda Guidelines
Individuals should be tested for MSI if at least one of the following is met:[157] 1) Colorectal cancer diagnosed <50 years of age 2) Presence of synchronous or metachronous CRC, or other HNPCC-associated tumors[a], regardless of age 3) Colorectal with the MSI-H histology[b], diagnosed <60 years of age 4) CRC or other HNPCC-associated tumor[a] diagnosed in ≥1 first-degree relative <50 years of age 5) CRC or other HNPCC-associated tumor[a] diagnosed in ≥2 first-degree relatives, regardless of age

[a]Hereditary nonpolyposis colorectal cancer (HNPCC)-related tumors include colorectal, endometrial, stomach, ovarian, pancreas, ureter and renal pelvis, biliary tract, and brain (usually glioblastoma as seen in Turcot syndrome) tumors, sebaceous gland adenomas and keratoacanthomas in Muir–Torre syndrome, and carcinoma of the small bowel.[157]

[b]Presence of tumor infiltrating lymphocytes, Crohn's-like lymphocytic reaction, mucinous/signet-ring differentiation, or medullary growth pattern.[157]

Recently, the use of clinical prediction models, such as MMRpro and PREMM, have gained popularity. These technologies are generally used to identify patients with a >5% likelihood of LS based on family history for subsequent genetic testing.[156] While these criteria are used for identifying patients who are likely to carry germline MMR gene mutations, the NCCN has recommended universal MMR or MSI testing for all patients with CRC or EC.[159] Reflex testing for MMR-d is important for two reasons. Firstly, it allows the identification of patients with somatic inactivation of MMR genes who could benefit from targeted therapies. Secondly, although more sensitive than the Amsterdam criteria, it has been shown that up to 50% of patients with LS do not even meet the revised Bethesda guidelines.[119] Likewise, the NCCN

has recommended that clinicians consider testing pancreatic tumors for MMR-d, as several studies have shown that up to 62.5% of patients with MMR-d pancreatic cancer do not meet genetic testing guidelines.[148,160]

1.3.2.3 Diagnostic Methods for MMR-d

Microsatellite Instability Testing

The first major assay for the diagnosis of MMR-d is microsatellite instability testing (MSI). Microsatellites are short, tandem repeats of 1 to 6 nucleotides scattered throughout the genome.[161] Due to their repetitive nature, these areas are more susceptible to insertions and deletions caused by slippage during replication. The failure of the DNA mismatch repair system to correct these "loop outs" of unpaired bases results in alterations in the length of these microsatellites which is a hallmark of MMR-d.[161] In the original Bethesda guidelines, released in 1997, a panel of five microsatellite markers, two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D5S346, D2S123 and D17S250) was proposed for the assessment of MSI.[162] Tumors with alterations in 2 or more markers were classified as MSI-H (high), those with instability at one marker as MSI-L (low) and those without any changes as MSS (stable).[162] Further research revealed that mononucleotide repeats had higher sensitivity and specificity in identifying MMR-d, compared to dinucleotide markers.[158,163] Therefore, in 2002, the NCI suggested testing a secondary panel of nucleotide markers, such as BAT-40 in samples that displayed instability at dinucleotide markers only.[162] Subsequently, the MSI Analysis System (Promega Corp.) was developed; a multiplex fluorescence assay which analyzes five quasi-monomorphic mononucleotide markers (BAT-25, BAT-26, NR-21, NR-24 and MONO-27) for MSI determination and two polymorphic pentanucleotide markers (Penta C and Penta D) for

sample identification.[163] MSI testing is highly reproducible however, optimal PCR product quality is essential in obtaining interpretable results.[161] Furthermore, due to the partial functional redundancy of MSH6 and MSH3, patients with mutations in MSH6 may still be microsatellite stable.[161]

Immunohistochemistry (IHC)

Immunohistochemistry staining for the four major MMR proteins: MLH1, MSH2, MSH6 and PMS2 was made possible by the development of monoclonal antibodies against the MMR proteins in the late 1990s.[164] Tumors with MMR-d demonstrate absence of nuclear staining for one or more of these proteins. Individuals with mutations in *MLH1* tend to lose expression of both MLH1 and PMS2, as PMS2 is degraded in the absence of its heterodimer binding partner, MLH1.[164] On the other hand, individuals with mutations in *PMS2* demonstrate absent staining for PMS2 only.[164] Likewise, lack of staining in both MSH2 and MSH6 indicates a mutation in *MSH2* as it is responsible for the stability of MSH6, while absent staining for MSH6 alone is suggestive of a mutation in *MSH6*.[164] IHC is a quick and simple assay, however there are caveats. In terms of biological considerations, IHC is reliable for mutations that result in protein degradation or truncation. However, missense mutations which alter the protein function without resulting in protein degradation will not be detected by IHC.[164] While the majority of mutations in *MSH2* are protein truncating, up to one-third of *MLH1* mutations are missense mutations which have the potential to result in a false-normal staining pattern.[164] There are also technical considerations when using IHC for MMR-d testing due to variability in staining quality.[164] These are often due to differences in tissue fixation, staining procedures, tissue source as well as the

specific antibodies used for staining.[160] Therefore, care must be taken in developing standardized and optimized protocols for using IHC as a diagnostic tool for MMR-d.

Sequencing Technologies

Recently, the use of next-generation sequencing technologies for the diagnosis of MMR-d has started gaining attention.[105] Cancer-associated gene panels such as MSK-IMPACT, a hybridization capture-based NGS assay, and whole-genome sequencing (WGS) of tumor samples can be used to identify oncogenic drivers, including MMR gene mutations.[165] Furthermore, because these technologies sequence a significant portion of the tumor's genome, computer algorithms such as MSIsensor can be used to assess the length of all microsatellite loci sequenced and classify them as MSI-H, MSI-L and MSS.[166] Tumors are generally considered MSI-H when >10% of the sequenced microsatellites are unstable.[132,149] Mutational burden can also be used as a surrogate marker of MMR-d. Not only is the number of single-nucleotide variants (SNVs) across the genome elevated in cases of MMR-d, but the specific types of transversions and their abundance can be used to identify MMR-d tumors based on mutational signatures.[146] A landmark study by Alexandrov *et al.* identified mutational signatures found across human cancers and their associated etiologies.[167] Signatures 6, 15, 20 and 26 are characteristic of defective MMR across cancer types and are associated with high numbers of small (< 3bp) insertions and deletions, particularly at mono- and poly-nucleotide repeats.[167] Although these technologies avoid the technical limitations seen with IHC or MSI testing, they are more costly, less widely available and are do not necessarily predict the clinical phenotype associated with MMR deficiency, including response to immunotherapy.[160]

I.3.3. Immunotherapy for MMR Deficient PDAC

Cancer immunotherapy represents not only a promising advancement in cancer therapy, but one of the most significant breakthroughs in science in the past decade.[168] Of note, tremendous success has been made in using monoclonal antibodies to target negative regulators of the immune system, such as PD-1 and CTLA-4, a strategy known as immune checkpoint blockade.[169] By preventing the action of these inhibitory molecules, cytotoxic T cells are stimulated to attack tumor cells thereby resulting in disease regression.[169] In fact, the 2018 Nobel Prize in Physiology or Medicine was awarded to James P. Allison and Tasuku Honjo for their discovery of this treatment approach.[170]

Although immune checkpoint blockade was a novel discovery with tremendous potential, it was not immediately embraced by the scientific community due to its variable clinical efficacy.[171] It is now known that this variability can be partly explained by the mutational burden of the tumor type being treated.[172] For example, malignancies with a high number of somatic mutations, such as melanoma, achieve high response rates to anti-PD1 blockade of up to 40%.[172] In contrast, pancreatic cancers, which are considered "immune-cold" due to their exceptionally low mutational burden, seldom respond to immune-based therapy.[173]

Interestingly, colorectal cancers also display low mutational burdens and anti-PD-1 response rates.[172] However, MMR-d colorectal cancers achieve response rates similar to that of melanoma due to the increased mutational load associated with defective DNA repair.[172] The accumulation of mutations in MMR-d tumors results in the production of an increased number of neoantigens thereby increasing the immunogenicity of the tumor and providing a basis for treatment with immunotherapy.[174] Likewise, non-colorectal cancers associated with MMR-d also exhibit high mutational burdens and response rates, suggesting immunotherapy could be a highly

effective therapy for this subtype.[172] In fact, in May 2017, the FDA granted accelerated approval for the PD-1 inhibitor, pembrolizumab, in advanced solid tumors displaying MMR-d, regardless of cancer type.[175] A study by Le *et al*. evaluated response to PD-1 blockade in 86 patients with MMR-d, across 12 tumors types.[176] Overall, 54% of patients achieved partial or complete responses.[176] Of note, all 6 patients with MMR-d pancreatic cancers responded to treatment, with two achieving complete responses, supporting the use of anti-PD-1 treatment in MMR-d pancreatic cancer.[176]

I.4 Tissue Microarrays

I.4.1 Utility of tissue microarrays

The use of tissue microarrays (TMA) is a modern revolution in pathology research first developed by Kononen *et al*. in 1998.[177] A microarray contains hundreds of representative tissue samples assembled on a single histological slide.[178] TMAs are constructed by extracting cyclindrical tissue cores, ranging from 0.6mm to 2mm in diameter, from multiple donor formalin-fixed paraffin-embedded (FFPE) blocks and re-embedding these into a single recipient block at a specified location.[179] This approach allows the high throughput analysis of up to 1000 or more samples for a factor of interest, at a substantially faster pace, and with lower costs compared to studies on whole-sections.[178] Furthermore, since all the samples can be analyzed by processing a single slide, there are significant savings in the amount of reagents needed as well as the amount of work and time required to prepare and analyze the slides.[180] There are several other advantages associated with the use of TMAs in cancer research. First, only a small amount of tissue is required allowing maximal preservation of limited or precious samples.[178] This is particularly important when using patient-derived samples for example, tumor biopsies.

Furthermore, the original block used for diagnosis remains intact and therefore can be returned to if needed.[178] Additionally, because the analysis is carried out on a single slide, conditions are standardized across samples, an attractive feature for a wide range of research projects, particularly those involving immunohistochemistry.[178] The use of TMAs prevents any variability in staining results caused by differences in antigen retrieval, temperature, incubation times or washing procedures.[178,179] Recently, automated systems have been developed for the construction of TMAs. These systems are faster, more accurate and produce TMAs with higher sample density and quality than manual approaches.[179] Despite the innumerable advantages, the use of TMAs has been at times criticized as the small tissue sample may not adequately represent the entire patient tumor, particularly in heterogeneous samples.[178,179] However, it is thought that the use of multiple tissue cores, along with the use suitable controls ensures accurate representation of the original patient tumor.[179,181,182]

I.4.2 Considerations in the construction of microarrays for pancreatic cancer

The construction of microarrays involves a few basic steps.[178] Hematoxylin and eosin (H&E) slides from each block are collected and examined by an experienced pathologist to identify regions of interest.[179] Tissue cores from these regions are then removed and arrayed on the donor block.[178] Following heating of the block to prevent core loss, the TMA is sectioned for subsequent molecular and immunohistochemical analysis.[178] However, there are several important considerations in the construction of TMAs, particularly in studies using pancreatic tumor specimens. Although 0.6mm cores are most commonly used in the construction of TMAs, pancreatic tumors display low cellularity compared to other cancer types due to its abundant and desmoplastic stroma.[183] Therefore, larger core sizes of 1.5-2 mm are preferable.[184] Similarly,

there is variability in the number of tumor cores considered to be representative in oncological studies employing the use of TMAs.[184] Although not explicitly researched in pancreatic cancer, studies of breast, prostate and bladder tumor microarrays have generally established that 1 to 4 0.6mm cores yield results comparable to standard tissue sections.[181,182,185] However, due to the potential for core loss, it is better to err towards the higher end of this estimate.[186] In the construction of TMAs for any cancer type, the inclusion of appropriate control tissue is crucial.[184] Generally, a variety of tissue samples from tumors other than the one being studies are included as a control for staining as well as for orientation purposes.[184] Furthermore, it is highly advised to include matched normal tissue for each tumor specimen as an intra-patient control.[184] Finally, in order to ease sectioning, the spacing between samples should be at least 0.1mm and a margin of paraffin should be left around the samples.[184] Following these guidelines, the use of TMAs in studies of pancreatic cancer, including those assessing the prevalence of MMR-d, has become commonplace.[141-143,146-148]

I.5 Rationale

PDAC is a highly lethal disease with limited treatment options. In the era of genomic-based medicine, interest has grown in subtyping PDAC based on underlying genetic mechanisms, which may be useful in individualizing treatment and improving patient outcomes. MMR-d PDAC is one of such subtypes, which has been shown to exhibit an exceptionally high mutational burden, as well as increased numbers of neo-antigens, suggesting that these patients may benefit from immunotherapy. Given the recent approval of the PD-1 inhibitor, pembrolizumab, for tumors displaying MMR-d and/or MSI, characterizing the prevalence of MMR-d/MSI in PDAC is essential to decide if clinical reflex testing for MMR-d in all incident PDAC cases should performed and to guide development of clinical trials for immunotherapy in MMR-d PDAC.

I.6 Hypothesis

Considering the published data and ad-hoc testing at the MUHC, I hypothesize that the incidence of MMR-d in PDAC is closer to 1%, as previous sequencing studies have concluded.

I.7 Specific Aim

To construct tumor microarrays and perform clinical-grade IHC using the QPCS resource in order to determine the prevalence of MMR-d in PDAC at our center.

Table 4. Overview of studies of MMR-d prevalence in PDAC.

		MSI					IHC				Sequencing		
Reference	**# patients**	**Type**	**# total markers**	**# mono nucleotide**	**MSI-H**	**Prevalence**	**Type**	**Prevalence on TMA**	**Confirmation**	**Prevalence after confirmation**	**Type**	**Prevalence**	**Concluded Prevalence**
Goggins *et al.*, 1998[191]	82	MSI PCR	6	1	≥ 2 (40%)	3.7% (3/82)	---	---	---	---	---	---	**3.7%**
Ghimenti *et al.*, 1999[144]	21	MSI PCR	10	0	≥ 4 (40%)	0% (0/21)	---	---	---	---	MLH1, MSH2	0% (0/21)	**0.0%**
Yamamoto et al., 2001[139]	100	MSI PCR	5	2	≥ 2 (40%)	13% (13/100)	---	---	---	---	MLH1, MSH2, MSH3, MSH6	not stated	**13.0%**
Nakata *et al.*, 2002[140]	46	MSI PCR	8	0	≥ 3 (37.5%)	17.4% (8/46)	---	---	---	---	---	---	**17.4%**
Maple *et al.*, 2005[150]	35	MSI PCR	10	4	≥ 3 (30%)	5.9% (2/34)	Whole section	---	---	8.6% (3/35)	---	---	**8.6%**
Ottenhof *et al.*, 2012[141]	78	---	---	---	---	---	TMA 3 x 0.6mm	12.8% (9/78)	No	---	---	---	**12.8%**
Laghi *et al.*, 2012[145]	338	MSI PCR	5	5	not stated	0.3% (1/338)	Whole section	---	---	0.6% (1/182)	---	---	**0.3%**
Riazy *et al.*, 2015[142]	265	---	---	---	---	---	TMA 2 x 0.6 mm	not stated	Yes	15% (41/265)	---	---	**15.5%**
Eatrides *et al.*, 2016[143]	109	---	---	---	---	---	TMA 1 x 1.0mm	22% (24/109)	No	---	---	---	**22.0%**
Connor et al., 2017[146]	597	---	---	---	---	---	TMA 2-4 x 1.5mm	1.7% (10/597)	No	---	---	---	**1.7%**
Humphris *et al.*, 2017[147]	385	MSI sensor	---	---	> 3.5%	0.8% (3/385)	TMA 3 x 1.0mm	not stated	Yes	1% (4/385)	WGS/WES	0.8% (3/385)	**1.0%**
Lupinacci *et al.*, 2018[148]	428	MSI PCR	6	6	not stated	0.9% (4/428)	TMA	13% (56/428)	Yes	1.4% (6/428)	---	---	**1.6%**
Hu *et al.*, 2018[149]	833	MSI-PCR MSI sensor	5	5	≥ 2 (40%) > 10%	0.5% (4/833)	Whole section	---	---	0.5% (4/833)	MSK-IMPACT	0.6% (5/833)	**0.8%**
Latham *et al.*, 2018[132]	824	MSI sensor	---	---	> 10%	0.7% (6 of 824)	Whole section	---	---	0.6% (5 of 824)	MSK-IMPACT	0.6% (5 of 824)	**0.7%**

Chapter II: Methods

Patient Selection:

The case series consisted of all patients consecutively enrolled in the Quebec Pancreas Cancer Study between April 1st 2012 and June 30th 2018 who met the following eligibility criteria: proband, confirmed pathological diagnosis of PDAC and availability of adequate tumor and normal tissue for analysis. Patients who were enrolled in the QPCS more than 1 year after diagnosis were excluded to prevent survival bias. Patients who were self-referred or referred from medical genetics were also excluded to control for selection biases. Based on these criteria, 97 patients were included in the study including 79 resection specimens and 18 biopsy samples. Pathology reports were carefully reviewed for each patient to confirm the diagnosis of PDAC and identify relevant tissue samples for assessment. Ethics approval for the study was provided through institutional ethics approval of the QPCS.

Assessment of tissue samples:

All available hematoxylin and eosin (H&E) pathology slides with corresponding formalin-fixed paraffin-embedded (FFPE) tissue samples were collected and microscopically examined to identify block(s) with appropriate tumor and normal tissue and high cellularity. Areas of interest were outlined using a fine-line marker. The chosen areas were reviewed by a clinical pathologist. Following confirmation, the regions were then marked on FFPE blocks using a fine-line marker.

TMA construction:

TMAs were constructed using a TMA Grand Master (3DHISTECH Ltd.) automated system. Triplicate TMAs were created using 1.5 mm diameter cores from selected tissue regions. Each TMA copy contained one tumor core arrayed next to one matched normal sample. Therefore, 3

tumor and 3 normal cores were used for each patient. Each TMA block contained control tissues from the pancreas, liver, stomach, duodenum and spleen. In addition, each TMA block contained matched tumor and normal tissue cores from a patient with clinically confirmed MMR-deficiency as well as cores from a patient who tested negative clinically (MMR-proficient). Spleen cores were placed at the bottom left of each block for block identification and orientation purposes. The layout of the TMA block is shown in **Figure 3.**

IHC staining:

TMA blocks were sectioned at 4uM for IHC analysis. IHC staining for MLH1, MSH2, MSH6 and PMS2 was performed clinically by the MUHC's pathology department (CLIA-certified) using a BenchMark ULTRA IHC Staining Module (Roche Diagnostics). Standard protocol using OptiView DAB IHC Detection Kit and the following primary antibodies were used: mouse monoclonal antibody G186-15 against MLH1 (Biocare Medical), rabbit monoclonal antibody EPR3947 against PMS2 (Cell Marque), mouse monoclonal antibody G219-1129 against MSH2 (Cell Marque), knockout tested rabbit recombinant monoclonal antibody EPR3945 against MSH6 (Abcam).

IHC analysis:

Stained TMA slides were imaged at 40X using an Aperio Scanscope XT system (Leica) and the results analyzed using ImageScope software. Mismatch repair protein expression was considered intact (MMR proficient) if any area of at least one TMA tumor core displayed positive nuclear staining of tumor cells. Cases that displayed the absence of staining for one or more mismatch repair proteins were selected for examination of IHC staining on whole 4uM sections. Cases

were considered MMR deficient if one or more proteins displayed complete loss of nuclear staining in tumor cells with positive staining in adjacent stroma and/or matched normal cores.

MSI testing:

Samples from patients confirmed to be MMR deficient on whole slides were tested for microsatellite instability. Matched tumor and normal tissue was removed using an 18-gauge needle from the identified regions on the FFPE blocks. DNA was extracted using the gSYNC DNA extraction kit (GeneAid) according to suggested protocol and quantified using a ND-1000 spectrophotometer (Thermo Scienfic). Extracted DNA was assessed using the MSI Analysis System V1.2 (Promega Corp.) according to the manufacturer's directions. Following amplification, fragment analysis was performed at the Quebec Genome Innovation Centre using an ABI-3700xL Genetic Analyzer (Applied Biosystems). Results were analyzed using Geneious software and its microsatellite plugin. Patients were considered MSI-H if ≥ 2 mononucleotide markers were altered in the tumor sample compared to the normal control sample, MSI-L if one mononucleotide marker was altered and MSS if none of the loci displayed instability.

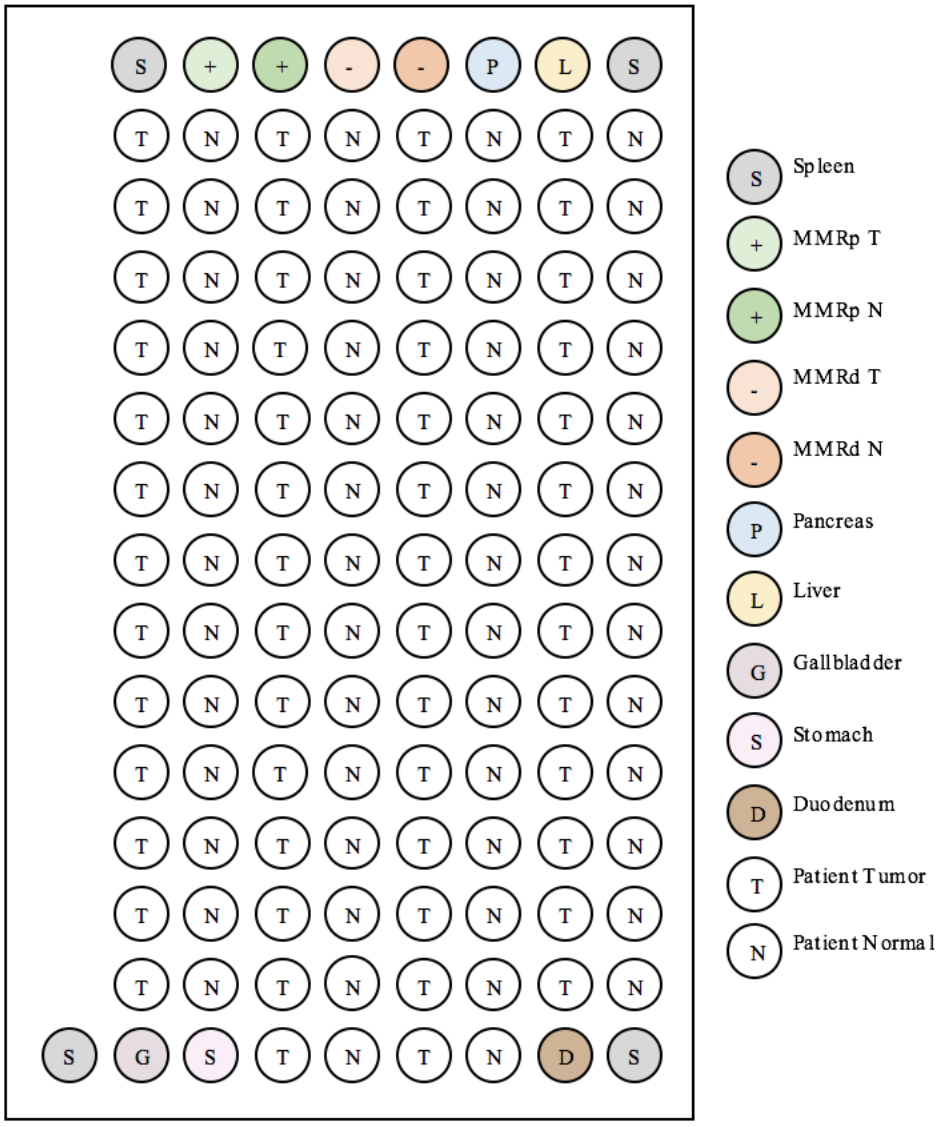
Sequencing:

Both patient 750 and his mother underwent clinical genetic testing through consultation with medical genetics. His mother was seen at the Centre Hospitalier de l'Université de Montréal (CHUM) following the IHC results of her colorectal cancer. Lymphocyte DNA was isolated and sequencing was performed for the *MSH2* gene. Subsequently, patient 750 underwent targeted genetic testing at the Jewish General Hospital to determine if his colorectal cancers were associated with the inheritance of his mother's pathogenic *MSH2* mutation.

Clinical Data Collection:

Family history and epidemiological data were collected from the Quebec Pancreas Cancer Study database. This information is collected by a genetic counsellor during the QPCS enrollment process. Clinical data parameters including sex, age of diagnosis, tumor pathology and survival data was obtained from the hospital's clinical database and chart review. Tumor staging was based on the 7th edition of the AJCC cancer staging manual.

Figure 3. Tumor microarray design and layout. TMAs were constructed using 1.5mm cores arrayed in 9 columns and 15 rows. Matched tumor and normal samples were arrayed side-by-side for each patient. Control tissues included spleen, pancreas, liver, gallbladder, stomach and duodenal samples. Tumor and normal tissue from a patient with confirmed MMR-d (orange) and from a patient with MMR-p (green) were also included as controls.



Chapter III: Results

TMA construction:

Triplicate tumor microarrays were constructed and stained for 97 patients. A total of 7 biopsy specimens could not be analyzed due to core loss during TMA construction and sectioning. Of the remaining cases, all three tumor cores were able to be assessed for each protein in 78 patients, two of the three cores in 10 patients and a single core in 2 patients. Therefore, a total of 90 patients were included in the final patient cohort.

Patient Characteristics:

The average age of diagnosis for the 90 patients included in the study was 65.5 years and the average overall survival was 28.1 months. A little more than half (58.9%) of patients were male. The patient cohort was enriched in stage II tumors, representing 73.3% of cases. Approximately two-thirds (67.8%) of patients had tumors located in the head of the pancreas. Consistent with these observations, the majority of specimens were obtained from a pancreaticoduodenectomy (Whipple's) procedure. Surgical pathology revealed positive margins in 28.9% of patients and lymph node involvement in 65.6% of patients. The majority of specimens displayed lymphovascular and perineural invasion (62.2% and 77.8%, respectively). Tumors cells were most often moderately differentiated (71.1%). Approximately one-third (34.4%) of patient samples exhibited PanIN3 precursor lesions while another 6.7% displayed IPMN. Chronic pancreatitis was present in nearly half of resected specimens. Patient characteristics are presented in more detail in **Table 5**.

IHC staining of TMAs:

Upon examination of the TMA results, 4 patients were flagged for absent staining of one or more MMR proteins, representing 4.4% of cases (95% CI 0.2%-8.7%). Patients 198 and 750 showed lack of staining in MSH2 and MSH6, patient 177 demonstrated isolated loss of MSH2 staining and patient 31 showed lack of staining for all 4 proteins (MLH1, MSH2, MSH6 and PMS2). All three tumor cores were able to be assessed in these four patients. Representative images of cores from each patient are shown in **Figure 5**.

Whole section IHC staining:

The results for these 4 patients were confirmed using IHC staining on 4uM whole tissue sections from new, separate FFPE blocks. Upon examination of the stained whole sections, only patient 750 demonstrated MMR-deficiency with a complete absence of staining for MSH2 and MSH6 in the malignant ductal cells, representing a prevalence of 1.1% (95% CI 0-3.3%). Whole slide sections for the other three patients had positive signals for all four MMR proteins tested. Of note however, a heterogeneous staining pattern was observed in all three patients with the majority of malignant ductal cells demonstrating intact nuclear staining and smaller regions displaying complete absence of staining. Whole slide staining for patient 31 revealed absent staining in certain regions for both MLH1 and MSH2 with intact, albeit weak staining for PMS2 and MSH6. On the other hand, staining patterns for both patient 177 and 198 revealed areas with complete absence of staining for MSH2 with extremely weak, focal staining for MSH6. In both patients, strong nuclear staining was observed for both MLH1 and PMS2. Representative images are presented in **Figure 6**.

Assessment of flagged patient pedigrees:

Patient 31 reported that her mother was diagnosed with ovarian cancer at age 71 and her aunt with breast cancer at age 65. Her daughter developed a benign pituitary prolactinoma at the age of 34. The patient reported no past personal history of cancer although did develop thyroid cancer two years after her diagnosis of pancreatic adenocarcinoma. Patient 177 reported several aunts with a history of cancer however, most of these cases were of unknown origin and were not confirmed. His mother did have two breast cancer diagnoses, one at age 34 and another at age 64. The patient reported no other personal or family history of cancer. Patient 198 had a limited family history however, she did not report any malignancies. The patient developed a squamous cell carcinoma of the skin two years following her diagnosis of pancreatic adenocarcinoma. In all three cases, histories obtained are not suspicious for Lynch Syndrome. On the other hand, patient 750 had a significant personal and family history of colorectal cancer, the hallmark of Lynch Syndrome. The patient was diagnosed with colorectal adenocarcinoma at age 33 with a recurrence at age 43. His mother had three diagnoses of colorectal cancer as well as a rectal cancer which required a colostomy. Of the patient's three siblings, one developed colorectal cancer in his 50s and another rectal cancer in his 40s. Based on this history, the patient meets the Amsterdam I, Amsterdam II and Revised Bethesda Guidelines for the diagnosis of Lynch Syndrome. Pedigrees for each patient are shown in **Figure 6**.

MSI analysis

Fragment analysis confirmed that patient 750 was MSI-H with instability at 4 of the 5 microsatellite loci. Deletions in BAT-25, NR-21, NR-24 and MONO-27 resulted in additional

peaks confirming deficiency of mismatch repair. A chromatogram of the capillary electrophoresis result is shown in **Figure 7**.

Chart Review of Patient 750:

Chart review revealed that following resection of the pancreatic mass, immunohistochemistry showed lack of staining for MSH2 and MSH6, confirming MMR-deficiency. Immunohistochemistry staining was performed on both his pancreatic and colorectal tumors in order to determine if the pancreatic mass was a colorectal metastasis or a discrete entity. On H&E, the PDAC tumor did show an intestinal appearance however, there were clear PanIN-3 precursor lesions. Furthermore, the colorectal cancer tumor cells were positive for CK7 in 10% of cells, CK19 in 100%, CK20 in 30% and CDX2 in 100%. On the other hand, the pancreatic cancer showed positive staining in 100% of tumor cells for CK7, 100% for CK19, 100% for CK20 and 100% for CDX2. Subsequent DNA sequencing for patient 750 and his mother revealed they were both carriers of a pathogenic *MSH2* mutation (MSH2 c.942+3A>T).

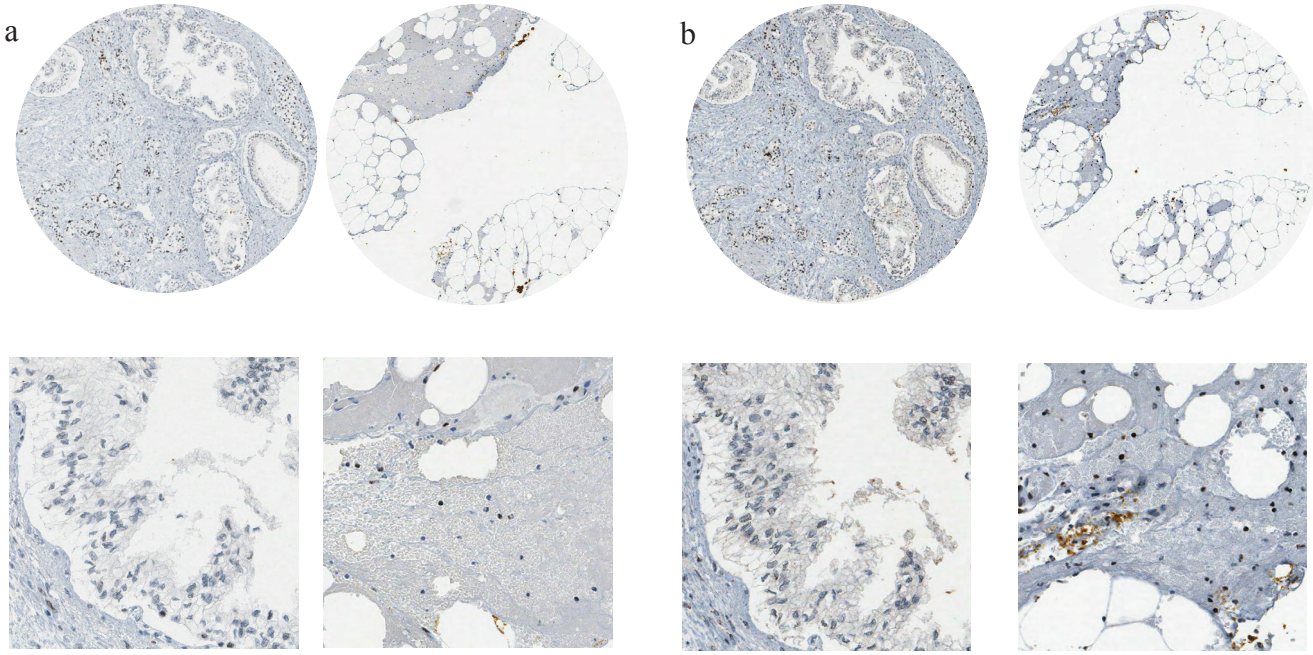
Table 5. Characteristics of Patient Cohort. Clinical data parameters for patients included in the study (n=90). 'n' denotes number of patients in each category. Cases in which certain pathological details were not reported are listed as not assessed (NA). Staging is based on AJCC cancer staging manual (7th ed.)

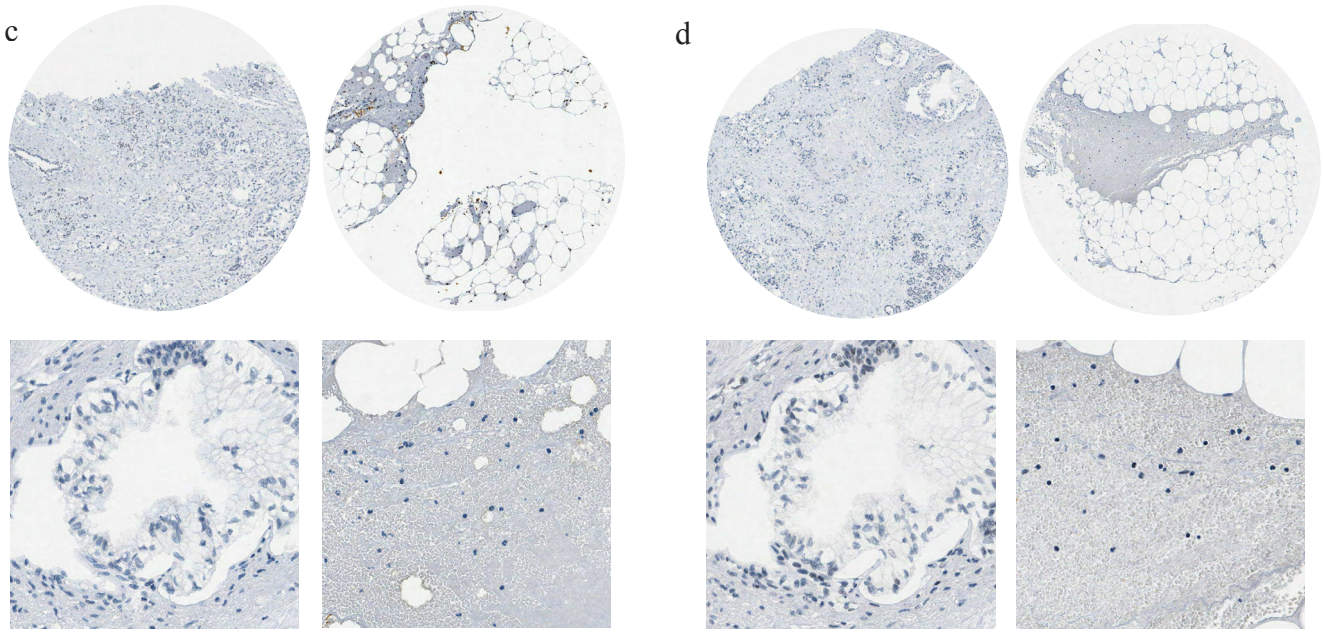
Average Age of Diagnosis (years)	**65.5**	
Average Overall Survival (months)	**28.1**	
	n	**%**
Sex		
Male	53	58.9
Female	37	41.1
Stage at Diagnosis		
Stage IA	0	0.0
Stage IB	2	2.2
Stage IIA	11	12.2
Stage IIB	55	61.1
Stage III	9	10.0
Stage IV	13	14.4
Location of Tumor		
Head	61	67.8
Body	17	18.9
Tail	12	13.3
Specimen		
Whipple	53	58.9
Distal Pancreatectomy	22	24.4
Total Pancreatectomy	4	4.4
Liver Biopsy	9	10.0
Pancreas Biopsy	2	2.2
Margins		
Positive	26	28.9
Negative	51	56.7
NA	13	14.4

Lymph Nodes		
Positive	59	65.6
Negative	19	21.1
NA	12	13.3
Lymphovascular Invasion		
Present	56	62.2
Absent	22	24.4
NA	12	13.3
Perineural Invasion		
Present	70	77.8
Absent	9	10.0
NA	11	12.2
Differentiation		
Well	11	12.2
Moderate	64	71.1
Poor	12	13.3
NA	3	3.3
Highest degree of PanIN		
PanIN1	6	6.7
PanIN2	12	13.3
PanIN3	28	31.1
Not present	31	34.4
NA	13	14.4
IPMN		
Present	6	6.7
Absent	71	78.9
NA	13	14.4
Chronic Pancreatitis		
Present	37	41.1
Absent	39	43.3
NA	14	15.6

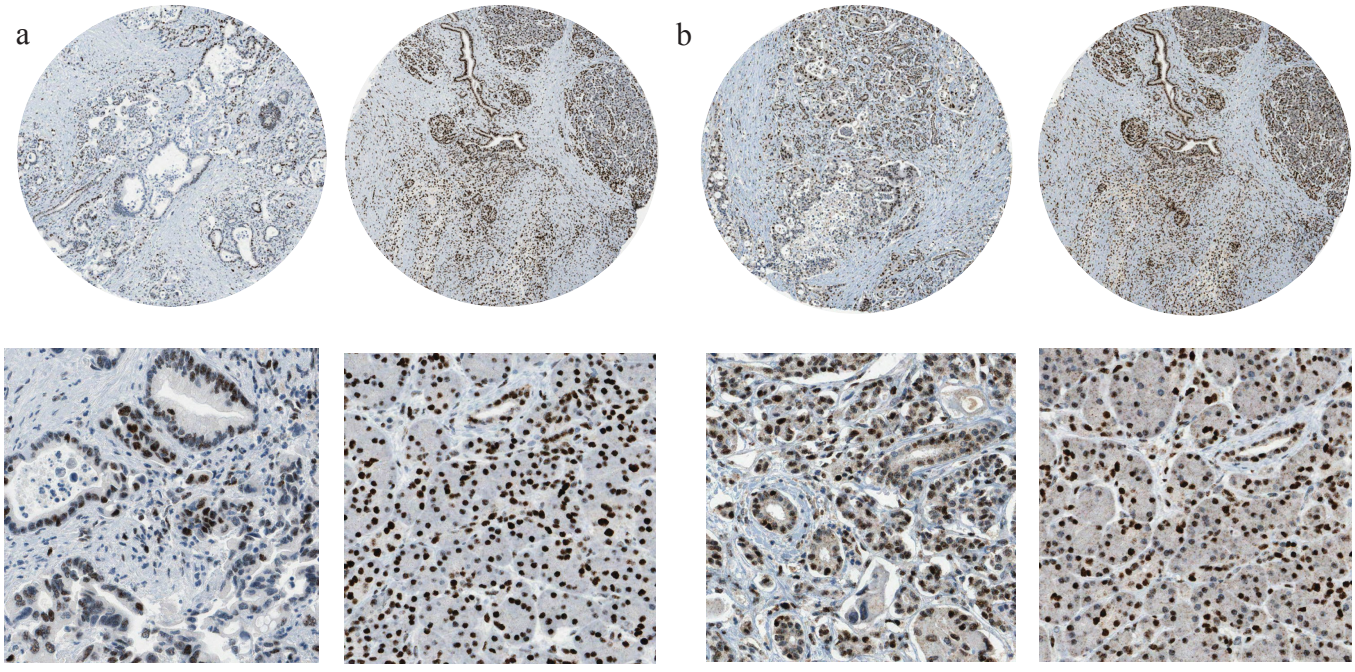
Figure 4. Representative images of tumor microarray IHC staining results (2X (top) and 10X (bottom)). Matched tumor (left) and normal (right) pancreatic epithelium cores stained for MLH1 (a), PMS2 (b), MSH2 (c) and MSH6 (d). Cores from patient 31 revealed absent staining for all four proteins. Although the normal cores were difficult to interpret due to presence of fatty tissue, the tumor stroma showed positive staining acting as a control. Patient 177 demonstrated lack of staining for MSH2 with intact staining for the remaining proteins as well as in normal control samples. Patients 198 and 750 demonstrated lack of staining for both MSH2 and MSH6 with intact staining in tumor samples for MLH1 and PMS2 as well as normal staining in control tissues.

Patient 31





Patient 177



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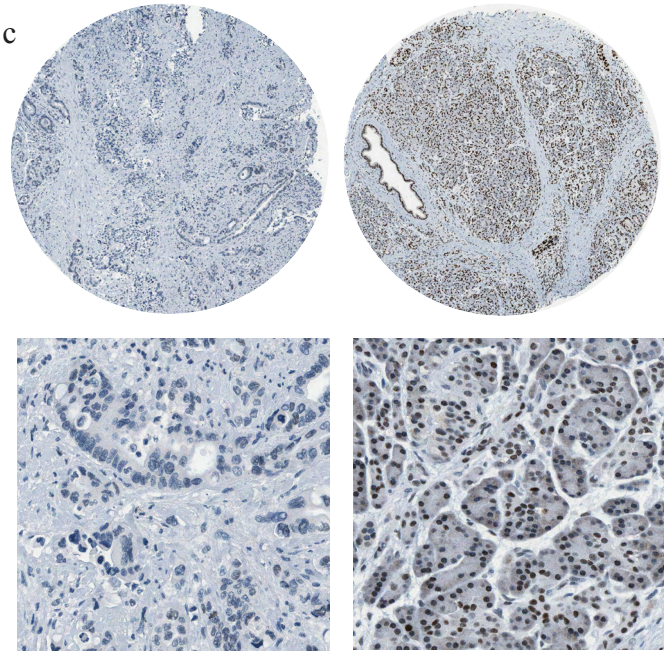

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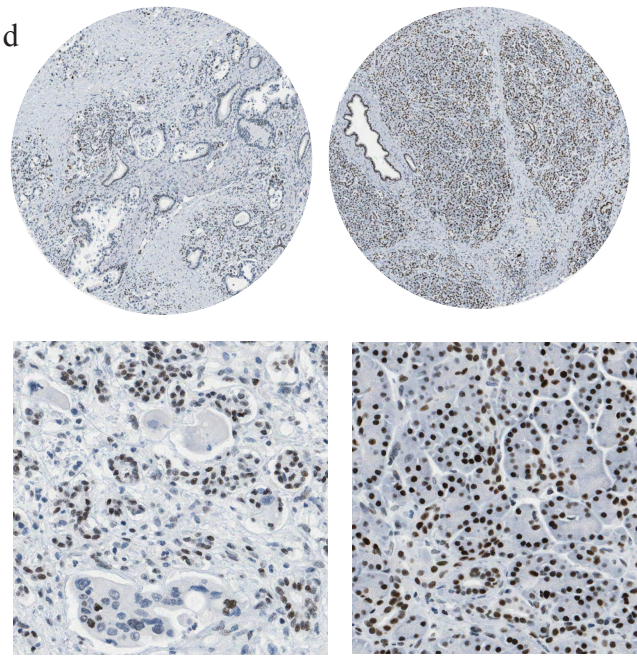
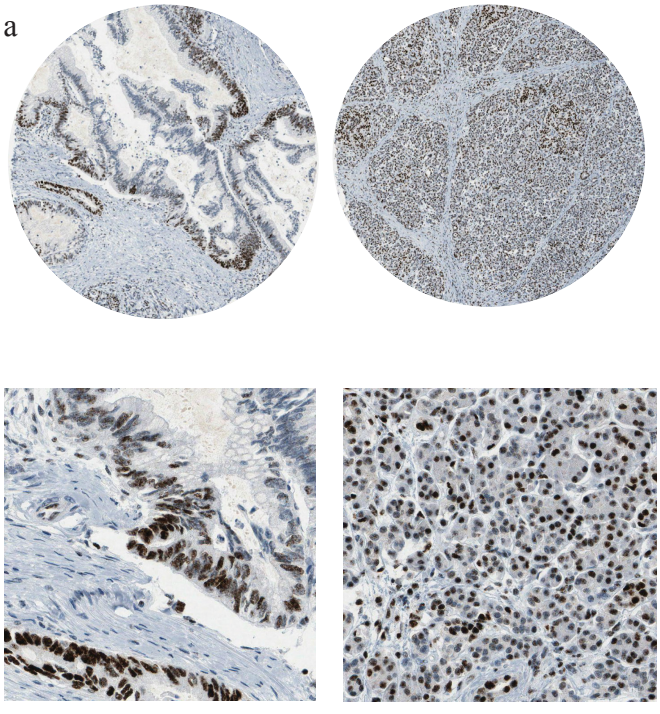
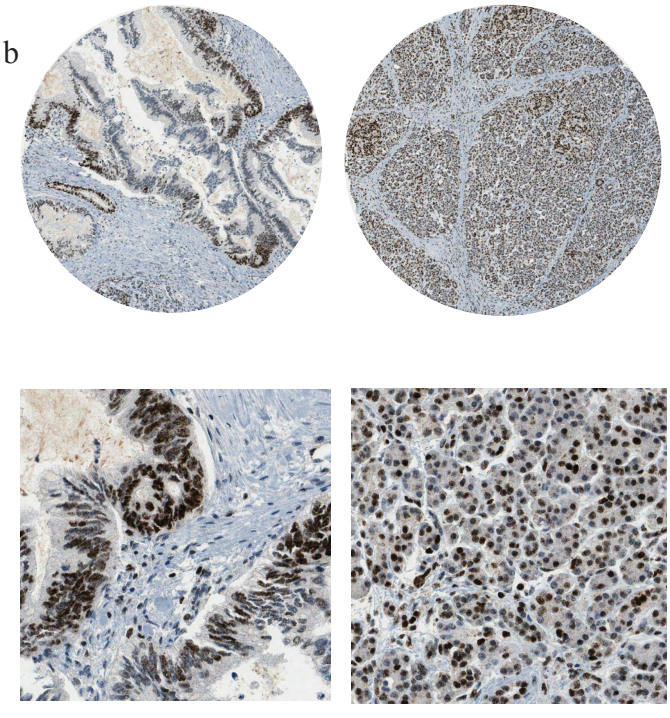


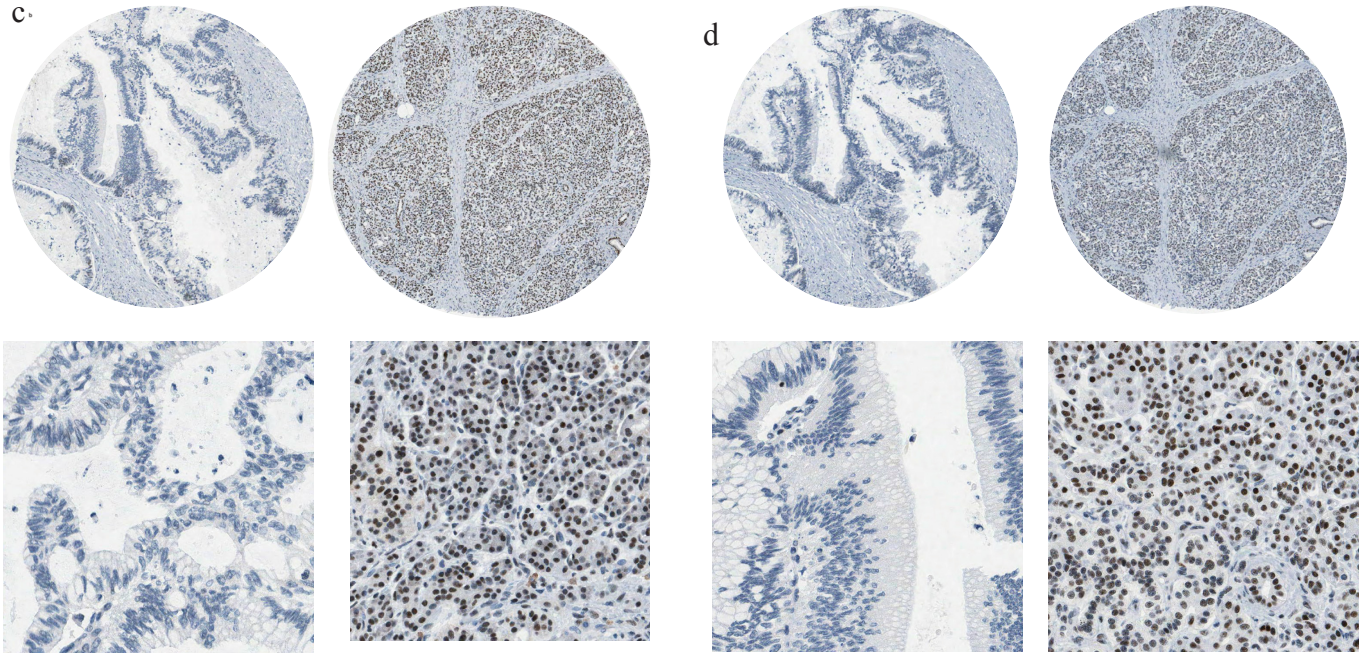
Patient 198

a

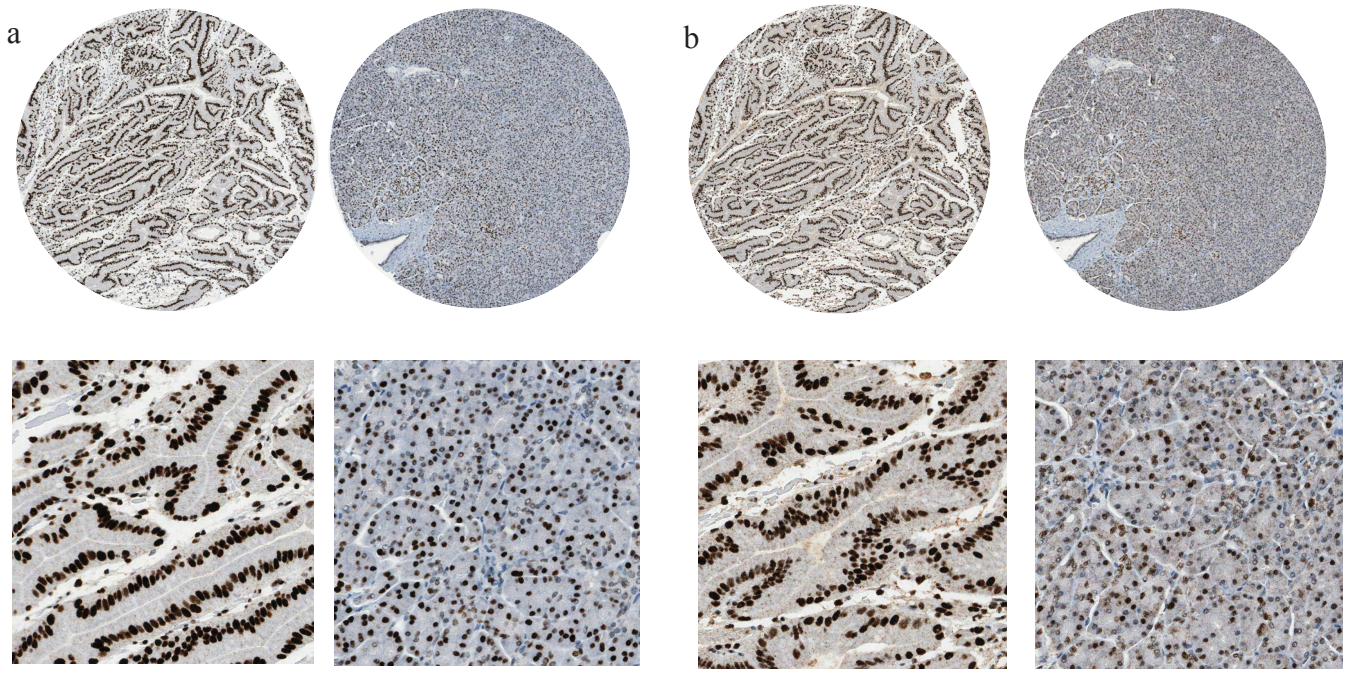


b





Patient 750



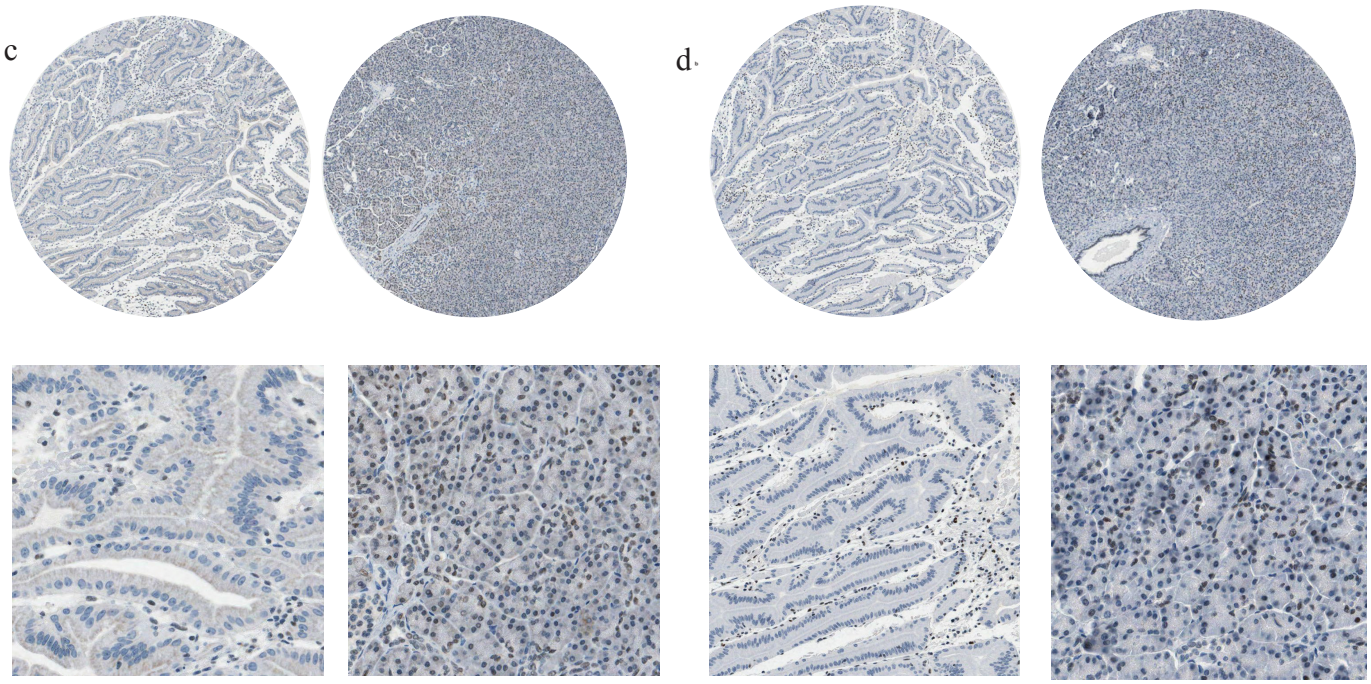
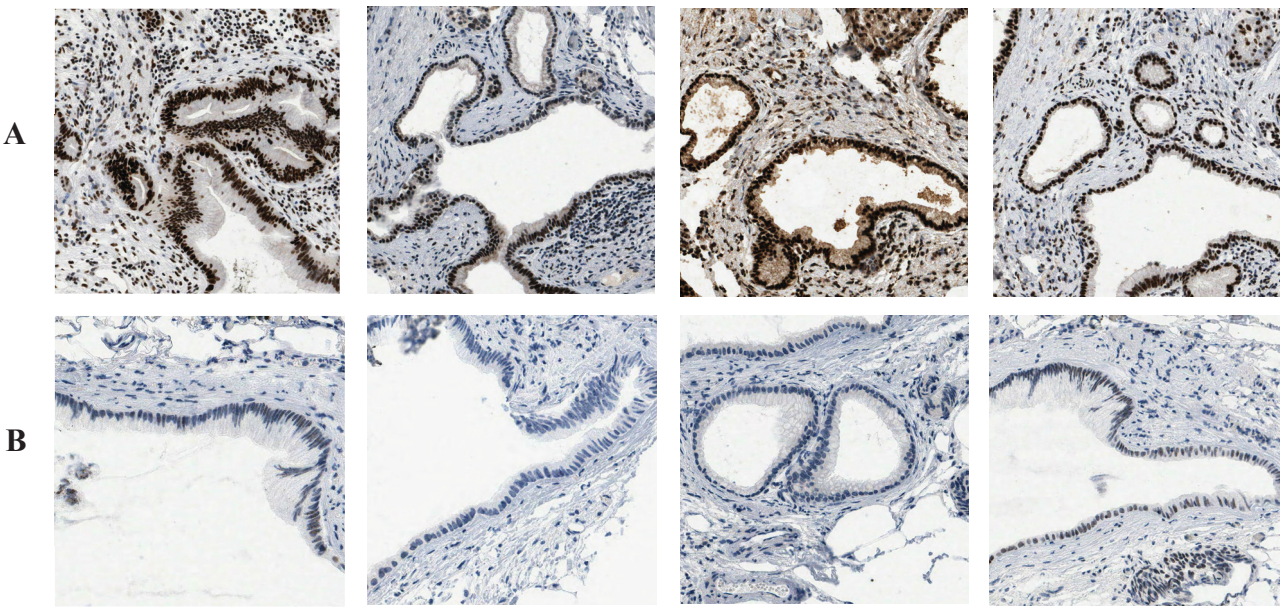
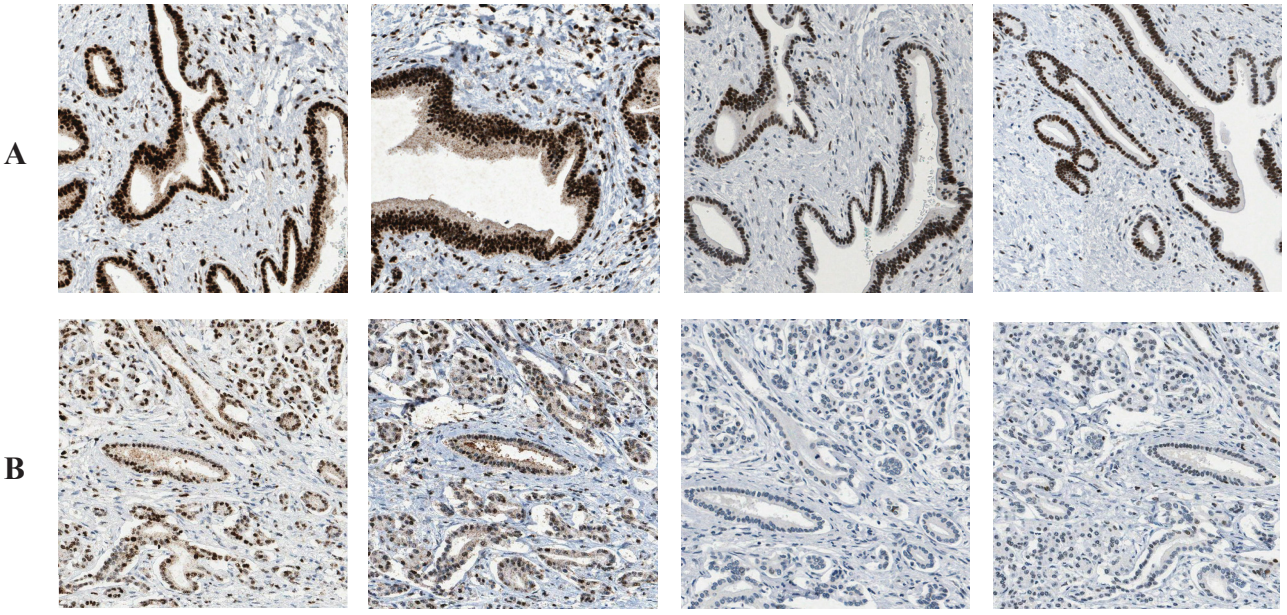

c
d

Figure 5. Representative images of IHC staining results (10X magnification). **A)** Regions on whole sections with intact nuclear staining for MLH1, PMS2, MSH2, MSH6 (left to right). Internal control tissue (stroma) demonstrates intact staining. **B)** Regions on same slide as **A** with lack of nuclear staining for at least one of: MLH1, PMS2, MSH2, MSH6 (left to right). For patient 31, there is complete absence or very weak staining for all 4 proteins. Patients 177 and 198 demonstrate intact staining for MLH1 and PMS2, absent staining for MSH2 and very weak staining for MSH6. Of note, regions with absent nuclear staining tend to have weak or absent stromal staining **C)** Intact nuclear staining for MLH1 (left) and PMS2 (right) for patient 750. **D)** Absent nuclear staining for MSH2 (left) and MSH6 (right) with intact stromal staining for patient 750, indicating mismatch repair deficiency.

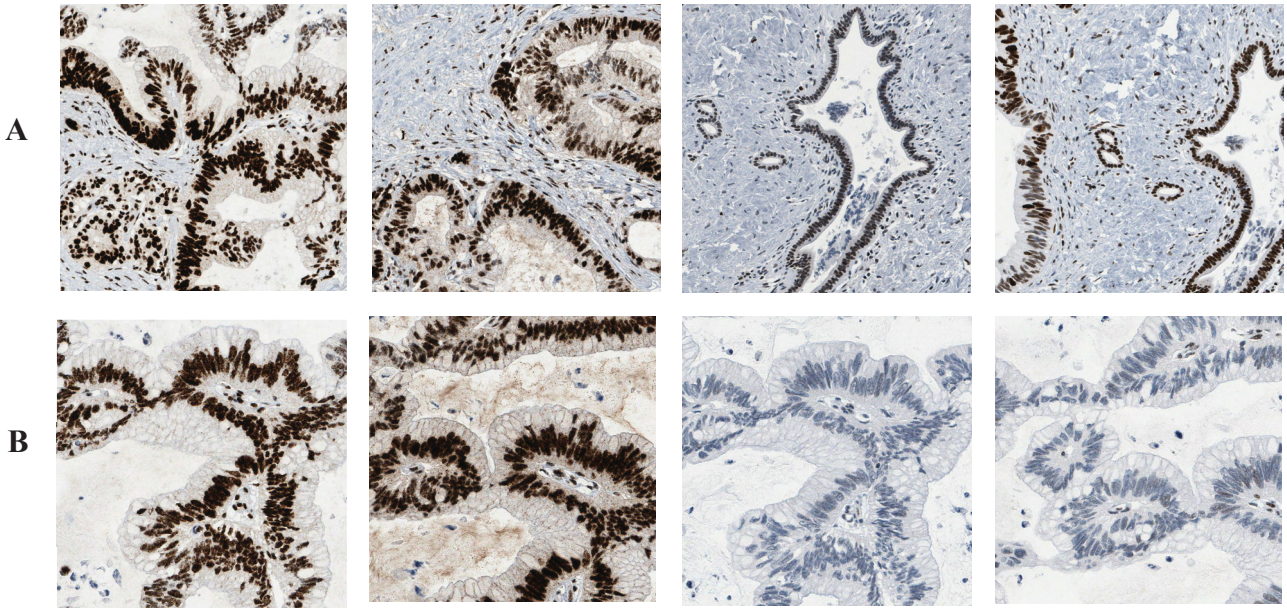
Patient 31



Patient 177

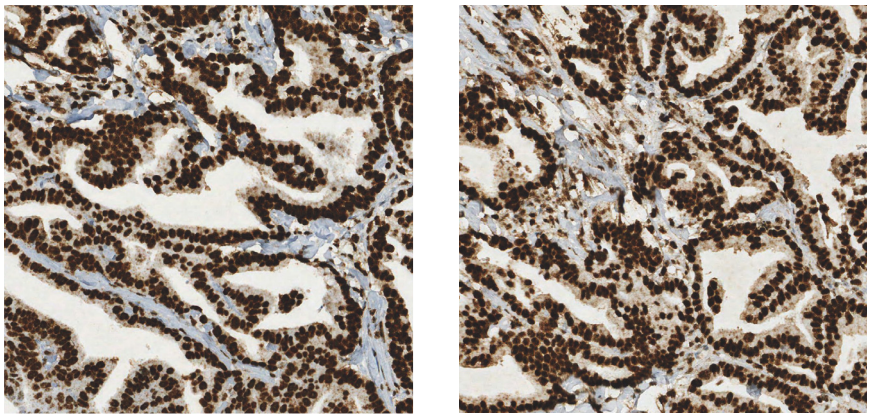


Patient 198



Patient 750

C



D

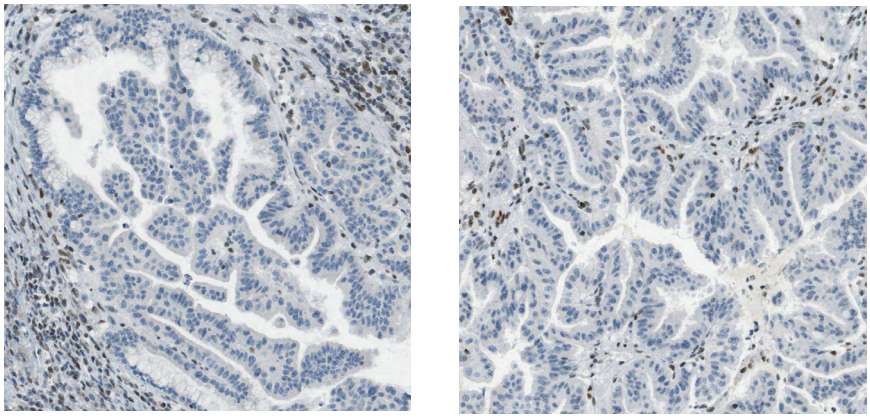
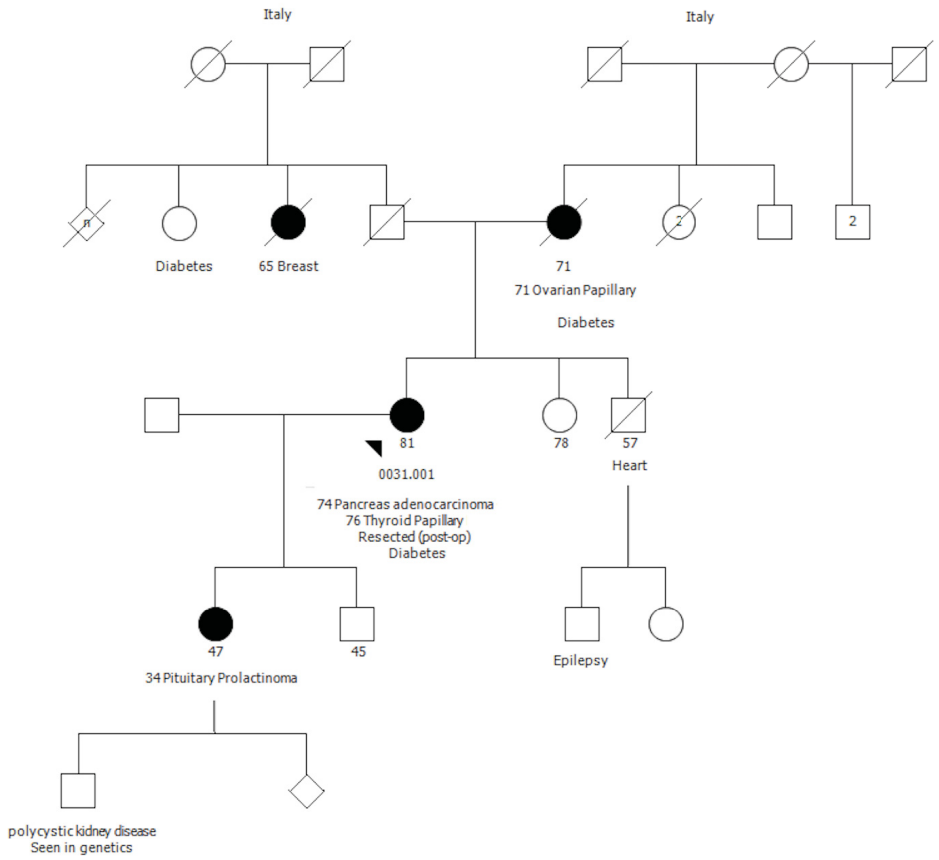
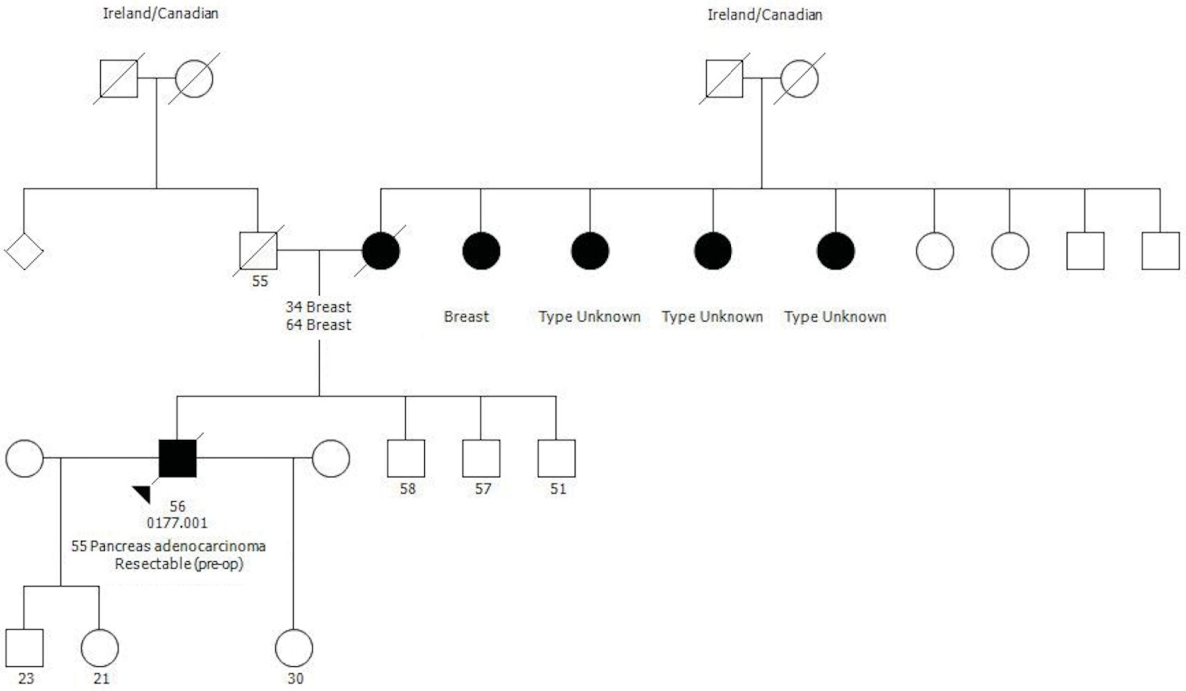


Figure 6. Pedigrees from patients with absent MMR staining on TMA. Individuals with a diagnosis of cancer are shaded in black. Patient 31 was diagnosed with PDAC at age 74. Her mother was diagnosed with ovarian cancer and her aunt with breast cancer. Patient 177 was diagnosed with PDAC at age 55. Although he reported several aunts with cancer, they were of unknown origin and unable to be confirmed. His mother did have two breast cancers, at the ages of 34 and 64. Patient 198 was diagnosed with PDAC at age 72. She did not report any family history of cancer. Patient 750 has an extensive personal and family history of colorectal cancer and meets both the Amsterdam I/II and Revised Bethesda Guidelines for a diagnosis of LS. Both he and his mother were confirmed to be *MSH2* mutation carriers (MSH2 c.942+3A>T) and are represented in the pedigree by green dots.

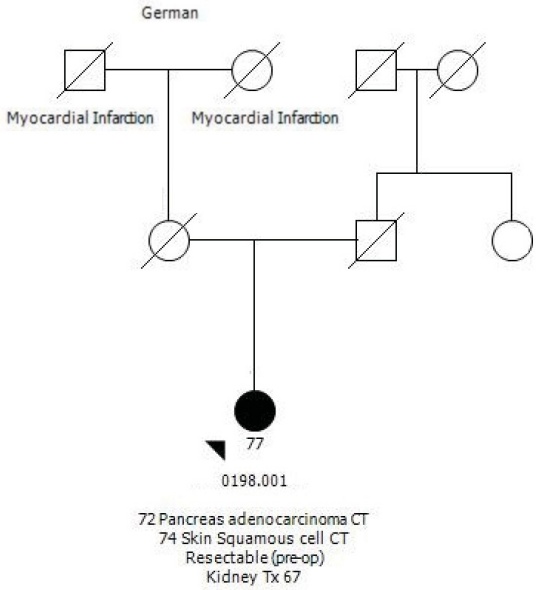
Patient 31



Patient 177



Patient 198



Patient 750

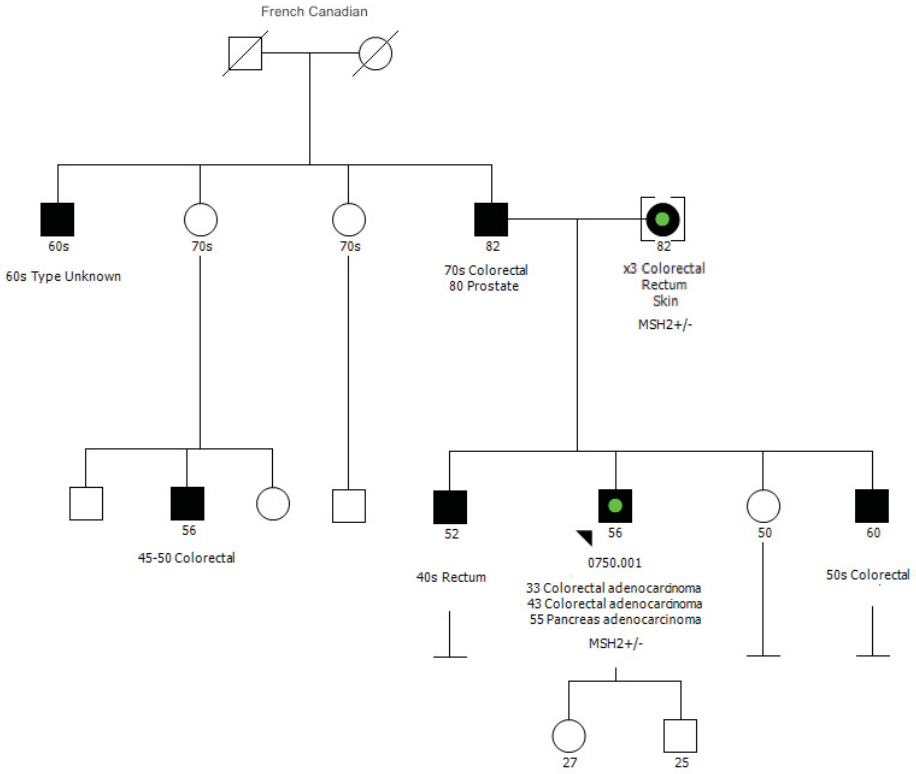
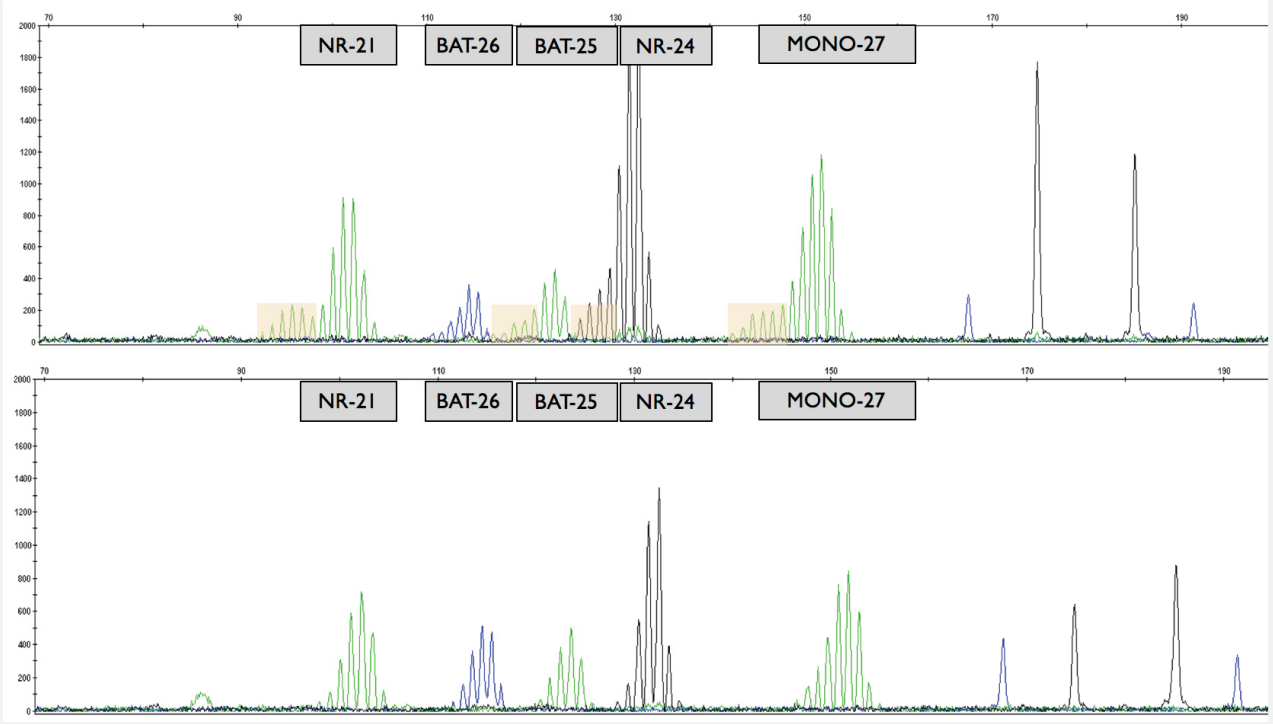

French Canadian
60s
60s Type Unknown
70s
70s
82
70s Colorectal
80 Prostate
82
x3 Colorectal
Rectum
Skin
MSH2+/-
56
45-50 Colorectal
52
40s Rectum
56
0750.001
33 Colorectal adenocarcinoma
43 Colorectal adenocarcinoma
55 Pancreas adenocarcinoma
MSH2+/-
50
60
50s Colorectal
27
25

Figure 7. Microsatellite instability results. Matched tumor DNA (top) and germline DNA (bottom) for patient 750. Fragment analysis revealed additional peaks in tumor DNA compared to germline DNA (highlighted in yellow) in 4 of the 5 microsatellite markers (NR-21, BAT-25, NR-24 and MONO-27) indicating the patient's tumor is MSI-H.



Chapter IV: Discussion

PDAC is a deadly disease with limited treatment options. In this study, we characterized the prevalence of MMR-d at our center using the QPCS resource in order to: 1) consolidate the estimates of MMR-d PDAC in the literature; 2) decide if clinical reflex testing for MMR-d should be performed in all incident PDAC cases. As predicted, MMR-d is rare in PDAC occurring in approximately 1% of patients. A limitation of the present study is its small sample size. Despite this, our results agree with several recent large-scale studies which have also concluded a prevalence of MMR-d in PDAC of 1-2%.[132,147,149] Another limitation is the enrichment of resected specimens in our patient cohort. This is largely due to the limited availability of tissue from patients with metastatic disease, which is generally diagnosed through fine-needle aspiration, rather than core needle biopsy. However, studies of colorectal cancer have shown that loss of mismatch repair is an early step in tumorigenesis.[187-189] In fact, a study by *Kloor et al.* identified loss of MMR protein expression in crypt foci of normal mucosa, suggesting MMR-d occurs even prior to adenoma formation in patients with LS.[188] Although similar studies have not been performed in pancreatic cancer, this evidence supports loss of MMR as a driver event in tumorigenesis and therefore, the prevalence in patients with early- versus late-stage disease is likely to be similar.

Despite the NCCN's recommendation to consider MMR-d testing in pancreatic tumors, only 5% of Canadian pathologists routinely test, or would consider testing for MMR-d in PDAC.[190] Furthermore, tests used for the diagnosis of MMR-d, including IHC and MSI were developed for specifically for colorectal tumors.[149] As a result, testing for MMR-d for PAC is not standardized and may explain, at least in part, the discrepancy in estimates of MMR-d prevalence in PDAC in the literature.

Early studies of MMR-d in PDAC implemented MSI testing using primarily dinucleotide markers in order to estimate the prevalence of this subtype.[139,140,144,150,191] In 1998, the National Cancer Institute attempted to standardize MSI testing by recommending a reference panel, referred to as the Bethesda panel, consisting of two mononucleotide markers (BAT-25 and BAT-26) and three dinucleotide markers (D2S123, D5S346 and D17S250). However, they also recommended dozens of alternative microsatellite markers.[162] As a result, both the number and specific panel of microsatellites analyzed was not consistent across these studies, which likely explains the large variability in the concluded estimates of MMR-d in PDAC obtained of 0% to 17.4%.[139,140,144,150,191] Furthermore, in 2002 it was determined that there were important limitations to the markers recommended by the NCI, due to the inclusion of dinucleotide repeats which were found to have poor sensitivity and specificity compared to mononucleotide markers.[158,163] In 2004, Bacher *et al.* screened a set of 266 mono-, di-, tetra- and penta-microsatellite markers in order to identify those with the greatest accuracy in identifying MMR-d. They confirmed the superiority of mononucleotide repeats and developed a fluorescent multiplex assay, known as the MSI analysis system, using the best mononucleotide markers identified: BAT-25, BAT-26, NR-21, NR-24, MONO-27.[192] Studies of MMR-d in PAC utilizing this panel obtained much more consistent results with estimated prevalences between 0.3% and 0.9%.[145,148,160] In our study, the MSI analysis system correlated with the IHC staining results confirming MMR-d in one patient.

As with any diagnostic test, there are certain limitations in using MSI to identify patients with MMR-d. MSI analysis is highly dependent on DNA and PCR product quality.[161] Consequently, it can be difficult to obtain MSI testing results when using archived samples such

as FFPE blocks. Secondly, MSH6 mutations tend to be associated with a lower level of MSI compared to other MMR mutations. Therefore, MSI testing can be normal despite loss of MMR function.[164] Finally, although MSI has the potential to identify mutations in the mismatch repair system outside of *MLH1, MSH2, MSH6* or *PMS2*, it does not indicate the specific gene mutated.[160]

For these reasons, some researchers and clinicians prefer the use of IHC staining of the MMR proteins in order to diagnose MMR-d. However, due to the abundance of dense stroma in pancreatic tumors, tumor cellularity can be low.[183] Therefore, it is essential to use a large enough tissue section in order for results to be interpretable. The use of tumor microarrays for research has become rapidly popularized due to the possibility of assembling a large number of representative samples from a defined patient cohort on a single block.[184] This allows convenient screening of a marker of interest while avoiding experimental variability and minimizing the use of material and costs.[184] We demonstrated that the construction of TMAs for studies of pancreatic cancer is feasible. However, we did note certain limitations such as the loss of cores, particularly in biopsy samples, during construction and sectioning. As a result, 7 patients out of our initial cohort of 97 could not be assessed for MMR-d, which is in keeping with previous studies estimating data loss in 5-7% of cases when using triplicate microarrays.[186] In order to prevent this, samples with limited tissue availability, especially biopsy specimens, should be analyzed in research studies using whole sections rather than being included in TMAs. Alternatively, a greater number of smaller diameter 0.6mm cores should be considered as smaller cores are associated with a lower incidence of core loss during TMA sectioning.[186] However, due to the sclerotic nature and low cellularity of PDAC tumors, an increased number of cores would

need to be used in order to guarantee the analysis of an adequate number of malignant duct cells.[184]

Although tumor microarrays are a good screening tool, they display a high degree of false positives. Based on the results of our TMAs, we identified 4 patients with MMR-d, reflecting a prevalence of 4.4%. However, on whole section staining, only 1 patient demonstrated lack of staining representing a prevalence of 1.1%, a four-fold difference. Our results are reflected by those of a recent study by Lupinacci *et al.*, in which 13% of patients showed lack of staining for at least 1 MMR protein on TMA cores. However, when these were confirmed on whole sections, only 1.4% of patients were confirmed to be MMR deficient.[148] This may explain why previous studies using tumor microarrays without the use of a secondary confirmation method concluded high rates of MMR-d in PAC of 12.8% and 22%, respectively.[143,148] On the contrary, studies implementing IHC staining on whole sections obtained much lower estimates of MMR-d in PAC of less than 1%.[132,145,160] The discrepancies between studies analyzing TMAs compared to larger tissue sections may be explained by the heterogeneous staining pattern we observed on whole section IHC staining. As cancer progresses, cells acquire additional mutations which results in the development of genetically distinct subclones, otherwise known as tumor heterogeneity. However, because TMAs only sample a very small portion of the tumor, certain populations of cells may not be sampled resulting in the misrepresentation of the entire tumor.

It is unclear whether the staining pattern observed in our study reflects a technical limitation of IHC or true tissue heterogeneity with a subset of tumor cells exhibiting MMR-d and others with intact mismatch repair. An important observation is that areas with absent nuclear

staining also demonstrated weak or absent internal positive control staining in the surrounding stroma, a pattern which is usually considered uninterpretable. Several mechanisms have been suggested to explain this staining pattern including differences in the local tumor microenvironment, regional hypoxia or ischemia leading to activation of pancreatic enzymes and subsequent autolysis resulting in protein degradation.[164,193]

On the other hand, there were associations between protein loss on TMAs compared to whole sections. In the case with loss of expression for all four proteins on the TMA, focal areas with weak or absent nuclear staining were also found on whole sections stained for each protein. Likewise, in the two cases with loss of MSH2 & MSH6 on TMA cores, areas on whole sections with complete loss of expression for MSH2 & MSH6 did stain for MLH1 and PMS2. This would support the hypothesis that there may be true tumor heterogeneity in these patients. Previous studies in endometrial and colorectal cancers have reported clonal loss of MMR protein expression.[194,195] A study by *Watkins et al.* assessed IHC staining for MMR proteins in 125 endometrial carcinomas. They identified nine patients with abrupt subclonal loss of MMR staining across or within tumor glands.[195] In most cases, MSI testing confirmed microsatellite instability in deficient subclones with the remaining MMR-intact portions remaining stable.[195] Notably, these cases were associated with epigenetic silencing events and were therefore somatic losses without any underlying germline mutation.[195] Similarly, a study by *Watson et al.* assessed 1003 CRCs for MSI.[194] They identified 75 MSI-H cases, of which 10% demonstrated heterogeneous zonal loss or intraglandular variations in MMR expression.[194] Three of the seven patients were found to have germline MMR mutations, while the remaining four cases were caused by somatic events.[194] MSI testing will be considered to further investigate the cases with

heterogeneous staining in our study. Although MMR-d PDAC is most commonly due to germline mutations, *MLH1* and *MSH2* hypermethylation has been reported in pancreatic tumors.[196] Furthermore, mutations in *EPCAM* result in the epigenetic silencing of *MSH2*, another mechanism that would be consistent with our IHC results and may account for the heterogeneous staining pattern seen in our cohort.[86] The clinical significance, specifically the responsiveness to checkpoint blockade therapy, of tumors with focal areas of MMR-d merits further investigation.

Due to the technical limitations of IHC and MSI, recent studies have implemented sequencing technologies including gene panel testing or WGS in order to estimate the prevalence of MMR-d in PDAC. These studies obtained consistently low estimates of MMR-d prevalence between 0.7% and 1.0%.[132,147,160] Although these tests are more expensive and have a longer turn-around time, they allow the determination of the precise mutation involved, whether it is somatic or germline and can also be used to assess the mutational load and microsatellite status of the tumor.[149]

Although a recent large-scale study analyzing whole-exome data from 183 pancreatic tumors failed to identify any patients with MMR-d PDAC, we identified one case in our cohort.[117] Because of his strong personal history of colorectal cancer, it was speculated that it could represent a colorectal metastasis rather than a true pancreatic cancer. However, histological assessment identified carcinoma *in situ* in the surrounding normal pancreas strongly favoring a diagnosis of PAC. Furthermore, the pancreatic tumor cells stained positively for CK7, which is typical for PDAC and rarely seen in CRC.[8] Although PDACs are typically negative for CK20

staining, approximately 25% of PDACs stain positively and therefore a diagnosis of PDAC is consistent with the IHC results.[8]

Based on our findings MMR-d PAC is rare, but does exist, accounting for approximately 1% of cases. Despite the low prevalence, clinical reflex testing may still be warranted. Although the patient with MMR-d in our study had an extensive personal and family history of colorectal cancer, obtaining a thorough family history is crucial in identifying which patients to test for MMR-d. Practically, this would mean having access to a genetic counsellor in clinic which is costly or having a clinician take the family history which is not always practical. In fact, a study by Hampel *et al.* assessed the feasibility of screening all incident CRC cases using IHC and MSI testing.[197] Only one out of 153 individuals identified as having LS had been previously diagnosed or referred to medical genetics, reflecting how rarely thorough family histories are obtained and assessed in practice.[197] Furthermore, patients with somatic MMR gene mutations, such as hypermethylation would not be identified based on family history. Several studies have established the cost-effectiveness of clinical reflex testing for MMR-d in all incident CRC cases diagnosed under the age of 70.[198-200] Although the rate of MMR-d is not nearly as high in PDAC as compared to CRC, the incidence of PDAC is much lower and treatment options are exceptionally limited. Therefore, IHC testing for MMR-d in all incident PDAC cases may be justified. Furthermore, mutations in other cancer predisposition genes are found in up to 30% of patients diagnosed with pancreatic cancer.[201] The lack of effective predictors of patients harboring mutations and the limited treatment options for PDAC have led several studies to suggest the use of a gene-panel reflex testing approach for all incident PAC cases.[202,203] Furthermore, identification of a germline mutation in a patient with pancreatic cancer may have cancer screening implications for asymptomatic at-risk relatives.[203]

In summary, my dissertation research suggests that MMR-d in PDAC is approximately 1%. Discrepancies in estimates of MMR-d in the literature can be largely attributed to differences in diagnostic methods. Despite the low prevalence of MMR-d, clinical reflex testing may still be warranted as there are several clinical implications in identifying such patients, including precision oncology treatment opportunities with immunotherapy, the identification of at-risk family members and the subsequent screening of these patients for prevention and early detection of Lynch-associated malignancies.

Chapter V: Bibliography

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