Universitat de Girona
Facultat de Medicina

FINAL DEGREE PROJECT

GUT MICROBIOTA ANALYSIS OF INTRADUCTAL PAPILLARY MUCINOUS NEOPLASM PATIENTS, A STEP BEFORE PANCREATIC CANCER:

A cross–sectional study

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A la iaia Pepi, que sé que sempre m'acompanya en tot.

A la meva família, per compartir amb mi aquest camí tan bonic que pot ser la vida.

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ABSTRACT

BACKGROUND: Intraductal papillary mucinous neoplasm is currently one of the most frequently diagnosed premalignant lesions of pancreatic cancer. Pancreatic cancer is known to be a major health problem worldwide due to its poor prognosis, and high mortality rates. Research efforts have been focused for years on determining risk factors and risk groups, early diagnosis, possible screening techniques, and improving treatments. Recently, both pancreatic and intestinal microbiota have been linked to pancreatic cancer, its carcinogenesis, and the progression of this pancreatic malignant disease. And it has been hypothesized that gut dysbiosis could be present in the earliest stages of the disease.

OBJECTIVE: The aim of this study is to determine which specific bacterial taxonomic changes in the gut microbiota, together with their relative abundances, are present in untreated IPMN patients compared to healthy controls. Investigated by means of a presence/absence study, identifying which bacteria with a relative abundance of less than 5% in healthy controls are present with an abundance of > 15% in IPMN patients, using 16s rRNA sequencing analysis of stool.

DESIGN AND SETTING: This study is designed as multicenter, cross-sectional study. It will be performed at Hospital Doctor Josep Trueta de Girona (with the participation of Institut Català de Salut patients and hospitals) over a period of three years and four months.

PARTICIPANTS AND METHODS: A total of 415 participants will be enrolled over 1.5 years using a consecutive sampling. This population (meeting inclusion and none of the exclusion criteria) will compromise patients recently diagnosed with IPMN in participating hospitals (83 patients) and their matched controls (332 controls, who have undergone abdominal CT scan for other medical reasons) for age, gender, and hospital. Their microbiota composition and structure will be analysed in faecal samples using 16s rRNA gene sequencing (MiSeq System) to assess the main objective. The primary dependent variable will be the bacterial taxonomy of the microbiota and its relative abundances, which will be compared between both groups. The secondary variables will include the characterization of bacterial taxonomy in IPMN patients and the assessment of alpha diversity in both groups. Additionally, certain covariates have been included.

KEY WORDS: Intraductal papillary mucinous neoplasm (IPMN), pancreatic cancer, gut microbiota.

ABREVIATIONS AND ACRONYMS

AEG: Asociación Española Gastroenterología

ATM: Ataxia Telangiectasia Mutated

BD-IPMN: Branch Duct – Intraductal Papillary Mucinous Neoplasm

BI: Bioinformatician

BMI: Body Mass Index

BRCA1/2: Breast Cancer genes 1 / 2

CAF: Cancer associated fibroblasts

CA 19.9: Carbohydrate antigen 19-9

CDKN2A: Cyclin – dependent kinase inhibitor 2A

CDX2: Caudal – type homeobox transcription factor 2

CEA: Carcinoembryonic antigen

CEIC: Comitè Ètic d'Investigació Clínica

CFU: Colony-forming units

Cls: Clinicians

CP: Chronic Pancreatitis

CRAMP: Cathelicidin – related antimicrobial peptide

CRG: Centre for Genomic Regulation

CT: Computed Tomography

DCA: Deoxycholic acid

DNA: Deoxyribonucleic acid

ERCP: Endoscopic Retrograde Cholangiopancreatography

EUS-FNA: Endoscopic Ultrasonography and Fine Needle Aspiration

GNAS: G – protein alpha – subunit

HC: Hospital coordinator

ICS: Institut Català de la Salut

IDIBGI: Institut D'Investigació Biomèdica de Girona

IgA: Immunoglobulin A

IPMN: Intraductal Papillary Mucinous Neoplasm

IL: Interleukin

KRAS: Kristen rat sarcoma viral oncogene

LPS: Lipopolysaccharides

LTA: Lipoteichoic acid

MAPK: Mitogen – activated protein kinases

MD-IPMN: Main Duct – Intraductal Papillary Mucinous Neoplasm

MI: Main Investigator

MLH1: MutL protein homolog 1

MR: Microbiology Researchers

MRCP: Magnetic Resonance Cholangiopancreatography

MRI: Magnetic Resonance Imaging

MUC: Mucin

NF-κB : Nuclear factor kappa – light – chain – enhancer of activated B cells

NRL: Nod-like receptor

OTUs: Operational Taxonomic Units

PanIN: Pancreatic Intraepithelial Neoplasm

PC: Pancreatic Cancer

PCR: Polymerase Chain Reaction

PDAC: Pancreatic ductal adenocarcinoma

PPAR-γ: Peroxisome proliferator – activated receptor gamma

PRSS1: Protease Serine 1

PSC: Pancreatic Stellate cells

Pts: Patients

QIIME: Quantitative Insights Into Microbial Ecology

RNF43: RING finger protein 43

SCD: Societat Catalana Digestologia

SCFAs: Short Chain Fatty Acids

STAT3: Signal transducer and activator of transcription 3

Sts: Statistician

TLR: Toll-like receptor

TP53: Tumour protein p53

16s rRNA: 16s ribosomal RNA

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1 INTRODUCTION

1.1 Pancreatic Cancer and Intraductal Papillary Mucinous Neoplasm (IPMN)

Pancreatic cancer (PC) is the seventh leading cause of cancer death in both sexes worldwide (466,000), despite relatively low incidence rates (496,000) (*Figure 1*), mainly because of its poor prognosis due to late diagnosis and limited therapeutic options (1,2). These limitations in its management result in a high mortality rate, which accounts for 83% of the incidence in Spain (3).

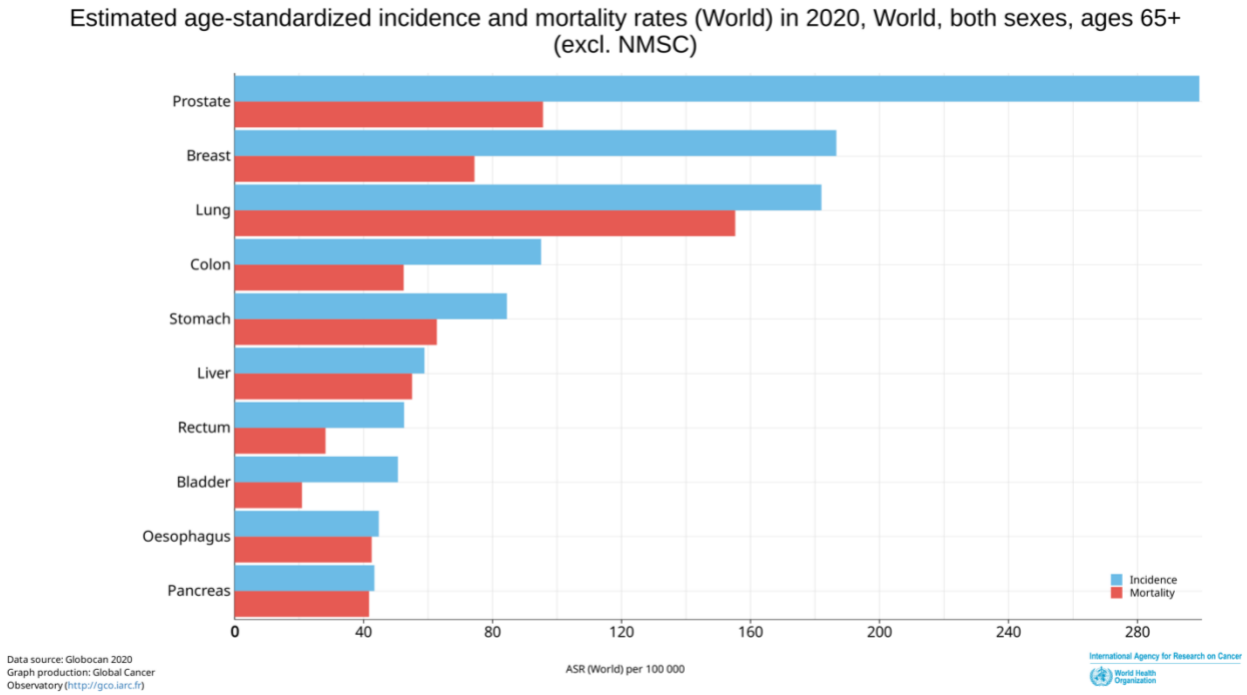

Figure 1. Estimated age-standardized incidence and mortality rates in 2020, World, both sexes, ages 65+ (excl. NMSC) *(4). As can be seen in this graph, pancreatic cancer is characterized by a high mortality in both sexes worldwide despite low incidence rates.*

In Spain, PC is estimated to be the third most common cause of cancer death in 2023 (7,663 in 2021) as well as the seventh more common cause of more incidence (9,280 new cases estimated in 2023). In Spain, as in the rest of the world, PC is characterized by a poor prognosis, resulting in almost the lowest five-year survival rates in both sexes compared to other types of cancer. It was observed that the five-years net survival rate for patients diagnosed with a PC between 2008 and 2013 was only 7.2 and 10.0 for men and women, respectively (3).

In order to reverse these trends and improve the prognosis of PC, an effective way is to understand the risk factors that influence the development of the disease, which provides suggestions for its prevention (5). As described by Hu JX *et al.*(5), risk factors for this malignant disease include **diabetes mellitus type II** (2-3 times greater risk of PC) (6), **obesity** (a 5 kg/m^2 increase in body mass index (BMI) was associated with a 12% increased risk of PC) (7), **smoking** (it causes an approximately 75% increase in the risk of PC) (8), **alcohol** (heavy alcohol consumption is a major cause of chronic pancreatitis, which has been linked to PC) (9,10), **chronic pancreatitis** (there is an increased risk of PC and it increases with the duration of disease) (11), and **family history** and **genetic susceptibility** (some germline variants and high-risk genes have been identified, such as BRCA1/2, ATM, TP53, MLH1, CDKN2A and PRSS1, among others) (12,13). In addition, changes in the **gut** (14) and **pancreatic** (5) **microbiota** have recently been included as risk factors, as well as shifts in the **oral** microecosystem (15) (although there are inconsistencies in this regard between multiple studies in which a significant association between PC and oral microbiota was not confirmed (2)).

Pancreatic ductal adenocarcinoma (PDAC) is the most common pancreatic neoplasm (16), representing a malignancy of the exocrine pancreas, while a minority present with neuroendocrine tumours (17). PDAC arises from non-invasive precancerous lesions that are curable if diagnosed and treated early enough (18). Most of them arise from pancreatic intraepithelial neoplasms (PanINs) (which progress from stage I to III accumulating different genetic mutations), while a smaller proportion (< 10%) evolve from intraductal papillary mucinous neoplasms (IPMNs), and mucinous cystic neoplasms (the least common) (18,19). As mentioned, IPMNs are not a major precursor of invasive cancer; however, their diagnosis is increasing due to incidental detection in abdominal imaging studies, as they are larger cystic neoplasms (≥ 5mm) (18–20), and subsequently, the number of pancreatic resections for IPMN has increased to the level of 20% of all pancreatic resections (21). In contrast, PanINs are rarely detected incidentally, despite being the most common precursor, because of their microscopic diameter, which prevents them from being seen through imaging (19).

IPMN is classified as a Pancreatic Cystic Neoplasm in the WHO classification (22) and is characterized by several aspects (*Table 1*) that define it as distinct lesion. IPMN is described as a

radiologically or grossly recognizable cystic neoplasm arising within and communicating with the pancreatic ductal system (23). This communication with the Wirsung duct and the absence of ovarian-type stroma is what differentiates it from other cystic neoplasms (24).

***Table 1. General features of pancreatic cystic neoplasms** (PCN) that permit differentiation between PCN, and define IPMN (24).*

Variables	*IPMN*	*MCA*	*SCA*	*SPN*
Age	60-70	40-50	50-70	20-40
Gender	Slightly more prevalent in males (60%)	Females (> 90%)	Females (75%)	Females (> 90%)
Pancreatic location	Anywhere	Distal (body and tail)	Anywhere	Distal (body and tail)
MD communication	Present	Typically absent (85%)	Absent	Absent
Macroscopic appearance	Macrocystic (BD)	Oligocystic or macrocystic	Microcystic (honeycomb pattern)	Macrocystic
Malignant potential	Low (BD) High (MD)	Low	Very low	Low
Predictors of malignancy	Size > 3 cm MD > 6 mm Thickened and irregular walls Mural nodules/septae	Mural nodules Peripheric calcifications Irregular walls		Big size

IPMN: Intraductal papillary mucinous neoplasm; MCA: Mucinous cystadenoma; SCA: Serous cystadenoma; SPN: Solid pseudopapillary neoplasm; MD: Main duct; BD: Branch duct.

IPMNs are usually diagnosed between the ages of 60 and 70 and affect men and women almost equally (21). Although their diagnosis is increasing, the real prevalence of these pancreatic cystic lesions remains unknown (25), mainly because of their incidental diagnosis due to the large proportion of asymptomatic patients. It is known that cystic pancreatic lesions have a prevalence of 2.4-16%, of which 10% are cystic neoplasms. Diagnosed IPMNs account for 15% of these cystic neoplasms (26).

IPMNs are characterized by papillae (protruding into the cyst), arising from intraductal proliferation of neoplastic mucinous columnar cells that produce thick and colloid-like mucin (21,22). IPMNs can be subclassified according to the duct they arise from (main pancreatic duct or MD-IPMN, and side branch duct or BD-IPMN, or mixed type), their histological characteristics or their grade of dysplasia.

Classification based on the duct in which they arise, determines three IPMN subtypes based on imaging studies and/or the histology (27):

- MD-IPMNs originate from the epithelium of the main pancreatic duct (25), usually occur in the head of the pancreas (23), and are characterized by dilation of the main pancreatic duct of > 5 mm without other causes of obstruction (27). This type is associated with a higher risk of progressing to carcinoma (25).
- BD-IPMNs develop from the side-branch duct, resulting in pancreatic cysts of > 5mm in diameter that communicate with the main pancreatic duct (27).
- Mixed type meet the criteria for both MP-IPMNs and BD-IPMNs, but has the same clinicopathological behaviour as MD-IPMNs (25).

This distinction according to the duct of origin is of clinical relevance because main duct involvement is a predictor of malignant transformation (27).

Based on histological features and mucin markers, four subtypes have been described that correlate with the risk of developing malignancy and its subsequent prognosis (25):

- Intestinal IPMN is the most common subtype and arises from the main pancreatic duct, produces MUC (intestinal mucin) 2+ and expresses transcription factor CDX2. If it progresses to malignancy, it develops into colloid carcinoma which has a better prognosis than PDAC due to its slow growth (28).
- Pancreato-biliary IPMN shows the phenotype of pancreatic and biliary duct cells expressing the mucin markers MUC1 and MUC5AC. If it progresses to carcinoma it has a poor prognosis similar to PDAC (28).
- Gastric IPMN has its origin in a branch duct resulting in a BD-IPMN and its phenotype resembles gastric antral mucosa expressing MUC5AC and MUC6. It seems to be the less aggressive (28).
- Oncocyte IPMN is a rare subtype that does not correspond to any of the cells of the gastroenteropancreatic epithelial system. Its prognosis is poor, and it usually presents with an invasive component (28). This subtype of lesion has recently been categorized by *The 2019 WHO Classification of tumours of the digestive system 5th edition* as an independent entity due to its distinct genomic and morphological features, being defined as Intraductal Oncocyte Papillary Neoplasm (IOPN) (29).

As stated, epithelial subtypes are correlated with a prognosis, but their utility is clinically limited because these phenotypes may be found in isolation, but may also mixed within a single lesion (30).

Based on the degree of dysplasia, IPMNs can be classified into **low-grade** and **high-grade dysplasia**, representing a spectrum that progresses to carcinoma formation. Cytologic and architectural atypia increases as IPMN progresses (23).

Some genetic alterations in IPMNs have been identified by different studies, revealing that the most common mutations correspond to **KRAS mutations** (the most important driver genes during IPMN development and one of the earliest mutations) or **GNAS oncogene mutations** (specific to IPMNs, so its presence in cystic fluid is diagnostic of an IPMN; it is more frequent in the intestinal subtype). These mutations are found in > 96% of IPMNs (> 50% of IPMNs have both mutations) (23,25,30,31). RNF43 inactivation has also been found in a high proportion of IPMNs. Furthermore, it has been shown that inactivation of both TP53 and CDKN2A is associated with transformation of IPMN into invasive carcinoma (23). The clinical relevance of these findings in its molecular profile lies in its use to assess IPMN progression and to provide additional information for accurate preoperative diagnosis that helps make treatment decisions (21).

As mentioned above, many IPMNs are diagnosed incidentally in asymptomatic patients during imaging studies, but a proportion of them present with clinical symptoms (30). The most common symptom is acute pancreatitis with a long-standing asymptomatic hyperlipidaemia due to ductal mucus obstruction or the papillary proliferation of the ductal epithelium (21). Other less frequent symptoms are abdominal pain, anorexia and weight loss, diarrhoea (secondary to exocrine pancreatic insufficiency), jaundice, nausea/vomiting, and new-onset diabetes mellitus (secondary to endocrine pancreatic insufficiency) (30).

Radiological features that suggest a diagnosis of IPMN include involvement of the duct system, **dilatation of the main pancreatic duct** or branch ducts (seen as cyst clusters), and surrounding parenchymal atrophy (30). Transabdominal ultrasound is a useful basic test that can help to differentiate between solid and cystic lesions, but it does not allow a complete examination due to the presence of bowel gas obscuring the view (22). **Magnetic resonance**

cholangiopancreatography (MRCP) is considered to be the best imaging technique for detecting and characterizing IPMNs, it is the most sensitive and can therefore determine the presence or absence of a communication between an IPMN and the main pancreatic duct (25). However, depending on local conditions and preferences, CT with contrast medium may also be useful. The diagnosis may be supplemented by endoscopic ultrasound and fine needle aspiration (EUS-FNA), which allow diagnostic imaging (dilation of the Wirsung duct, presence of wall nodules inside the duct, atrophy of the pancreatic parenchyma, or multiple cysts with the appearance of "grape clusters" in the subtype affecting collaterals), cytological diagnosis (epithelial cells, with more or less atypia, floating in abundant mucin), and laboratory chemical analyses of cystic fluid (amylase, CEA > 110ng/mL, GNAS positivity). Another option could be the use of endoscopic retrograde cholangiopancreatography (ERCP), which enables tissue sampling, and where mucin extrusion from the dilated ampulla of Vater is pathognomonic of IPMN; however, this procedure has inherent risks, such as pancreatitis or, in the worst cases, perforation (21,25,32,33).

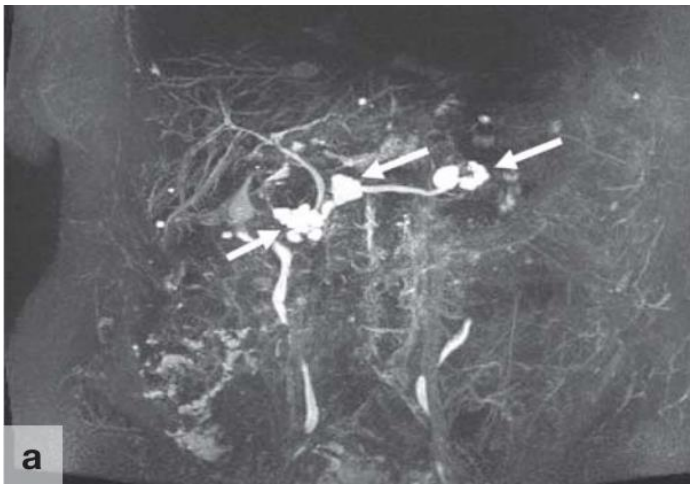

Figure 2. Magnetic resonance cholangiopancreatography of a multifocal MD-IPMN *(arrows) with lesions throughout the entire pancreas (32).*

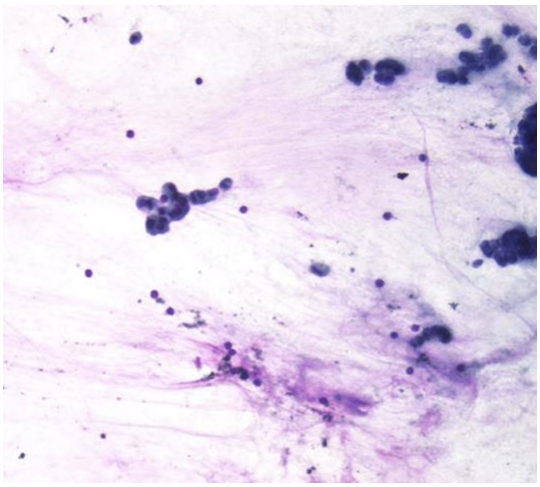

Figure 3. Cytomorphology of Intraductal Papillary Mucinous Neoplasm *consisting on clusters of mucinous epithelium in a background of abundant clean mucin (x200, Diff-Quik stain) (21).*

Some criteria for malignancy have been described to define the most appropriate management in each case, taking into account the risk of malignancy. These criteria are classified as worrisome

features (findings that require further investigation with EUS-FNA) and **high-risk stigmata** (findings that should lead to referral for surgical resection due to the risk of harbouring a carcinoma) (33).

- Worrisome features: **cyst size > 3cm**, **thickened** or **enhancing cyst walls**, **main pancreatic duct size 5 – 9 mm**, **enhancing mural nodule** ≤ **5 mm**, **CA 19.9 level > 37 U/mL**, and **abrupt tapering of the pancreatic duct**.
- High-risk stigmata: presence of **obstructive jaundice**, **enhancing mural nodule ≥ 5 mm**, and **main pancreatic duct ≥ 10 mm**.

If none of these features are observed, clinical management is based on the size of the cyst. However, due to the lack of sufficient long-term evidence, there are controversial points between management guidelines, so more research is still needed to understand the natural history of IPMN (25,33).

As a result of these discrepancies between the guidelines, treatment decisions should be individualized, taking into account the risks and benefits of surgical resection versus continued surveillance (33). If surgery is the procedure elected, it should be performed according to the location of the lesion: cephalic pancreatoduodenectomy is indicated for head or uncinate lesions, and distal pancreatectomy for neck, body, or tail lesions, both with regional lymphadenectomy. Enucleation or middle-segment pancreatectomy could be an option for a selected group of patients (33).

1.2 Intestinal microbiota

Nowadays, many sources define the human microbiome as a human organ from a physiological perspective (34,35). This point of view is gaining in importance due to the effects of its alteration in pathological conditions (34) and has recently become a popular topic of research. For this reason, it is important to understand the definition of the most commonly used terms in this context:

- **Microbiota** is defined as the community of living microorganisms (bacteria, archaea, eukaryotes and viruses) present in a specific environment (36,37).

- **Microbiome** refers to the entire habitat, including the microorganisms, their genomes, and the surrounding environmental conditions, including metabolites (signalling molecules, toxins, organic and inorganic molecules), structural elements (proteins, lipids, nucleic acids, polysaccharides), and molecules produced by coexisting hosts (36,37).

The human body is composed of more microbial cells (10 times greater) than human cells (38,39) forming diverse microecosystems (40). In particular, the intestinal tract harbours the largest amounts of microorganisms and the greatest number of species compared to other parts of the body (41), containing at least 1,500 different species of bacteria, 95% of which are microbial anaerobes (41,42). Although bacteria predominate, archaea, eukaryote and viruses are also represented (35), although less is known about their activities (41).

The composition of gut microbiota changes with age, from a sterile intestine in utero to maintaining phylum stability in adults (43). The colonization of the gastrointestinal tract by these microorganisms begins immediately after birth and has a variable structure influenced by the mode of delivery, level of hygiene, medication and the infant diet (35,44). This early colonization probably serves to attract other microbial partners, successively creating novel niches for other organisms (34). During the first postnatal years, bacterial diversity and functional capacity expand rapidly, tending to slow down in early childhood (1 – 5 years) and reaching **stability and maximum diversity in adulthood** (43).

The distribution of these microorganisms throughout the digestive tract is uneven due to the varying conditions in the different sections of the gastrointestinal tract (39). The stomach and duodenum are host to the lowest concentrations of bacteria (10^3 colony-forming units/mL), with concentrations increasing in the jejunum and ileum (10^4 – 10^8 CFU/mL) and reaching the **highest concentrations in the colon** (10^{14} CFU/mL) (39). Due to their abundance in the digestive tract, bacterial species account for up to 60% of the faecal dry mass (41).

To describe the composition of the microbiota, it is important to understand that microbes are taxonomically classified as other organisms by phylum, class, order, family, genus, and species.

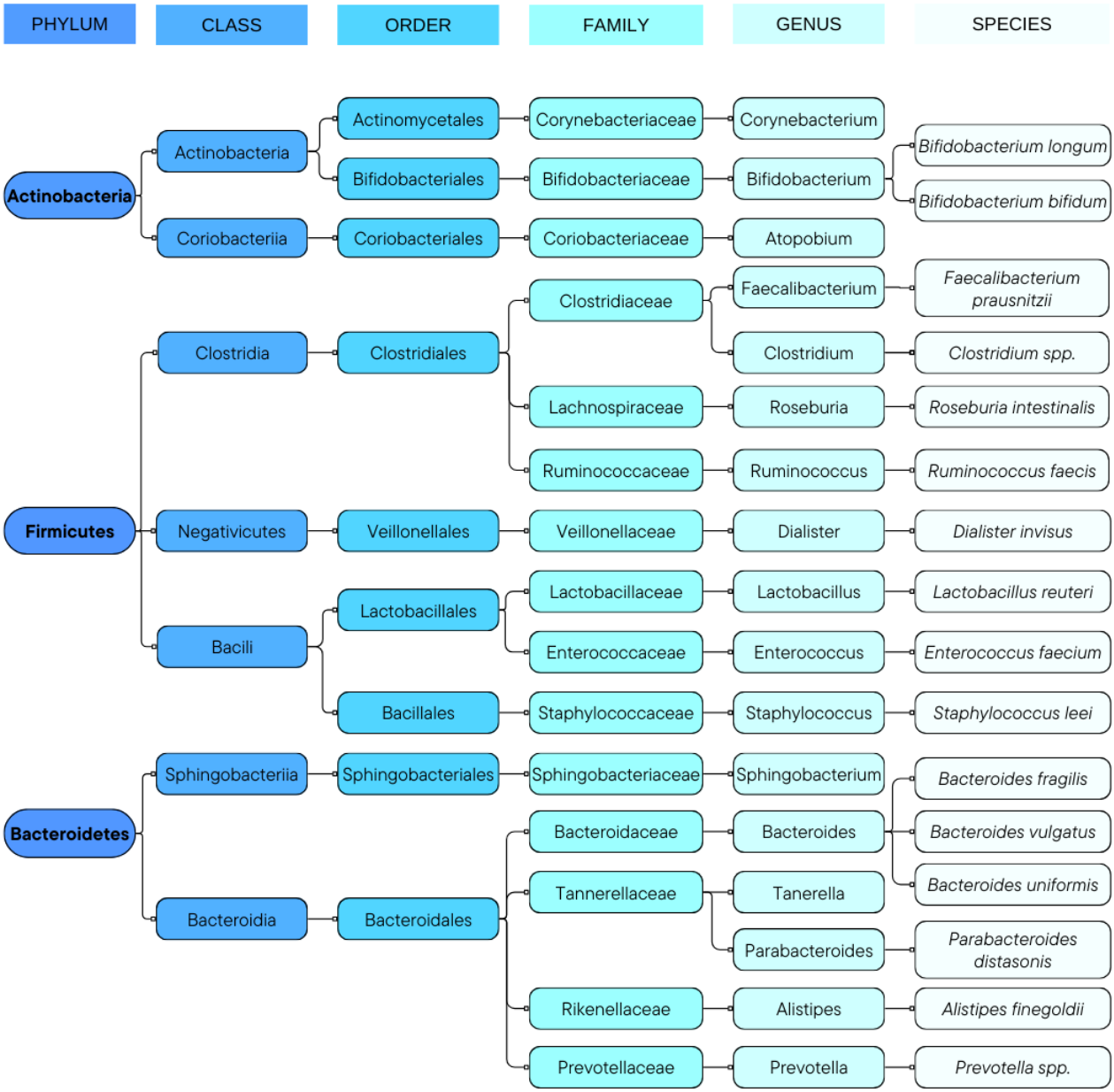

***Figure 4. Examples of taxonomic gut microbial community**; modified form "What is the Healthy Gut Microbiota Composition? A Changing Ecosystem across Age, Environment, Diet, and Diseases" (45).*

The composition of gut microbiota in a healthy population is dominated by the Firmicutes and Bacteroidetes phyla, followed by Actinobacteria and Verrucomicrobia. Proteobacteria represent only a small proportion of the gastrointestinal bacteria in healthy individuals; however, they account for higher proportions in patients with gastrointestinal diseases (40). The most common genera of bacteria found are the ***Bacteroides*** (30% of bacteria in gut), *Clostridium*, *Peptococcus*,

Bifidobacterium, *Eubacterium*, *Ruminococcus*, *Faecalibacterium* and *Peptostreptococcus* (41). The proportions of different genera vary significantly in a healthy population, describing three basic enterotypes: enterotype 1 contains a high proportion of *Bacteroides*, enterotype 2 has a high proportion of *Prevotella*, and enterotype 3 has a predominance of *Ruminococcus* (40).

Differences in microbiota's structure between individuals can be observed as a result of specific factors such as **diet** (long-term diets with a high intake of animal fat tend to produce enterotype 1, whereas carbohydrate-rich diets are associated with enterotype 2; also, a high-fat diet induces microbial dysbiosis and enhances low-grade inflammation) (38,46,47); **age** (40); **host genetics** (the composition of the microbial community is influenced by specific host genomic loci) (38); **delivery pattern** (43,48); **geographic location** (especially due to specific diets or extreme environments such as high altitude) (47), among others. However, the composition of an individual's microbiota can vary under certain circumstances, such as being under **antibiotic** treatment (affects some specific taxa and decreases bacterial diversity, requiring months for recovery) (38), **anti-acid treatment** and other drugs (43), **immunosuppression** (43), **smoking** (an increase in Firmicutes and Actinobacteria and a decrease in Bacteroidetes and Proteobacteria was observed in healthy individuals who quit smoking) (41), or **pathological conditions** (such as infections) (39). Despite these variations in an individual's flora, its compositional pattern usually remains constant, and this constant pattern is referred to as the "core microbiota" (37,48).

The gut microbiota plays an important role in the proper functioning of the host organism (41), performing its functions in four different aspects: metabolic, protective, structural and neurological (47).

- Metabolic: Gut microbes utilize dietary fiber (which is resistant to digestion by the host) through a series of enzymes that provide a source of energy and homeostasis (thanks to short-chain fatty acids, among others), break down undigested protein into peptides and amino acids (major metabolites that can have effects on the microbiota), transform a portion of the unabsorbed bile acids to increase its reabsorption, activate inactive polyphenols, synthesize certain vitamins (such as B12 and K), and metabolize dietary carcinogens (35,39,41,47).

- Protective: Mucus, antimicrobial peptides, secretory IgA, and metabolites produced by the microbiota constitute the intestinal immune barrier, whereby components of the microbiota (immunologically tolerated) occupy stable intestinal surfaces and prevent from the invasion of pathogenic microorganisms. In addition, microbiota metabolites such as short-chain fatty acids (SCFAs), aryl hydrocarbon receptor ligands, and polyamines have anti-inflammatory and chemo-preventive properties (39,41,47).
- Structural: SCFAs and active polyphenols restrict the infiltration of luminal content (by increasing the production of tight junction proteins), thus avoiding paracellular permeability, whereas the enterotoxins of some bacterial pathogens are able to weaken these junctions, thus increasing the permeability by cytokines (41,47). In addition, SCFAs stimulate epithelial cell proliferation and differentiation in the bowels (48).
- Neurological: Gut microbiota activity (production of neurotransmitters and metabolites, maintenance intestinal impermeability, modulation of enteric sensory afferents, and mucosal immune regulation) can control the enteric nervous system which is bidirectionally connected to the central nervous system by means of the gut-brain axis (47).

Although not so long ago the study of microbiota in some diseases was approached from a "one microbe – one disease" viewpoint, it is now beginning to be recognized that some diseases are the result of dysbiosis (38). Dysbiosis is defined as an imbalance of the relative abundance of phylum or genera that promotes disease in combination with genetics and environmental factors (49,50). Furthermore, in addition changes in relative abundance, it has been shown to coincide with loss of commensalism and diversity (51). Recently, many diseases have been linked to intestinal dysbiosis, such as inflammatory bowel disease (52), obesity (50), neurological and psychiatric disorders (53), colorectal cancer, and other oncologic diseases such as PC (14,41).

It has been observed that oncologic diseases are, in part, exacerbated by compounds produced by the microbiota that are harmful to DNA, as well as indirectly by the persistent pro-inflammatory microenvironment maintained by some bacteria (41).

Despite unanswered questions about PC and gut microbiota, recent studies have provided some insight into the relationship between gut microbiota and pancreatic cancer.

Firstly, it is important to understand the presence of the **gut-pancreas axis** as described by Zhang et al. 2022, and supported by experimental evidence that describes (54):

- The direct role of gut microbiota in shaping the pancreatic immune microenvironment, demonstrated, for example, by the regulatory effect that SCFAs have on the production of antimicrobial peptides (such as CRAMP) by insulin-secreting beta cells (54,55).
- The capacity of pancreatic acinar cells to shape intestinal microbiota mediated by the secretion of antimicrobials (such as Orai1) (54,56).

However, it is also essential to note that the **pancreas harbors its own microbiome** (in both, healthy and diseased individuals), which may be involved in PC tumorigenesis and progression, and which is closely linked to the gut microbiota. In fact, it has been shown that gut microbiota can migrate between the intestine and the pancreas, but the mechanisms remain elusive. However, translocation pathways such as the pancreatic duct reflux route, mesenteric venous drainage, and mesenteric lymphatic drainage, have been proposed (54).

The influence of gut microbiota in PC carcinogenesis was summarized by Li et al. 2020 on the following fourth pillars:

A. **Alterations of microbial metabolites**. Due to the changes in the gut microbiota there is a decrease in the thickness of the mucus layer, antimicrobial peptides and SCFAs, which consequently lead to chain reactions such as PPAR-γ inactivation (promotes the proliferation of Enterobacteriaceae due to the increased demand for available oxygen), and the increase in intestinal permeability of bacterial metabolites that reach the circulation and distant organs such as lipopolysaccharides (LPS, which interact with Toll-like receptor (TLR) pathways accelerating inflammation transmission and tumorigenesis), lipoteichoic acid (LTA, which promotes the over-secretion of proinflammatory factors), deoxycholic acid (DCA, which enhances the induction of PC), among others (57).

B. **Alterations of specific microbial species** of the gut microbiota influence the specific microbial taxa and diversity of the pancreatic tissue (as mentioned above, migration mechanisms still under investigation). The process of carcinogenesis has been correlated

with the bacterial composition of pancreatic tissue, rather than with bacterial abundance, as demonstrated in the study by Li et al. 2017 (where the microbes analysed were present in the pancreatic cyst fluid) or by Kartal et al. 2022 (in which it was found that PDAC harbors characteristic bacteria, consistent with the oral and gut microbiome communities) (2,57,58).

C. Microbiota-driven chronic inflammation, mainly due to the transport of bioactive molecules into the tumor microenvironment, has been confirmed. Specifically, it highlights the contribution of mutant KRAS (present in 90% of PDAC) influenced by inflammation (KRAS mutation requires the hyperstimulation by LPS-driven inflammation) to the acceleration of carcinogenesis (57). In addition, inflammation of the pancreatic tissue leads to hyperactivation of the pancreatic stellate cells (which mediate the necrosis and fibrosis processes) and cancer-associated fibroblasts, which perpetuate the disease and influence resistance to treatment by secreting inflammatory signals (IL-1a perpetuates KRAS activation, which in turn activates STAT3, thereby increasing IL-6 secretion, perpetuating disease development and progression) (59).

D. Microbiota-mediated immunoregulation. The structure of the microbiota is influenced by the immune system, and the gut microbiota continuously modulates the host immune system, contributing to the maturation and modulation of the homeostatic state when there is an imbalance. Immune system receptors recognize microbial molecules and trigger a chain of reactions that promote the transmission of inflammation and the development and progression of tumorigenesis (57):
    a. TLRs are activated in response to microbiota-derived products (LPS, LTA…) activating NF-κB and MAPK pathway, resulting in a proinflammatory state that promotes cancer development (57).
    b. NRLs recognize microbial signals and subsequently activate the release of interleukins that maintain the inflammatory state (57).

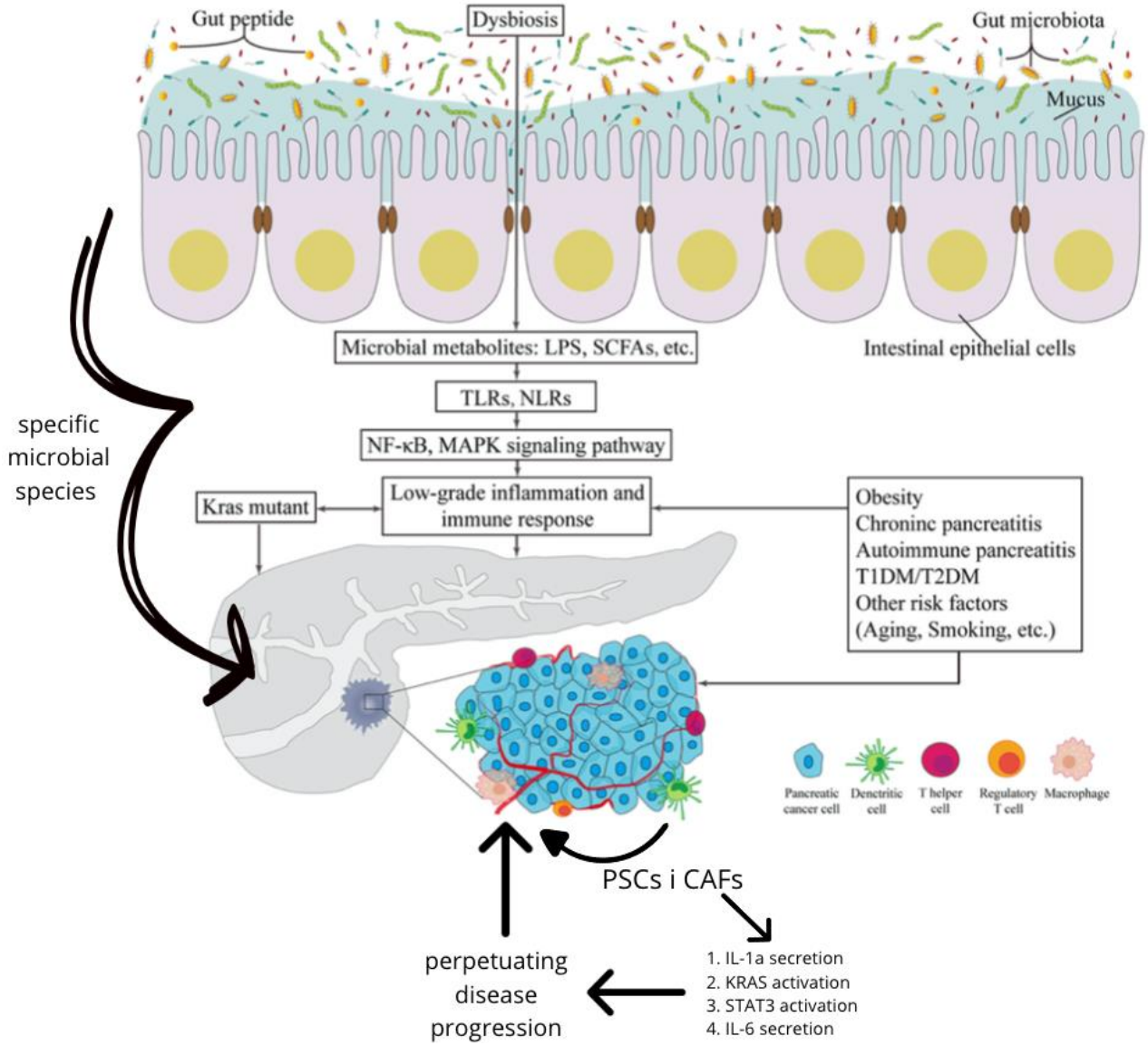

Figure 5. Influence of gut microbiota in the carcinogenesis of pancreatic cancer*, figure modified from "Gut microbiota: its potential roles in pancreatic cancer" (57). Dysbiosis contributes to changes associated with pancreatic cancer. Locally it triggers a decrease in intestinal mucus thickness and antimicrobial peptides. In addition, a decrease in the production of SCFAs allows for increased intestinal permeability leading to the passage of microbial metabolites (SCFAs, LPS…) to the circulation and distant organs. These metabolites activate TLRs and NLRs, initiating a cascade of inflammation and activating the immune response, thus favouring the process of tumorigenesis and disease progression. In addition, pancreatic cancer risk factors that interact in the process are also shown. LPS, lipopolysaccharides; SCFA, short-chain fatty acid; TLR, Toll-like receptor; NLR, Nod-like receptor; T1DM, type 1 diabetes mellitus; T2DM, type 2 diabetes mellitus; IL-1a and IL-6, Interleukins 1a and 6 respectively; PSC, Pancreatic stellate cells; CAF, cancer associated fibroblasts; NF-κB, Nuclear factor kappa-light-chain-enhancer of activated B cells; MAPK, Mitogen-activated protein kinases; KRAS, Kirsten rat sarcoma viral oncogene; STAT3, Signal transducer and activator of transcription 3.*

To arrive at this theoretical understanding that correlates gut microbiota with pancreatic cancer, several studies have highlighted the association between the two terms. In 2017, the results of a Chinese study were published, illustrating for the first time a gut microbial profile using MiSeq sequencing, which revealed a significant decrease in alpha diversity compared to matched controls, as well as an increase in some potential pathogens and LPS-producing bacteria and a decrease in some probiotics and butyrate-producing bacteria (60). Moreover, studies on preclinical models have demonstrated that gut microbiota can have an impact on pancreatic carcinogenesis from a distant site and not just within the pancreas, suggesting that this may be secondary to regulation of intratumoral cancer pathways or innate immune suppression by the microbiota (14). Zhou et al. showed that Firmicutes phylum is reduced in PDAC patients due to a reduction in butyrate-producing bacteria, which favors an inflammatory status and a dysregulated gut barrier that promotes pancreatic oncogenesis and tumor progression (61). Finally, a study was published in 2022 describing a faecal microbiome signature with high specificity for pancreatic cancer that, in combination to existing markers (CA19-9), could be useful as a screening tool and provide information on bacterial species that may be relevant for future research on disease prevention and therapeutic intervention (2).

With respect to IPMN, there are also studies that correlate it with the microbiota, especially the pancreatic microbiota, but also the oral microbiota. Halimi et al. conducted a study in which they cultured cyst fluid during surgical resection of pancreatic cystic neoplasms suspected of malignancy. They found that the cases positive for bacterial culture were mainly from pancreatic cysts histologically classified as IPMN, and that some of the bacteria were capable of inducing cell damage through DNA damage. This is of particular interest because IPMN is a precursor of pancreatic cancer (62). These results are in line with other studies such as the one by Gaiser RA et al., who found a significant increase in bacterial DNA and IL-1β concentrations in IPMNs, in contrast to other cystic pancreatic neoplasms. Furthermore, this specific detection correlates with increased levels of LPS in the cyst fluid, establishing that the microbiome harboring the pancreatic cyst plays a role in modulating the local tumor environment (63). Finally, some studies have compared the diversity of the oral microbiota between PDAC, IPMN and healthy controls without finding significant differences. However, there are conflicting results among studies on the relative

abundance of phyla and taxa, which have shown that intratumorally there are some known bacterial species inhabiting the human oral cavity, but with the impediment of determining a specific signature of individual species or multispecies models in saliva (2,63,64).

Knowledge of the microbiota has evolved greatly in recent decades, mainly due to the use of new molecular techniques independent of bacterial culture (which limited the study due to the difficulty in culturing anaerobic microorganisms). The main molecular methods correspond to 16S rRNA-based methods and shotgun metagenomics (42). The 16S rRNA Amplicon Sequence is a next-generation sequencing method for the determination of metataxonomy, which allows phylogenetic and taxonomic classification, as well as microbial relative abundance, by detecting the 16S rRNA gene which, due to its stability over time, size, distribution in all bacterial species, and ease of amplification, is suitable for bioinformatic analysis (42,65). Mass sequencing with this method requires a previous PCR amplification step, and the selection of primers that amplify a specific region of the gene. The most commonly used primers correspond to the V3-V4 region, which is considered the most universal for most bacterial species (65). This allows mass PCR amplification of the specific region of the 16S gene of many bacteria while allowing simultaneous sequencing analysis on a single sample (66). Each sequence is assigned a taxonomic group from public databases and the results are then analysed using bioinformatics tools (65). Unlike 16s rRNA sequencing, shotgun metagenomics amplifies total DNA, allowing the study of how alterations in microbial composition influence gene content and expression, characterizing not only bacteria but also other microorganisms such as viruses and their relationship with the microenvironment. It is a method that requires more data computation, making the process more complex and expensive (65).

Given the close relationship between gut microbiota and pancreatic cancer, it may be interesting to learn which gut microbiome-targeted therapies are available today, although most of them still need a lot of research.

- Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” (67). They modify the gut microbiota, so it is thought that they may have an impact on pancreatic carcinogenesis and on the effectiveness of its

treatment. The molecules resulting from probiotics may also play a role against pancreatic cancer (68).

- Prebiotics are nutrients that are degraded by the gut microbiota and confer health benefits, including phenols, polyunsaturated fatty acids, conjugated linoleic acids, etc. (54). Increased expression of genes associated with improved survival outcomes in PDAC models receiving prebiotics, as well as down-regulation of genes involved in carcinoma development and inflammation, among others, have been demonstrated in preclinical models (68).
- Symbiotics are products consisting of both probiotics and prebiotics (68). In studies of chronic pancreatitis, symbiotics have been shown to improve biochemical and hemogram parameters as well as intestinal barrier function. No significant studies have been conducted in PDAC (54).
- Postbiotics are "a preparation of inanimate microorganisms and/or their components that confer a health benefit on the host" (69), which may include bacterial metabolites such as SCFAs, vitamins, phenols, enzymes, among others. It is accepted that the beneficial effects of some postbiotics may lie in strengthening the integrity of the intestinal barrier, reducing inflammation, or eliminating some selective cytotoxicity against tumor cells (68).
- Faecal microbiota transplantation aims to restore a more favorable microbial composition by transplanting stool from a healthy donor into another patient's intestine. In one study, human faecal microbiota were transplanted into mice, and 5 weeks after the procedure, it was concluded that human microbiota were present in the gut and the pancreatic tumour of the mice, and there was a reduction in the size of the pancreatic cancer (54,68,70).

Many of the conclusions reached today indicate that there is still a long way to go in research to determine exactly the mechanisms by which the gut microbiota influences disease in the pancreas, as well as to understand the mechanisms of therapeutic interventions on the microbiota to improve their outcomes and applicability.

2 JUSTIFICATION

Despite its low incidence, PC is the seventh leading cause of cancer death globally, mainly because of its poor prognosis due to late diagnosis (symptoms do not appear until the most advanced stages) and limited therapeutic options, especially at these stages when the disease has progressed and where most PCs are diagnosed. Survival rates 5 years after diagnosis are among the lowest (< 10%) of any cancer (1–3).

Various medical interventions are being studied that could improve these rates, including the identification of various risk factors (mostly associated with pro-inflammatory states, including smoking, pancreatitis, obesity, etc., as well as individual genetic predisposition) (5). In addition, research is progressing towards the identification of a risk group (currently limited to genetic factors, without taking into account other risk factors) with the intention of being able to carry out screening and facilitate early diagnosis, and trying to find minimally invasive diagnostic tools (e.g., detection of circulating tumour DNA). On the other hand, the current trend also focuses on finding more effective therapeutic interventions (such as advances in surgical techniques, the use of preoperative treatments, or finding more effective systemic therapies).

However, the definition of the risk group is complicated by the presence of a high percentage of spontaneous PC, and universal screening is not appropriate due to the low incidence of this malignant neoplasia. In addition, early diagnostic tools, including some biological markers, are not as sensitive and specific enough (e.g., circulating tumour DNA is detectable in only 50% of patients with localized disease) (16,17).

In conclusion, current research on PC focuses on many points throughout the natural history of the disease (early diagnosis, diagnostic tools, therapeutics, etc.) and, as with any disease, it is also important to focus on its prevention, thus avoiding its development and consequences. For these reasons, this study proposal aims to determine the taxonomic profile of the intestinal microbiota using faecal samples (non-invasive) in patients with the most frequently diagnosed pancreatic cancer precursor lesion (IPMN) with the aim of identifying significant changes in microbial taxa and their relative abundance in order to generate new hypotheses aimed at finding new possible non-invasive tools for early detection of pancreatic disease, and to influence the prevention of

progression from IPMN to PC through treatment of the intestinal microbiota, given that the microbiota has been determined as a risk factor (a causal relationship between it and carcinogenesis has been demonstrated, mediated by the innate immune system and the dysregulation of the oncogenic pathways) and changes in the intestinal microbiota with treatment intention have been shown to influence the composition of the pancreatic microbiota in preclinical models (57,70).

3 HYPOTHESIS

- In individuals diagnosed with untreated IPMN (premalignant pancreatic lesion), we will find changes in the taxonomy and relative abundance of gut microbes compared to healthy controls.

4 OBJECTIVES

The main objective of this study is to determine which specific taxonomic changes in the gut microbiota, together with their relative abundances, are present in untreated IPMN patients compared to healthy controls.

The main objective will focus using a presence/absence study to determine which bacteria with a relative abundance of less than 5% in healthy controls are present with an abundance of > 15% in IPMN patients, using 16s rRNA sequencing analysis of stool.

Secondary objectives will be as follows:

- To determine the presence/absence of each taxonomic phylum, genus and species in the intestinal microbiota by 16s rRNA analysis of faecal samples in patients with treatment-naïve IPMN.
- To determine the presence/absence of each phylum, genus, and species in the IPMN group in contrast to healthy controls using 16s rRNA analysis of faeces.
- To compare the relative abundance of each species in the gut microbiota by 16s rRNA analysis of faeces between healthy controls and the IPMN group.
- To determine the diversity of the gut microbial community of untreated IPMN patients and to compare it with healthy controls.

5 MATERIALS AND METHODS

5.1 Study design

This study is designed as a multicenter, cross-sectional study, with the aim of comparing the gut microbiota by comparing faecal samples from untreated patients diagnosed with IPMN and from healthy controls using a microbial presence/absence study. It will be conducted at the Hospital Doctor Josep Trueta de Girona (with the collaboration of ICS patients and hospitals) over a period of three years and four months.

5.2 Population

The study population includes patients between 30 and 75 years of age with a recent anatomopathological diagnosis of IPMN by EUS-FNA at ICS hospitals. Recruitment will be performed over a year and a half, as 10 patients per year are diagnosed in Hospital Doctor Josep Trueta de Girona, and 83 patients with newly-diagnosed IPMN are needed. Accordingly, the following hospitals (ICS dependent hospitals) are asked to participate in the study (hospital catchment area in brackets):

- Hospital Universitari Doctor Josep Trueta de Girona (800,000)
- Hospital Universitari de Bellvitge (201,192)
- Hospital Universitari Germans Trias i Pujol (800,000)
- Hospital Universitari Vall d'Hebron (430,000)
- Hospital Universitari Arnau de Vilanova de Lleida (450,000)
- Hospital Universitari Joan XXIII de Tarragona (600,000)
- Hospital Verge de la Cinta de Tortosa (175,000)

5.3 Inclusion and exclusion criteria

INCLUSION CRITERIA:

- Untreated patients with EUS-FNA diagnosis of IPMN, aged 30 – 75 years
- Healthy controls cases matched for age, gender, and hospital

EXCLUSION CRITERIA:

- Patients with a history of chemotherapy or abdominal irradiation
- Presence of other active diseases, including hepatic disease, intestinal disease (such as inflammatory bowel disease), metabolic syndrome, diabetes, or immunosuppression
- Patients with a colectomy or other previous surgical interventions that modifies the intestinal anatomy
- Diagnosis of pancreatic adenocarcinoma or presence of other oncologic pathologies.
- Patients with a diagnose of chronic pancreatitis
- Antibiotic treatment in the last two months
- Probiotic treatment in the last two months
- Treatment with immunosuppressive therapy
- Pregnancy

5.4 Sample

5.4.1 Sampling method

A consecutive non-probabilistic sampling method will be used for those patients diagnosed with IPMN by magnetic resonance cholangiopancreatography with anatomopathological confirmation by EUS-FNA at one of the ICS centers participating in the study: Hospital Universitari Doctor Josep Trueta de Girona, Hospital Universitari de Bellvitge, Hospital Universitari Germans Trias i Pujol, Hospital Universitari Vall d'Hebron, Hospital Universitari Arnau de Vilanova de Lleida, Hospital Universitari Joan XXIII de Tarragona, Hospital Verge de la Cinta de Tortosa.

All patients diagnosed with IPMN who meet the inclusion criteria and none of the exclusion criteria will be informed about the study by means of the Participant Information Sheet (*annex I*) provided by the Digestive Unit of the corresponding hospital, which will explain what the study consists of, its objectives, its relevance, what implications it will have for the patient, how the confidentiality of the participant's personal data will be guaranteed, and will emphasize the voluntary nature of participation as well as the possibility of dropping out from the study if desired. After a few days (before receiving treatment for IPMN but without delay), the patient will be asked if they wish to participate and, if so, will be asked to sign the informed consent form (*annex II*).

The healthy control group will be recruited, matched for age (with an interval of ± 5 years), gender and hospital, after undergoing an abdominal CT scan for other reasons without suspicion of pancreatic lesions and meeting the inclusion criteria and none of the exclusion criteria. Participation in the study will be proposed to these individuals who will be informed about it by means of the Participant Information Sheet (*annex I*) and asked few days later if they wish to participate and, if so, to sign the informed consent form (*annex II*).

5.4.2 Sample size

The sample size was estimated using GRANMO software, specifically the setting for two independent proportions.

In a two-sided test, assuming an alpha risk of 5% and a statistical power of 80%, **83** subjects in the IPMN group and **332** subjects in the healthy control group are required to recognize a difference of greater than or equal to 10% as statistically significant, considering that the proportion in healthy control group was estimated to be 5%, and that this was as a cross-sectional study. As this is a cross-sectional study, where only one sample will be taken with no follow up of the patients, the drop-out rate is expected to be zero.

5.5 Variables, methods of measurement and data collection

All the data collected will be organized in a database managed by the main investigator. All data will be previously pseudo-anonymized beforehand by an external team.

5.5.1 Independent variable: IPMN cases group

The diagnosis of IPMN will be defined as the independent variable and expressed as a dichotomous nominal qualitative variable: IPMN is present or not.

The **IPMN group** (IPMN is present) will be constructed through the Digestive Units of ICS participating hospitals in Catalunya, inviting to the study those patients with an IPMN diagnosis based on information provided by the Radiology Unit (MRI/CT: dilatation of the main pancreatic duct or branch ducts, and surrounding parenchymal atrophy; MRCP: communication between an IPMN and the main pancreatic duct), the Digestive Endoscopy Unit (EUS-FNA: dilation of the

Wirsung duct, presence of wall nodules inside the duct, atrophy of the pancreatic parenchyma) and the Anatomopathological Unit of each hospital (cyst fluid analysis: epithelial cells with more or less atypia floating in abundant mucinous content, CEA > 110ng/mL, possible GNAS positivity).

The **healthy control group** (IPMN is not present) will be recruited from patients who have undergone an abdominal CT scan for other medical reasons and matched with the IPMN group based on age (with an interval of ± 5 years), gender and hospital.

5.5.2 Dependent variable: Outcome

The study of gut microbiota characterization through faecal samples will be established as the dependent or outcome variable and will be measured in both groups (IPMN cases group and healthy control group).

The outcome variable will be studied using multiple analyses with the aim of characterizing the gut microbiota at the metataxonomic level. Since the main objective focuses on determining which microorganisms (and their respective abundances) have a relative abundance growth rate of 10% in patients not treated for IPMN based on their respective presence in healthy controls with an abundance of < 5%, the dependent variable will be defined as a dichotomous nominal qualitative variable (bacterial taxonomy) and it will be calculated by its relative abundance (first as a proportion (%) and then as a quantitative continuous variable, expressed as a mean with its standard deviation or as a median with its quartiles if data is not distributed normally). These abundances will be extracted from the OTUs generated (by the QIIME program). Secondary analyses will be carried out to describe changes in the microbiota, where the outcome variable will be defined as a:

- Dichotomous nominal qualitative variable to determine the presence/absence of each taxonomic phylum, genera and species in the gut microbiota of patients with treatment-naïve IPMN, and to compare it with healthy controls.
- Quantitative continuous variable (no. of each bacteria as a mean with its standard deviation, or as a median with its quartiles if data is not distributed normally; finally expressed as a percentage) to determine each bacterial abundance, and to compare it between groups.

- Quantitative continuous variable (alpha diversity, calculated using the Shannon index) will be used to determine the diversity of the gut microbial community of untreated IPMN patients and to compare it with healthy controls. It will be calculated using the QIIME Pre-processing and Visualization program.

To obtain these data, mass sequencing of stool samples from participants will be performed using 16s rRNA gene sequencing. Study participants will be instructed to collect the faeces samples themselves, in stool sample containers, and to freeze them immediately for 4 days. At that time, they will take the samples to the gastroenterology department of the reference hospital associated with the study to which they belong, where the stool samples will be stored at -20ºC.

All samples will be sent to the Microbiology Service in the Parc Científic i Tecnològic of the Universitat de Girona, where the microbiological analysis will be performed by microbiology researchers following a standardized protocol based on the instructions of the manufacturer Illumina MiSeq System (71). Initially, all patient data will be pseudo-anonymized by an external team. Stool samples will be stored and frozen at -80ºC for at least 24 hours (to preserve DNA integrity) on a sterile swab in a test tube labelled with an anonymous barcode corresponding to each patient until the DNA extraction process begins. DNA extraction will be performed using extraction and dilution solutions (NucleoSpin® 96 DNA Stool (Macherey-Nagel)) and sample processing by vortex mixing and boiling water bath. Next, an amplicon PCR will be performed. For first PCR reactions a PCR plate (MicroAmp Fast Optical 96-Well Reaction Plate), Amplicon PCR Forward Primer and Amplicon PCR Reverse Primer (Ion 16S™ Metagenomics Kit; targeting the V3 and V4 regions of the 16s gene; the specific primers will be: forward primer 341F, 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGA CAGCCTACGGGNGGCWGCAG-3', and reverse primer 805R, 5'GTCTCGTGGGCTCGGAGATGTGTA TAAGAGACAGGACTACHVGGGTATCTAATCC-3), and KAPA HiFi HotStart Ready Mix (DNA polymerase) will be required. To continue, a gel electrophoresis step will confirm the size of PCR product. The next step will be to purify the 16S V3 and V4 amplicon away from free primers and primer dimer species using LGC Biosearch Technologies sbeadex™ SAB (sequencing application beads) (Fisher Scientific). After that, dual indices and Illumina sequencing adapters will be set using the Nextera XT Index Kit performing an index PCR. In

addition, a second clean-up will be performed using LGC Biosearch Technologies sbeadex™ SAB (sequencing application beads) (Fisher Scientific). Finally, library quantification (using the Qubit® assay Kit in the Fluorometer Qubit® 4, with Wi-Fi to measure DNA concentration), pooling and denaturation will be performed prior to MiSeq sequencing. The MiSeq® System (next-generation sequencing) will be used to perform the mass sequencing of the bacterial genome of the stool sample (performed in CRG, Barcelona). This system includes the MiSeq Reporter Software which will allow a preliminary analysis of 16s rRNA amplicon sequencing data, providing raw classification results for the sample, taxonomic classification of 16S rRNA targeted amplicon reads (using a version of the Greengenes taxonomic database), and visualization of taxonomic classification and relative abundance. In addition, the QIIME Pre-processing and Visualizations program will allow a more detailed analysis, including the generation of OTU clustering and diversity analysis.

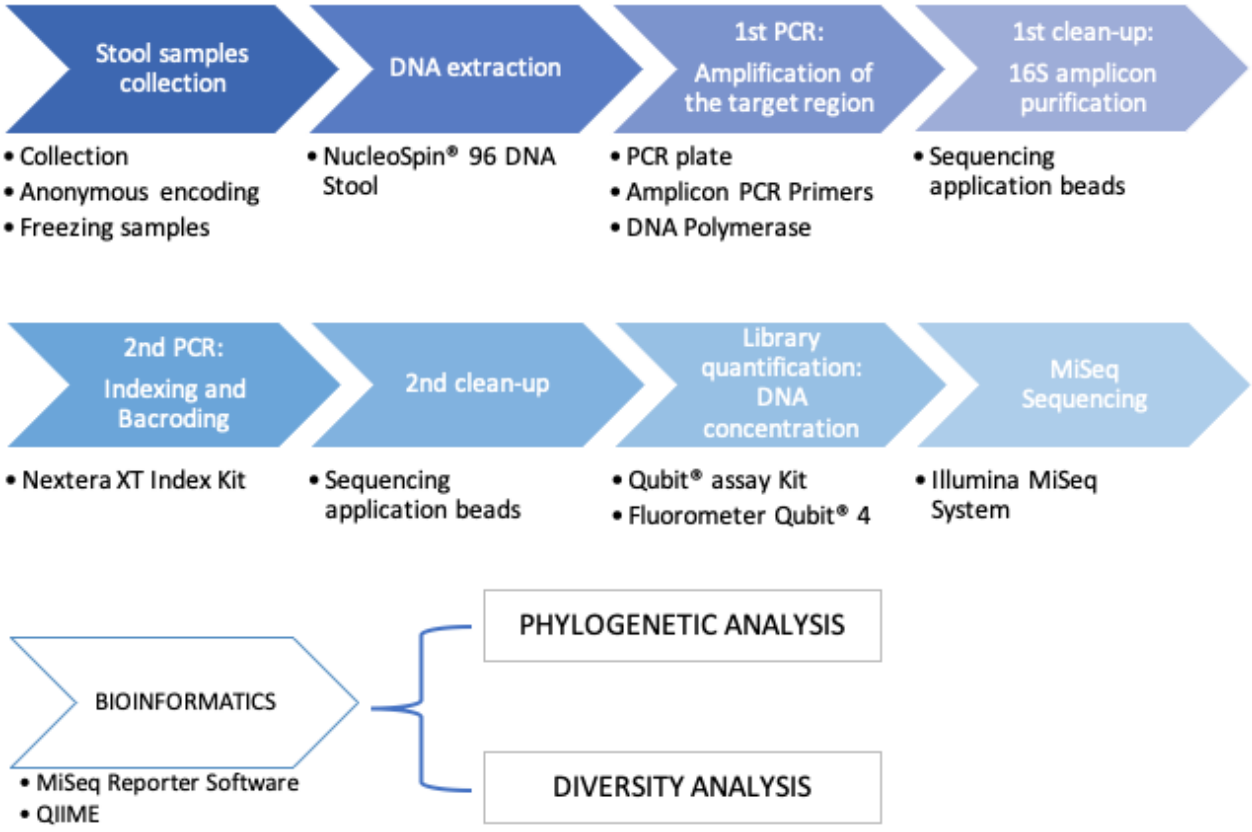

Figure 6. Data collection flow chart of the dependent variable (analysis of gut microbiota in faeces sample). *From samples collection to library preparation (DNA extraction, PCRs, cleans-up), MiSeq sequencing, and bioinformatics analysis. DNA, Deoxyribonucleic acid; PCR, Polymerase Chain Reaction; QIIME, Quantitative insights into microbial ecology.*

5.5.3 Covariates

Some variables will have to be considered due to their influence on the microbiota composition. Those covariates with significant differences will be analyzed with a multivariate analysis.

- Age. Quantitative continuous variable measured in years.
- Gender. Qualitative nominal variable: female or male.
- Body mass index. Quantitative continuous variable measured in kilos / metre^2.
- Diet: vegan, vegetarian, or omnivore. Qualitative polytomous nominal variable.
- Smoking: non-smoker, ex-smoker, active smoker. Qualitative polytomous nominal variable.
- Socioeconomic conditions represented by:
    - Education: years. Quantitative continuous variable.
    - Social class: qualitative polytomous ordinal variable. It will be categorized in five groups according to *"Sociedad Española de Epidemiología en base a la CNO-2011"* (72,73):
        - Class I: managerial staff in administration and in enterprises with more than 10 employees. Professions associated with second and third-cycle university degrees.
        - Class II: management staff in companies with fewer than 10 employees. Professions associated with first-cycle university degrees, artists and athletes.
        - Class III: administrative, security and safety services personnel, self-employed persons, and supervisors of manual workers.
        - Class IV: skilled and semi-skilled manual workers.
        - Class V: unskilled workers.

6 STATISTICAL ANALYSIS

The statistical analysis will be carried out by the statistical analyst. It will be done using the software "Statistical Package for Social Sciences (SPSS)" version 29.0.1. A statistically significant p-value of less than 0.05 is assumed and a 95% confidence interval is defined for all analyses.

6.1 Descriptive analysis

We will summarize the dependent qualitative variables (presence/absence of microbes in the IPMN group and in the healthy control group) using proportions and the dependent quantitative variables (bacterial abundance, and alpha diversity) using means and standard deviations, or medians and interquartile ranges (Q1 and Q3) if the data are not normally distributed.

We will stratify these descriptive analysis according to the groups defined by IPMN diagnosis (IPMN group) and healthy controls.

Additional stratification will be done with the covariables. Age will be categorized in quartiles as well as years of education. BMI will be categorized as underweight (< 18 kg/m^2), normal weight (≥ 18 kg/m^2 and < 25 kg/m^2), overweight (≥ 25 kg/m^2 and < 30 kg/m^2) and obese (≥ 30 kg/m^2).

6.2 Bivariate analysis

Differences in the proportions of the qualitative dependent variables between the IPMN and the healthy groups will be tested by chi-squared or Fisher's exact test if the number of expected cases in a cell is less than 5.

The difference of means (in case the distribution is normal) and medians (in case the distribution is not normal) of the quantitative dependent variables will be tested with the Student's t and the Mann-Whitney's U, respectively.

These tests will also be stratified by the covariables.

6.3 Multivariate analysis

To assess the difference in presence/absence by relative abundance and presence/absence of each microorganism detected between the IPMN and healthy groups we will use logistic regressions controlling for all the covariables.

In the case of the relative abundance of each species in the gut microbiota and the diversity of the gut microbial community, both quantitative variables, we will use linear regressions again adjusting for all the covariables. If non-normality were found in these dependent variables, an appropriate

transformation of the dependent variable (e.g., taking logarithms) will be performed before using the linear regressions.

7 ETHICAL AND LEGAL CONSIDERATIONS

This cross-sectional study will be conducted according to the ethical principles established in the "*Declaration of Helsinki of Ethical Principles for Medical Research Involving Human* Subjects" by the World Medical Association. For this reason, the research protocol will be presented and submitted for consideration and approval by the *Clinical Research Ethics Committee* (CEIC) of the Hospital Universitari Doctor Josep Trueta, and of the CEIC of the other participating hospitals, before the study begins, as well as at the end of the study, when final report will also be submitted to the CEICs. Throughout the study, all procedures will strictly follow the protocol approved by the CEIC. In addition, the benefits of the study outweigh the minimal risks to the patient, and at no time will the diagnosis and treatment times of the disease be altered.

In addition, all participants will be personally informed by the clinicians and will receive a Participant Information Sheet (*annex I*) that outlines the objectives of the study, all procedures, risks, efforts to protect personal information, and any other relevant information. Participants will asked to voluntarily sign the Informed Consent Form (*annex II*) after a few days (and after resolving any doubts, if necessary) before being included in the study, according to "*Ley 41/2002 básica reguladora de la autonomía del paciente y de derechos y obligaciones en materia de información y documentación clínica*". Furthermore, the patient will be informed that they can freely decide at any time not to participate in the study (even if they have previously signed the consent form), so that their data and samples will not be used for research.

In the event that patients agree to participate in the study, in order for biological samples to be used in the study, they must receive the Participant Information Sheet (*annex III*) and sign the specific Informed Consent Form (*annex IV*) of the corresponding Biobank (which can also be revoked at the patient's request), thus complying with "*Llei 14/2007, de 3 de juliol, de Investigació biomédica*" and "*Reial Decret RD 1716/2011 que aprova el reglament de desenvolupament de la Llei 14/2007 de Investigació biomédica*", establishing that the samples are provided to be used exclusively for this study, that the samples will be anonymized to guarantee the confidentiality of

the patient's data, that the patients will be informed of the general objectives of the project, as well as its benefits and possible drawbacks, and that the patient's will accept the possibility of being contacted in the event that more clinical data is needed in the future.

In addition, the confidentiality of personal and clinical data will be guaranteed at all times in accordance with "*Llei Orgànica 3/2018, de 5 de desembre, de Protecció de Dades Personals i garantia dels drets digitals*" and "*Reglamento (UE) nº 679/2016 del parlament europeu i del consell relatiu a la protecció de les persones físiques pel que fa al tractament de dades personals i a la lliure circulació d'aquestes"*, by anonymizing the data (by an external team), and limiting access solely to the data necessary for the study, and to the researchers carrying out the study. Patients will be allowed to modify or destroy any of their collected data, at any time.

As for the basic ethical principles (Belmont; Beauchamp and Childress), they summarize the ethics followed by this project and protected by law. In short, the principle of justice will be considered in the equitable distribution of the benefits (being general for the entire population), in the non-discrimination of participants, allowing equal access to participation to all subjects who meet the established clinical conditions. The patient's autonomy will be respected by ensuring the patient's voluntary and free participation through access to accurate information that allows the subject to make such a decision, as well as the protection of their personal data. As mentioned above, it will be processed by means of the Patient Information Sheet (*annex I*) and the Informed Consent Form (*annex II*). The principle of beneficence is the moral obligation to act for the benefit of others; this principle will be respected by focusing the study to allow the generation of new knowledge and/or new lines of research that will be of benefit to the global community. Finally, the principle of non-maleficence will be respected by not altering the timing of diagnosis and treatment of the patient's disease, by using a non-invasive technique of gut microbiota analysis, and by ensuring that the patient and their choices are not prejudiced at any time.

8 STUDY LIMITATIONS AND STRENGTHS

The main limitations of this study are:

- As a cross-sectional observational study, it is not possible to determine causal relationships between microbiota and IPMN. However, the aim of the project is not to establish causal

relationships but to describe the characteristics in terms of taxonomy, abundance and diversity of the microbiota in this group of patients.

- This study is based on assumptions made from studies conducted in patients with established PDAC, in preclinical models, or in patients with IPMN lesions but where the pancreatic microbiota was determined. However, there are currently no precedent studies linking gut microbiota to premalignant IPMN lesions. Therefore, the sample size has been determined on the basis of obtaining enough to answer the main objective; however, it could be limited to determine that the possible findings are statistically significant, since we do not know how many microorganisms that meet the conditions of the main objective will be compared and, therefore, the greater the number of microbes (greater number of comparisons), the greater the probability of committing a type I error. For this reason, this study is a hypothesis-generating study, so a future confirmatory study should be carried out with the sample adjusted according to each microorganism identified (meeting the main objective) to confirm that the findings in question have not been due to chance. This will also be the case for the secondary objectives established.
- As the sampling method is non-probabilistic, being a consecutive sampling, there could be a selection bias. Furthermore, the absence of randomization in the sampling process could introduce confounding bias through the presence of covariates that influence the dependent variable. In an effort to mitigate such concerns, a thorough examination of the inclusion and exclusion criteria for both groups has been conducted in order to preserve the representativeness of the sample and to control for confounding variables. Therefore, the presence of unmeasured confounding factors cannot be ruled out.
- In the case of a multi-center study, there could be possible interobserver variability that could lead to bias. To avoid this, a procedure manual will be developed to standardize the methods used for sample collection and determining IPMN diagnoses, as well as the training of professionals. In addition, the sequencing and analysis of the data will be centralized in the coordination center, located at the Hospital Doctor Josep Trueta in Girona. Taking these considerations into account will enhance the validity (accuracy) and reliability (precision) of the measurements conducted in the study.

- The tool used to study gut microbiota metagenomics, 16s rRNA gene sequencing, has its inherent limitations that could result in a misclassification bias (71,74). The mentioned limitations and its potential solutions for enhancing validity and precision are as follows:
    - Possible environmental contamination of DNA that may lead to false results. To avoid this, a standardized protocol (manual of procedures) will be established for sample collection, sample preservation, and laboratory processing.
    - Difficulty in identifying new microbes (as the reference sequence of those microorganisms is unknown), inability to assess the functionality of the microbial community and its metabolic activity (it exclusively determines the taxonomic composition of the microbial community), and limitation with regard to the identification of bacteria with very similar amplified sequence (limited taxonomic resolution). Since this study is an initial study of the microbiota in this specific population (diagnosed with IPMN), if significant changes are found, a new study should be carried out in the future using microbiota study techniques with greater metagenomic capabilities.
    - Specificity in the exclusion of bacteria does not allow the identification of other microorganisms such as viruses, fungi, prions, etc., or the microenvironment formed by metabolites, among others. In case of significant changes as a result of using this method, a metagenomic study with the corresponding techniques should be planned in the future.
    - PCR amplification bias may occur due to differences in amplification efficiency of certain fragments (more abundant sequences may amplify more than less abundant sequences, generating erroneous results) or due to cross-contamination between samples during the preparation and amplification process. To minimize the likelihood of these biases, quality controls (included in the procedure manual) and specific training for researchers will be performed. They will also be corrected by subsequent bioinformatic analysis.

9 WORK PLAN AND CHRONOGRAM

9.1 Work plan

9.1.1 Participating centres

The 7 centres proposed to carry out the study are as follows: Hospital Universitari Doctor Josep Trueta de Girona, Hospital Universitari de Bellvitge, Hospital Universitari Germans Trias i Pujol, Hospital Universitari Vall d'Hebron, Hospital Universitari Arnau de Vilanova de Lleida, Hospital Universitari Joan XXIII de Tarragona and Hospital Verge de la Cinta de Tortosa.

9.1.2 Research team members

The following professionals will be part of the research team:

- The **Main Investigator** (MI) is the professional responsible for leading the study, preparing the research protocol, procedure manuals and data collection sheets, as well as securing the participation of the various hospitals proposed and organizing the hospital coordinators.
- The **Hospital Coordinators** (HC) are the professionals in charge of overseeing that the procedures specific to each hospital are performed and complying with the protocols established by the study.
- **Clinicians** (Cls) are the health care professionals from each participating hospital who are responsible for participating in the study and informing the population in question about the Project. They will be the ones who recruit patients by means of their IPMN diagnosis as well as their healthy controls matched for age, gender, and hospital.
    - Gastroenterologists: they will be in charge of the diagnosis of IPMN based on the results of EUS-FNA and MRCP and will contact the patients corresponding to the group with a diagnosis of IPMN to enroll them in the study.
    - Radiologists: they will perform the MRCP diagnostic prior to the determination of the IPMN. They will also play a key role in the recruitment of healthy patients, who will be referred by this service after having undergone an abdominal CT scan without pathologies that would exclude them from the study.

  - Anatomopathologists: they will be essential for the diagnosis of IPMN (through the analysis of the material extracted by fine needle aspiration) in order to recruit the group in question.
- The **Microbiology Researchers** (MR) will be the professionals responsible for preparing the samples in libraries to be used to carry out mass sequencing using the MiSeq System.
- The **Bioinformatician** (BI) will responsible for the construction and subsequent analysis of the database created from the mass sequencing results. He/she will generate the OTUs and perform the subsequent diversity analysis using MiSeq Reporter software and QIIME. He/she will also prepare the data for subsequent statistical analysis by the statistician.

9.1.3 Stages of the study

The study will have a duration of three years and four months and will be divided into six stages with their corresponding activities:

STAGE 1: ELABORATION OF THE PROTOCOL AND STUDY DESIGN (duration of 3 months):

1. The main investigator will conduct bibliographic research, after which the preparation of the research protocol in CEIC format, as well as the procedure manual and data collection sheet (which will allow the unification of the procedures and help minimize biases) will commence.
2. The main investigator will also contact the 7 hospitals targeted to participate in the study, to encourage them.

STAGE 2: ETHICAL APPROVAL (duration of 4 months):

1. The research protocol, in the appropriate format, will be submitted to the CEIC of each participating hospital for ethical evaluation. Any suggestions will be considered and any modifications will be made accordingly.

STAGE 3: COORDINATION AND TRAINING OF THE MEMBERS OF THE TEAM (duration of 3 months):

1. Several meetings will be held with the research team. The study and its protocol will be presented, clinicians will be invited to participate, and the hospital coordinator will be identified.

2. The main investigator, together with the microbiology researchers, will organize the relevant laboratory materials.
3. A certain number of training sessions adapted to the specific tasks of the professionals will be held: on patient recruitment and data collection for the clinicians, and on how to work with the samples in the laboratory for the microbiology investigators. The aim is to standardize working methods by following the procedure manual and using the data collection sheets.
4. A pilot study of all the procedures to be carried out by each professional in the team will be carried out on the first patient recruited, by conducting a process analysis that will be shared with the other professionals involved, with the aim of defining each process in as much detail as possible and avoiding correctable errors.

STAGE 4: DATA COLLECTION (duration of 22 months):

1. Patients will be recruited in each hospital using a non-probabilistic consecutive method as described. They will have to meet the inclusion criteria and none of the exclusion criteria. It is essential that all participants sign informed consent forms (see *annex II* and *IV*) after carefully reading the Participant Information Sheets (see *annex I* and *III*).
2. Subsequently, the stool samples (as well as the other relevant clinical data specified in *Covariates* section) will be collected according to the procedure manual, before any treatments have been applied (and without delaying them). They will then be sent frozen by FedEx Custom Critical service to the Microbiology Service of the Parc Científic i Tecnològic of the Universitat de Girona.
3. Personal data corresponding to each sample will be pseudo-anonymized by an external team, and organized in a database by the main investigator.
4. Sample processing will be performed by microbiology researchers, who will prepare libraries for the next-generation sequencing MiSeq. Mass sequencing service will be outsourced to CRG Genomics Unit in Barcelona.

STAGE 5: DATA ANALYSIS AND FINAL EVALUATION (duration of 3 months)

1. The bioinformatician, in collaboration with the microbiology researchers, will create a database and generate the OTUs, and analyze diversity indexes using MiSeq Reporter software and QIIME. He/she will also prepare the data for the subsequent statistical analysis by the statistician.
2. Analysis, interpretation, and conclusion of the findings will be carried out by the statistician, microbiology researchers and the main investigator. The aim is to arrive at a conclusion that brings together the perspective of all disciplines.

STAGE 6: PUBLICATION OF RESULTS AND DISSEMINATION (duration of 7 months)

1. The main investigator will write the final article with the results and conclusions, which will be edited and supervised by English proofreaders. Finally, it will be published.
2. The written study will be presented at the Societat Catalana de Digestologia (SCD) congress and Asociación Española de Gastroenterología (AEG) congress.

9.2 Chronogram

Table 2. Chronogram. *MI, Main investigator; CEIC, Comitè Ètic d'Investigació Clínica ; Cls, Clinicians; MR, Microbiology researchers; HC, Hospital coordinators; Sts, Statistician; Pts, Patients; BI, Bioinformatician; CRG genomics, Centre for Genomic Regulation; M1, M2, M3, M4…, M12, refers to months.*

STAGE AND ACTIVITIES	STAFF	YEARS AND MONTHS																																					
		1st YEAR												2nd YEAR				3rd YEAR												4th YEAR			
		M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 1	M 4	M 8	M 12	M 1	M 2	M 3	M 4	M 5	M 6	M 7	M 8	M 9	M 10	M 11	M 12	M 1	M 2	M 3	M 4
STAGE 1: ELABORATION OF THE PROTOCOL AND STUDY DESIGN																																							
Bibliographic research	MI																																						
Protocol elaboration (in CEIC format)	MI																																						
Elaboration of the procedure manual	MI																																						
Elaboration of the data collection sheet	MI																																						
Participaiting hospital contact	MI																																						
STAGE 2: ETHICAL APPROVAL																																							
Ethical evaluation and approval	CEIC																																						
STAGE 3: COORDINATION AND TRAINING OF THE MEMBERS OF THE TEAM																																							
Meetings of research team	MI, Cls, HC																																						
Preparation of the laboratory workspace	MI, MR																																						
Training of clinician and researchers	Cls, MR																																						
Pilot study	Cls, MR, Sts																																						
STAGE 4: DATA COLLECTION																																							
Patient recruitment	Cls																																						
Stool sample collection	Pts, Cls, MR																																						
Coding of the data	External team																																						
Sample processing	MR																																						
Setting up of PCR	MR																																						
DNA extraction and amplification	MR																																						
Next-generation sequencing MiSeq	CRG genomic																																						
STAGE 5: DATA ANALYSIS AND FINAL EVALUATION																																							
Creation of database	MR, BI																																						
Statistical analysis	Sts, MR																																						
Analysis, interpretation, and conclusion of results	Sts, MR, MI																																						
STAGE 6: PUBLICATION OF RESULTS AND DISSEMINATION																																							
Scientific publications	MI																																						
Attendance at SCD and AEG congresses	MI																																						

10 BUDGET

***Table 3. Budget**. PCR, Polymerase Chain Reaction; DNA, Deoxyribonucleic acid; SCD, Societat Catalana de Digestologia; AEG, Asociación Española de Gastroenterología*

EXPENSES	UNIT COST	UNIT	TOTAL
Subcontracted services			**12,580 €**
Statistician	50 €/h	150 h	7,500 €
Sequencing service by CRG Genomics using MiSeq System		415 samples	3,300 €
Transport of frozen samples from hospitals to Parc Científic i Tecnològic of the Universitat de Girona (FedEx Custom Critical)	46€ per transport	From 6 different hospitals	280 €
Data pseudo-anonymization by external team	30 €/h	50 h	1,500 €
Material costs			**22,171 €**
Globe Scientific Faecal Collection Container	111 € / case of 500	415 samples	111 €
DNA extraction kit: NucleoSpin® 96 DNA Stool (Macherey-Nagel))	1,683 € / kit	415 samples, 2 kits	3,366 €
PCR Plate (MicroAmp Fast Optical 96-Well Reaction Plate (Roche))	57 € / plate	415 samples, 5 plates	285 €
Primers: Ion 16S™ Metagenomics Kit (Thermo Fisher Scientific)	1,208 € / kit (250 reactions)	415 samples	2,416 €
Polymerase: KAPA HiFi HotStart Ready Mix (Fisher Scientific)	166 € / kit (50 reactions)	415 samples, 9 kits	1,494 €

E-Gel™ 96 agarose gels with 1% agarose and SYBR™ Safe DNA gel stain (Thermo Fisher Scientific)	784 € / unit	415 samples, 6 units	4,704 €
Nextera® XT Index Kit (Illumina)	1,222 € / unit (384 samples)	415 samples, 2 units	2,444 €
Qubit® assay Kit	433 € / 500 reactions	415 samples	433 €
Fluorometer Qubit® 4, with Wi-Fi	3,655 € per device	1 device	3,655 €
LGC Biosearch Technologies sbeadex™ SAB (sequencing application beads), 100mL (Fisher Scientific)	1,626 € per unit	415 samples, 1 unit	1,626€
Standard laboratory supplies and reagents: pipettes, tips, storage tubes, gloves, etc.			1,500€
Printing costs	0.03 € / page	4565 copies	137 €
Meeting expenses			**2,100 €**
Hospital coordinator meetings (travel expenses, allowances, and meals costs)	100 € / attendee	3 meetings with 7 hospital coordinators	2,100 €
Insurance policy			**0 €**
Divulgation costs			**3,700 €**
Correction of the English version of the article	500 € / article	1 article	500 €
Open access article publication fees	2,000 € / article	1 article	2,000 €
Attendance at scientific meetings and congresses: SCD and AEG	600 € / meeting	2 meetings	1,200 €
TOTAL COST			40,551€

The members of the research team (the main investigator, hospital coordinators, clinicians, microbiology researchers, and the bioinformatician) will not incur any additional costs. They are employees of each participating hospital.

In addition, diagnostic tools (such as EUS-FNA, MRCP, and CT) have not been considered because the screening process occurs after obtaining this information which is routinely performed on patients who need it (and it is available at participating hospitals).

Since the MiSeq System for next-generation sequencing is not available in Girona, this process will be outsourced to the CRG in Barcelona, thereby avoiding the need to purchase the device.

Regarding insurance, in this study, it will not be contracted as no high-risk interventions are performed on the patient.

11 CLINICAL AND HEALTHCARE IMPACT

If the results obtained in this study support our hypothesis, changes in the taxonomy of the gut microbiota and their relative abundances in patients with IPMN compared to healthy controls will have been determined using non-invasive tools. This finding would open up new lines of research linking gut microbiota to one of the premalignant lesions of PC.

Based on these hypotheses, further confirmatory studies should be conducted and learning in this field should progress towards the determination of gut microbiota as a risk factor, the identification of possible specific pathogens responsible for cellular changes in the pancreas, the treatment of IPMN or the prevention of progression to PC through treatment of the microbiota, and/or the use of stool analysis to diagnose this type of pancreatic lesions in a non-invasive way and even as a screening tool. In fact, in future studies with a larger sample, it may even be possible to investigate, along the same lines, whether there are differences in the microbiota between those patients who develop PC from IPMN and those who do not progress.

As mentioned above, pancreatic cancer is a pathology with a very poor prognosis and progress is slow in this field of research. It is therefore particularly important to invest more resources in its study with the aim of achieving possible prevention, improving prognosis and quality of life, achieving higher cure rates and, above all, survival rates.

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13 ANNEXES

13.1 Annex I. Participant Information Sheet

FULL D'INFORMACIÓ AL/LA PARTICIPANT

Estudi: Anàlisis de la microbiota intestinal en pacients amb Neoplàsia Intraductal Papil·lar Mucinosa

Centre assistencial: .....

Investigador/a principal: .....

INFORMACIÓ GENERAL

Ens dirigim a vostè per transmetre-li la informació sobre un projecte liderat pel Servei de Digestiu de l'Hospital Doctor Josep Trueta de Girona, al qual se'l/la convida a participar.

L'estudi ha estat aprovat pel Comitè d'Ètica i Investigació Clínica (CEIC) dels hospitals participants en el projecte (Hospital Universitari Doctor Josep Trueta de Girona, Hospital Universitari de Bellvitge, Hospital Universitari Germans Trias i Pujol, Hospital Universitari Vall d'Hebron, Hospital Universitari Arnau de Vilanova de Lleida, Hospital Universitari Joan XXIII de Tarragona, Hospital Verge de la Cinta de Tortosa). L'aprovació s'ha realitzat d'acord amb la legislació vigent, i amb respecte als principis enunciats en la Declaració de Hèlsinki i a les guies de bona pràctica clínica.

La col·laboració que se li sol·licita al/la participant consisteix en la recollida d'una mostra fecal. Però abans de participar-hi, caldrà que llegeixi aquesta fulla amb atenció i resolgui els possibles dubtes que li sorgeixin al respecte de l'estudi. Posteriorment, caldrà el seu consentiment, de manera lliure i voluntària, aprovant conformitat amb les condicions que regeixen aquest estudi.

PARTICIPACIÓ VOLUNTÀRIA: Què passa si decideixo abandonar l'estudi?

La seva participació en l'estudi és voluntària, i pot decidir no participar o canviar la seva decisió i revocar el consentiment en qualsevol moment sense necessitat d'indicar-ne els motius per telèfon, carta o correu electrònic. No participar o deixar de participar no produirà cap perjudici en el seu tractament ni en la relació amb el seu metge/ssa.

DESCRIPCIÓ GENERAL DE L'ESTUDI

A. OBJECTIUS I FINALITATS DE L'ESTUDI?

El projecte pel qual se li sol·licita la participació es centra en l'estudi de la Neoplàsia Intraductal Papil·lar Mucinosa de pàncrees, una lesió quística del pàncrees que en ocasions pot precedir el càncer de pàncrees. Recentment, s'han determinat relacions entre diverses malalties del pàncrees (entre elles, el càncer) amb la microbiota intestinal, fet que obre línies d'investigació per determinar noves eines de diagnòstic centrades en la microbiota intestinal, tractaments de la microbiota que puguin tenir influència en la malaltia pancreàtica en qüestió associada, o determinar la microbiota intestinal com a potencial factor de risc permetent trobar noves estratègies de prevenció. És per això, que coneixent que la Neoplàsia Intraductal Papil·lar Mucinosa del pàncrees pot precedir en ocasions al càncer, volem determinar possibles canviïs en l'estructura de la microbiota intestinal en pacients amb aquesta lesió (en comparació amb persones que no hagin estat diagnosticades d'aquesta neoplàsia) que permetin generar noves hipòtesis sobre la relació d'aquests dos termes, i seguir així progressant en aquest camp.

B. QUANT TEMPS DURA L'ESTUDI?

L'estudi té prevista una durada total de 3 anys i 4 mesos.

C. QUINES CARACTERÍSTIQUES HAN DE REUNIR ELS PARTICIPANTS DE L'ESTUDI?

Per poder participar en aquest estudi cal complir amb un criteris d'inclusió, sense presentar cap dels criteris determinats com a excloents. Per a participar cal, o haver rebut el diagnòstic de Neoplàsia Intraductal Papil·lar Mucinosa de pàncrees sense haver rebut encara cap tipus de tractament, o haver estat sotmès a una tomografia computeritzada (TC) d'abdomen per altres qüestions mèdiques on no es determini lesió pancreàtica. A més, els pacients disposats a participar no poden complir amb cap dels següents criteris:

- Haver rebut quimioteràpia o radioteràpia abdominal
- Tenir un diagnòstic actualment de les següents malalties: malalties hepàtiques, malalties intestinals (ex: malaltia inflamatòria intestinal), síndrome metabòlica, diabetis, o trobar-se en estat d'immunosupressió.
- Haver rebut el diagnòstic d'adenocarcinoma de pàncrees o d'altres malalties oncològiques.
- Tenir diagnosticada una pancreatitis crònica

- Haver rebut tractament antibiòtic en els darrers sis mesos
- Haver rebut tractament probiòtic en els darrers dos mesos

D. EN QUÈ CONSISTEIX LA MEVA PARTICIPACIÓ EN L'ESTUDI?

El participant haurà de recollir una mostra de femta seguint les instruccions recomanades pel metge/ssa en el recipient que se li proporcionarà. Caldrà que congeli la mostra al seu domicili i que l'entregui a l'hospital de referència on se li ha proposat participar en l'estudi. La mostra s'etiquetarà amb un codi que s'assignarà anònimament a cada participant i serà traslladada al Parc Científic i Tecnològic de la Universitat de Girona on es durà a terme l'estudi.

A més, la mostra de femta recollida serà emmagatzemada en un establiment per a la seva conservació anomenat Biobanc (de l'hospital corresponent), pel que el participant haurà de signar un altre consentiment informat on confirmi que hi està d'acord.

E. DESTINACIÓ DE LA MOSTRA DESPRÉS DE LA SEVA UTILITZACIÓ EN AQUEST PROJECTE.

Una vegada hagi finalitzat aquest estudi de recerca, és possible que hagi quedat mostra de cada participant sense utilitzar. En relació a aquestes mostres, se li ofereixen les següents opcions que haurà de determinar en el full de consentiment:

a) Destrucció de la mostra
b) Utilització en futurs projectes relacionats amb aquesta línia de recerca
c) Permanència de l'excedent de la mostra al Biobanc de l'hospital corresponent

<u>BENEFICIS: Quins beneficis obtindré de la meva participació a l'estudi?</u>

És possible que vostè no rebi cap benefici directe amb la seva participació en aquest estudi. Però sí la satisfacció d'haver participat en una investigació que pot acabar millorant a la llarga procediments de prevenció, diagnòstic i tractament de la Neoplàsia Intraductal Papil·lar Mucinosa així com, de manera indirecta, en la prevenció del Càncer de Pàncrees.

Per altra banda, qualsevol troballa identificada en la seva mostra que pugui ser significativa per la seva salut, l'hi serà comunicada. Ha de saber que té dret a conèixer els resultats dels estudis obtinguts a partir de l'anàlisi de les seves mostres, així com també té dret a la no informació d'aquests resultats.

POSSIBLES RISCS: Quins riscs assumeixo si participo en l'estudi?

No es considera que s'assumeixi cap risc per a la pròpia salut en aquest estudi, ja que no es fa ús de cap tècnica invasiva, i el paper actiu del participant acaba amb l'entrega de la mostra.

PROTECCIÓ DE DADES PERSONALS I CONFIDENCIALITAT: Com s'assegurarà la confidencialitat de les meves dades?

Les dades recollides seran estrictament confidencials. Aquestes dades seran anonimitzades per un equip extern, evitant així el reconeixement del participant en tot moment. A més seran utilitzades exclusivament amb fins d'aquesta recerca (els excedents de la mostra podrien utilitzar-se en altres investigacions si així ho permet el participant indicant-ho al consentiment informat d'aquest estudi i al del Biobanc en qüestió). Per garantir la confidencialitat de les seves dades s'adoptaran les mesures establertes d'acord amb la "*Llei Orgànica 3/2018, de 5 de desembre, de Protecció de Dades Personals i garantia dels drets digitals"* i el "*Reglament (UE) nº 679/2016 del parlament europeu i del consell relatiu a la protecció de les persones físiques pel que fa al tractament de dades personals i a la lliure circulació d'aquestes"*.

DIFUSIÓ DELS RESULTATS: Què se'n farà dels resultats obtinguts de l'estudi?

Una vegada finalitzat l'estudi, s'elaboraran conclusions a partir dels resultats obtinguts. Aquests resultats i conclusions seran publicats a revistes científiques i presentats a congressos, tant si el resultat és positiu com si és negatiu. I sempre, la divulgació dels resultats es durà a terme respectant l'anonimat del/la participant.

COMPENSACIÓ ECONÒMICA: Tindré alguna compensació econòmica si participo a l'estudi?

La donació i utilització de mostres biològiques humanes es fa de manera voluntària i, per tant, no remunerada. Per tant, la seva participació en l'estudi no rebrà cap compensació econòmica.

DUBTES: Amb qui he de contactar davant de qualsevol dubte o problema que sorgeixi?

Davant de qualsevol dubte o pregunta sobre l'estudi, restem a la seva disposició. Pot posar-se en contacte amb l'equip investigador mitjançant el telèfon ..... o escrivint al correu electrònic .....

Moltes gràcies per la seva col·laboració.

13.2 Annex II. Informed Consent Form

CONSENTIMENT INFORMAT

Estudi: Anàlisis de la microbiota intestinal en pacients amb Neoplàsia Intraductal Papil·lar Mucinosa

Centre assistencial: .....

Investigador/a principal: .....

Jo, ... amb DNI ....., declaro que:

- He llegit la fulla informativa sobre l'estudi que se m'ha entregat.
- He pogut fer les preguntes que m'han sorgit i se m'han respost de forma satisfactòria.
- He rebut informació suficient sobre l'estudi.
- He estat informat/da per l'investigador ..... de les implicacions i finalitats de l'estudi.
- Entenc que la meva participació és voluntària.
- Entenc que les mostres obtingudes seran etiquetades amb un codi amb la finalitat de mantenir la confidencialitat de les meves dades i que, d'acord amb la Llei 14/2007 de Investigació Biomèdica, la mostra excedent de l'estudi serà utilitzada per futures investigacions relacionades amb aquest projecte o amb la línia de recerca, o serà destruïda, segons la meva voluntat.
- Entenc que puc revocar el meu consentiment de participació a l'estudi en qualsevol moment, sense haver de donar explicacions, i sense que això afecti la meva assistència sanitària.

Així doncs, lliurement dono la meva conformitat per participar en l'estudi amb mostres de:

☐ Femta

Autoritzo ser contactat/da en el cas de necessitar més informació o mostres biològiques addicionals:

☐ SÍ ☐ NO ☐ Contacte (telèfon/e-mail): .....

En cas que resulti excedent de la mostra, afirmo haver estat informat/da sobre les opcions de destinació en finalitzar el projecte de recerca. En aquest sentit:

A. Sol·licito la destrucció de la mostra excedent ☐

B. Permeto que les meves mostres siguin utilitzades en investigacions futures relacionades amb la meva malaltia

☐ SÍ ☐ NO

C. Permeto que les meves mostres siguin introduïdes en el Biobanc corresponent

☐ SÍ ☐ NO

Dono lliurement la meva conformitat per participar en l'estudi i dono el meu consentiment per l'accés i utilització de les meves dades en les condicions detallades en el full d'informació.

Firma del / la participant

Data: ___ / ___ / ___

Firma del / la investigador/a

Data: ___ / ___ / ___

13.3 Annex III. Biobank Participant Information Sheet

FULL D'INFORMACIÓ AL/LA PACIENT

A la majoria d'hospitals, a més de la tasca assistencial, també es realitza investigació biomèdica. Les mostres i dades clíniques obtingudes per al diagnòstic o control de les malalties, una vegada utilitzades amb aquesta finalitat, resulten útils i necessàries per a la investigació. Per aquest motiu, sol·licitem que llegeixi detingudament aquest document d'informació i el consentiment informat que si li adjunta al final per a la seva firma, si està d'acord en participar en aquesta proposta.

QUÈ ÉS UN BIOBANC? És una plataforma de suport a la recerca que treballa per obtenir, emmagatzemar, gestionar i distribuir mostres biològiques humanes amb la finalitat de fomentar la recerca biomèdica d'excel3lència. El nostre objectiu és contribuït a la millora del coneixement, prevenció, diagnòstic, pronòstic i/o tractament de les malalties. Les mostres i/o dades incloses en el Biobanc podran ser cedides per realitzar estudis d'investigació, sempre amb la prèvia avaluació d'un comitè científic i d'un comitè d'ètica. Tota l'activitat del Biobanc es realitza amb el compliment de la Llei 14/2007, de 3 de juliol, d'Investigació Biomèdica i el Reial Decret 1716/2011, de 18 de novembre, de regulació dels Biobancs.

MOSTRES BIOLÒGIQUES I INFORMACIÓ ASSOCIADA. Depenent de la situació, les mostres podran procedir d'excedents de proves i/o intervencions quirúrgiques que se li han realitzat o se li realitzaran al seu centre de salut o, aprofitant el procés assistencial pel qual ha acudit al centre de salut se li recollirà una mostra addicional. Aquestes mostres podran recollir-se en els diferents centres de salut que integra l'IDIBGI: Hospital Universitari de Girona Dr. Josep Trueta (Institut Català de la Salut), Institut d'Assistència Sanitària, Institut Català d'Oncologia, Institut de Diagnòstic per la Imatge i els centres d'Atenció Primària de l'Institut Català de la Salut de Girona.

En tractar-se d'una extracció de femta, l'obtenció es realitzarà per mètodes naturals no invasius, pel que no existeixen riscos associats.

Una vegada obtingut, el material biològic passarà a formar part del Biobanc fins que s'esgoti, sense que això comprometi el procés assistencial habitual. Tot i així, vostè podrà disposar de les seves mostres quan sigui necessari per motius de salut, sempre que encara estiguin disponibles. La identificació de les mostres biològiques i la informació associada serà codificada. Únicament el personal degudament autoritzat del Biobanc podrà accedir a les dades personals, a l'historial clínic i als resultats de les proves, quan sigui necessari. Per tal de completar la informació clínica relacionada amb la malaltia Neoplàsia Intraductal Papil·lar Mucinosa, és possible que els investigadors hagin de recopilar dades accedint al seu historial clínica amb la prèvia aprovació del projecte de recerca per part del Comitè d'Ètica d'Investigació Clínica.

PROTECCIÓ DE DADES I CONFIDENCIALITAT. En compliment del Reglament (UE) 2016/679, del Parlament Europeu i del Consell de 27 d'abril del 2016, reglament general de protecció de dades, i de la Llei Orgànica 3/2018, de 5 de desembre, de protecció de dades personals i garantia dels drets digitals, l'informem que el tractament de les seves dades passarà a formar part dels tractaments de l'IDIBGI, i se li aplicaran les mesures de control i seguretat aplicades a les categories especials de dades. L'IDIBGI, com a responsable del tractament, l'informa que podrà exercir els seus drets d'accés, rectificació, supressió, oposició, objecció i, si escau, portabilitat i limitació, dirigint-se al correu electrònic transparencia@idibgi.org o al Parc Hospitalari Martí i Julià, c/Dr. Castany, s/n (Salt). Pot consultar més informació sobre la protecció de dades a: www.idibgi.org.

DONACIÓ DE CARÀCTER ALTRUISTA. La donació que vostè realitza és gratuïta i altruista, pel que no obtindrà cap retribució econòmica. No es preveu que obtingui beneficis directes per la seva salut, ja que els resultats que s'obtindran seran amb finalitats d'investigació, però pot ajudar a obtenir informació que pot beneficiar a la societat en un futur.

PARTICIPACIÓ VOLUNTÀRIA. La seva participació és totalment voluntària. Si firma el consentiment informat confirmarà que desitja participar. Pot negar-s'hi o retirar-lo en qualsevol moment posterior a la firma, sense que això repercuteixi negativament en la seva assistència mèdica, present o futura. Podrà revocar el consentiment en qualsevol moment sense necessitat d'indicar-ne els motius i dirigint-se al Biobanc personalment, per telèfon, carta o correu electrònic.

INFORMACIÓ SOBRE ELS RESULTATS DE LA INVESTIGACIÓ I EL DESTÍ DE LES MOSTRES. En cas que ho demani, el Biobanc podrà proporcionar-li informació sobre quines són les investigacions en què s'han utilitzat les seves mostres. Si d'aquestes investigacions s'obtingués informació rellevant per la seva salut o la dels seus familiars, aquesta li serà comunicada si així ho estima oportú. En aquest cas, utilitzaríem les dades de contacte disponibles al seu historial clínic.

En cas d'eventual tancament del Biobanc, la informació sobre el destí de les seves mostres estarà a la seva disposició al Registre Nacional de Biobancs per Investigació Biomèdica.

Si us plau, consulti al Biobanc per qualsevol dubte que tingui ara o en el futur sobre aquest document. Mail: bioban@idibgi.org, telèfon: 872 98 70 87, o direcció (Parc Hospitalari Martí i Julià, c/Dr. Castany, s/n (Salt)).

13.4 Annex IV. Biobank Informed Consent Form

CONSENTIMENT INFORMAT

Consentiment informat per a l'obtenció I utilització de mostres biològiques i/o dades clíniques per a investigació biomèdica i la seva conservació en el Biobanc

Si ha comprès la informació que se li ha proporcionat en el document informatiu, resolt qualsevol dubte que pogués tenir i decideix col·laborar amb el Biobanc en els termes abans explicats, si us plau, llegeixi i firmi aquest full-.

Atorgo el meu **consentiment informat explícit** de forma lliure, específica i informada per obtenir i conservar el meu material biològic i la informació clínica associada al Biobanc IDIBGI. Aquest podrà emmagatzemar i utilitzar les mostres biològiques obtingudes, les dades clíniques i les proves d'imatge, amb la finalitat de desenvolupar projectes d'investigació biomèdica, sempre que aquests comptin amb l'aprovació d'un comitè d'ètica d'investigació competent.

Atorgo el meu consentiment explícit per:

- Utilitzar el material biològic excedent i/o dades associades per a investigació biomèdica:

  ☐ SÍ ☐ NO

- Obtenir i utilitzar material biològic addicional i/o dades associades per a investigació biomèdica:

  ☐ SÍ ☐ NO

- Utilitzar les mostres biològiques emmagatzemades prèviament a l'hospital:

  ☐ SÍ ☐ NO

- Rebre informació derivada de la investigació, si és rellevant per a la meva salut o la dels meus familiars:

  ☐ SÍ ☐ NO

- Ser contactat/da en el cas de necessitar més informació o mostres addicionals:

  ☐ SÍ ☐ NO

  En cas afirmatiu, telèfon o e-mail de contacte:

  .....

Desitjo incloure les següents restriccions sobre l'ús de les meves mostres i/o dades:

.....

.....

Firma del / la participant	Firma del / la professional
Nom i cognoms:	Nom i cognoms:
DNI:	DNI:
Data: ___ / ___ / ___	Data: ___ / ___ / ___