UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

CURRICULUM IN MEDICINA CLINICA E SPERIMENTALE

ENDOSCOPY-BASED INVESTIGATION OF NOVEL BIOMARKERS FOR PANCREATIC CANCER

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Tesi di DOTTORATO di RICERCA di Livia Archibugi

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Ciclo di dottorato XXXIV

SSD MED/12

Anno Accademico 2018/2019

CONSULTAZIONE TESI DI DOTTORATO DI RICERCA

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ENDOSCOPY-BASED INVESTIGATION OF NOVEL BIOMARKERS FOR PANCREATIC CANCER

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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both ‘I’ and ‘We’ interchangeably.

All the results presented here were obtained by myself, except for:

1) Microbiome NGS and bioinformatic analysis (Results, chapter 3.1, figure 1-15), were performed in collaboration with Dr. Loris Bertoldis and Eleonora Sattin, BMR Genomics, Padua, Italy.

2) NGS on RNA and bioinformatic analysis (Results, chapter 3.2.3, figure 21), was performed by Dr. Dejan Lazarevic, José Manuel Garcia Mantega and Marco Morelli, Center of Omics Sciences, San Raffaele Scientific Institute, Milan, Italy.

3) Some of these data (Results, chapter 3.2, Figure 16-20) were already published in the paper:
RNA Extraction from Endoscopic Ultrasound-Acquired Tissue of Pancreatic Cancer Is Feasible and Allows Investigation of Molecular Features. Archibugi L, et al. Cells. 2020 Nov 30;9(12):2561.

All sources of information are acknowledged by means of reference.

Acknowledgements

Prof. Claudio Sette, my Second Supervisor, and his collaborators Veronica Ruta and Valentina Panzeri for hosting me in Rome in their lab to teach me and let me perform PCR, and also for their fruitful collaboration throughout these 3 years

Miriam Redegalli and Sabrina Gloria Giulia Testoni, for their help with RNA extraction during the first year of PhD

Dejan Lazarevic, Fabio Simeoni, Giovanna Torri and all the staff from the COSR laboratories, for the hospitality to use their lab and support with reagents when performing RNA extraction and with the use of Bioanalyzer.

AIRC grant (IG 2015 17177 - PI Gabriele Capurso) for the support in buying the majority of the reagents.

Dedication

A dedication to my efforts, to my perseverance, resoluteness, commitment, gentleness despite anger, to my fortitude and idealism, to my Mr. Impostor (from the impostor syndrome all PhD students suffer from), to the things I have sacrificed, spending my free time and my nights and weekends working and studying, to fulfill the lack of knowledge on the fields we decided to walk on. A dedication to me, I deserve this.

A BIG dedication to my "unofficial" mentor for the past 10 years, in science and also in life, Gabriele Capurso, who, despite his working "in the shadow" and the absence of official acknowledgement he might get, has worked on the idea of the project and has helped me tremendously and continuously; also for trusting in me and my capabilities, even when I wouldn't. But also to all our fights, often fruitful (to me at least, but for sure also to him despite he won't admit it), on how to do things in this project, on which topic to choose, which patients enroll, which columns keep in the database, […], but above all a big thank for believing continuously in what I believe in, for believing in improvement, in doing things right, and for believing in all of us being able to turn into Übermensch and really take the big leap in finding the cure.

A dedication to my DoS, Prof. Arcidiacono, for all the teaching (quiet.. but continuous) and the guide he has given to me, for giving me the tools and really the possibility to work on the topic I liked the most, and for leaving me free to lead my project, trusting my judgement and my commitment, and yet supporting me anytime I needed a support.

A dedication to all my colleagues, doctors and nurses and OSS, to the friendship I've felt throughout these years, to the things you have taught me, to all our difficult times, to our love, to our fights and to our being true to ourselves and to each others, above all to Piera, Matteo and Giuseppe. To Mara, Alberto, Gemma, Laura, the residents and all the others..

A dedication to my family, to our quiet and shy love, despite distance, despite everything, strong and pure

To Gianmarco, for having followed me in Milan, for the support and patience throughout all these past 3 years, for the late hours, for understanding who I am and what I believe in, for the strength he has given me, for the vivid thinking and reasoning I needed in the darkest hours but also in the brightest, for the things that I've learnt, but especially for the love and warmth that I've felt, despite who I am and everything

Abstract

Introduction: Personalized medicine for pancreatic cancer (PDAC) has started seeing the light in the latest years, with guidelines acknowledging the importance of germline and somatic testing to help guide the treatment. Concerning transcriptome evaluations, two main molecular subtypes (classical and basal-like) have been identified on the basis of RNAsequencing performed on surgical samples. Nonetheless, patients amenable of surgical resection are less than 20%, while Endoscopic Ultrasound (EUS) is the gold-standard technique for tissue acquisition for the diagnosis of PDAC. Furthermore, studies trying to evaluate RNA extraction from EUS-acquired tissue are lacking and showed only scanty results.

The investigation of the microbiome is also increasingly recognized as relevant for PDAC growth. Microbiome signatures were first ascribed as factor associated with the occurrence of PDAC with periodontitis being associated with higher risk. The oral microbiome may reach the pancreas through the duodenum. Recently, microbiome changes in the duodenum have been associated with PDAC onset and with its response to chemotherapy in patients and animal models. Nonetheless, these studies have been performed mostly on PDAC obstructing the pancreatic and biliary duct, and the association between oral and duodenal microbiome has not been explored.

Aim: A) to optimize a methodology to obtain good quantity and quality RNA from EUS-acquired PDAC samples, in terms of best needle and sample conservation method. Furthermore, to evaluate whether RNA derived from EUS could be used for transcriptomics. B) to assess the oral and duodenal microbiome in a cohort of PDAC patients with lesions not obstructing the bile or pancreatic duct, as compared to matched healthy controls, and to investigate the association between microbiome and survival.

Materials and methods: For aim A) a set of PDAC patients undergoing EUS with tissue acquisition was enrolled, using 3 different needles (Menghini tip, Franseen tip or reverse-bevel tip) and 3 different conservation methods (snap frozen, RNALater or Trizol). RNA was extracted and quantity and integrity (RIN) analyzed and compared. Q-PCR was performed on adequate samples for evaluation of contamination and prognostic markers,

as also for splicing events. Clinical and survival data were collected. RNAsequencing was performed on a subset of patients with prognostic scores applied to define molecular subtypes. For aim B) patients with PDAC not obstructing the pancreatic/bile ducts were enrolled and matched to HC for gender and age after sample size calculation. Oral and duodenal (with brushing of the II portion duodenal wall) sampling for microbiome evaluation was performed during endoscopic procedure. Microbiome evaluation was carried out through Next Generation Sequencing and analyzed to evaluate alpha and beta-diversity. Furthermore, survival curves analyzing differences in alpha-diversity and bacteria abundance were performed to define microbiome signatures related to survival. All patients signed informed consents and studies were approved by local IRB.

Results: Concerning the RNA project, samples conserved in Trizol resulted in a significantly higher RNA concentration compared to other conservation methods. The different needle type did not affect neither quantity nor quality of RNA. Retrieved RNA was adequate for both qPCR for relevant contamination and prognostic biomarkers (GATA6 and ZEB1), as also for the evaluation of splicing variants. GATA6/ZEB1 ratio was correlated with survival of patients. RNAsequencing was feasible in a subset of 15 samples, with bioinformatics analysis retrieving one basal-like subtype, notably with a survival of only 3 months.

As for the microbiome project, 24 PDAC patients with no obstruction of the pancreatic/bile ducts were enrolled and compared with 24 HC. Alpha-diversity in terms of OTUs was borderline significantly lower in PDAC duodenal brushing compared to controls, as also compared to PDAC saliva samples. Furthermore, a significant difference in terms of beta-diversity Unweighted Unifrac was seen between brushing and saliva for HC, and for brushing and saliva for PDAC; also saliva between HC and PDAC was significantly different. Lastly, PDAC patients with lower alpha-diversity showed a significantly lower survival compared to those with a high alpha-diversity, with a specific microbiome signature discriminating the two groups.

Conclusions: EUS-acquired samples conserved in Trizol retrieve adequate quantity and quality of RNA, useful both for q-PCR and RNAsequencing able to evaluate biomarkers/signatures with a prognostic role. Duodenal microbiome of PDAC patients shows peculiar changes, with lower alpha-diversity and increased detection of peculiar

families linked to inflammation and cancer in other anatomical sites, as also linked to a reduced survival. These results support the use of endoscopy-based sampling to investigate biomarkers of PDAC diagnosis and clinical behavior.

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Acronyms and abbreviation

IVC: inferior vena cava

PDAC: Pancreatic Ductal AdenoCarcinoma

TNM: Tumor Nodes Metastases

R: resectable

BR: borderline resectable

LA: locally advanced

M: metastatic

CT: Computed Tomography

MRI: Magnetic Resonance Imaging

EUS: Endoscopic Ultrasound

PAXG: Gemcitabile, Nab-Paclitaxel, Capecitabine and Cisplatin

PARP: Poly (ADP-Ribose) Polymerase

OS: Overall Survival

RNA: RiboNucleic Acid

EUS-TA: Endoscopic Ultrasound Tissue Acquisition

FNA: Fine Needle Aspiration

FNB: Fine Needle Biopsy

DNA: DeoxyriboNucleic Acid

PDAC-NO: Pancreatic Ductal AdenoCarcinoma Not Obstructed

HC: Healthy Controls

OUT: Operational Taxonomic Units

ASV: Amplicon Sequence Variant

ANCOM: ANalysis of COmposition of Microbiomes

PLSDA: Partial Least Squares Discriminant Analysis

sPLSDA: Sparse Partial Least Squares Discriminant Analysis

ROC: Receiver Operating Characteristic

HR: Hazard Ratio

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1. Introduction

1.1 The human pancreas

The pancreas is an abdominal retroperitoneal organ, divided into the head, the body, and the tail. The function of this gland is both exocrine, with the production of digestive enzymes, and endocrine, with the production of hormones regulating glucose homeostasis and other relevant gastrointestinal hormones.(1)

The exocrine part of the pancreas accounts for the majority of the gland volume (around 85%), while the endocrine part represents less than 4% of the total pancreatic volume. The remaining 20% is essentially composed of mesenchyme (1). The islets of Langerhans, composing the endocrine part, are found scattered among the exocrine pancreas. These are composed of the beta cells, predominant, producing insulin, the alpha cells, producing glucagone, the F cells producing PP, the delta cells producing somatostatine and epsilon cells, producing ghrelin.(2)

The exocrine pancreas is composed only by two main types of cells, the acinar cells and the ductal cells.(1)

The head of the pancreas is vascularized by the superior and inferior pancreaticoduodenal arcades, respectively arising from the gastroduodenal artery (originating from the common hepatic artery and therefore the celiac trunk) and the superior mesenteric artery (1). The body and tail of the pancreas are instead vascularized by the splenic artery through multiple branches (1). Venous drainage of the pancreas follows the arterial pattern and drains into the portal system. (1).

Approximately 90% of the pancreatic blood supply is dedicated to the exocrine pancreas (1).

1.2 Pancreatic Ductal Adenocarcinoma

Among the solid tumors affecting the pancreatic gland, the most common one, originating from the ductal cells and representing about 90% of solid pancreatic tumors, is the so-called Pancreatic ductal adenocarcinoma (PDAC). This tumor has an incidence of about 12 cases out of 100.000 people and has a very aggressive behavior with a dismal

prognosis. Currently, it has a 5-year overall survival (OS) rate of less than 9% and is also predicted to become the 2nd leading cause of cancer-related mortality(3,4).

PDAC is staged with the TNM classification 8th edition (2) and can be also categorized as resectable (R), borderline resectable (BR), locally advanced (LA) and metastatic (M), based on the extension of disease (see Table 1). The main techniques to stage PDAC are CT scan, MRI and Endoscopic Ultrasound (EUS). EUS is currently the gold-standard technique to acquire tissue in order to define the histological diagnosis of the tumor.

Resectable	Borderline resectable	Locally advanced
No distant metastasis	No distant metastasis	No distant metastasis
No radiographic evidence of SMV or PV distortion	Venous involvement of the SMV or PV with distortion or narrowing of the vein or occlusion of the vein with suitable vessel proximal and distal, allowing for safe resection and replacement	Unreconstructible SMV/portal occlusion
Clear fat planes around celiac axis, hepatic artery and superior mesenteric artery	• Gastroduodenal artery encasement up to the hepatic artery with either short segment encasement or direct abutment of the hepatic artery without extension to the celiac axis • Tumor abutment of the superior mesenteric artery not to exceed 180° of the circumference of the vessel wall	• Any celiac abutment • Greater than 180° superior mesenteric artery encasement • Aortic or inferior vena cava invasion or encasement

Table 1. NCCN Criteria for pancreatic cancer resectability

The diagnosis of PDAC is unfortunately often delayed because of the non-specific symptoms and as first level imaging techniques such as abdominal ultrasound might not

be able to explore the pancreatic gland properly.(5,6) For this reason, about 70% of PDAC are already LA or M at diagnosis, and only 20-30% are amenable of curative surgical treatment. Furthermore, it is believed that PDAC is able to produce micro-metastatic sites even when the stage is defined as resectable.(5,6)

The management of PDAC consists mainly in surgery, chemotherapy or chemo-radiation but, also when surgery can be offered as the first upfront treatment, the 5-year OS of this cohort of patients is only around 25%. In this context, neoadjuvant treatment with newly developed chemotherapeutic regimens is slowly proving its efficacy, as also patients undergoing upfront surgery are not always amenable of undergoing a full adjuvant chemotherapy treatment. On the contrary, when chemotherapy is offered as neoadjuvant treatment, the rate of patients not undergoing surgical resection for the low performance status is low, but they do not undergo resection rather for the progression of disease under treatment.(5,6) This can in fact also allow a selection of patients that should not undergo surgery as probably already bearing a micrometastatic disease at diagnosis.(5,6)

Currently, main neoadjuvant treatment for PDAC consists in Folfirinox, Gemcitabine-Nab-Paclitaxel and PAXG (Gemcitabile, Nab-Paclitaxel, Capecitabine and Cisplatin).(5,6)

1.3 Microbiome and PDAC

The role of microbiome and its association with PDAC has been investigated widely especially for what concerns the oral microbiome. Periodontal disease has been, in fact, associated to the onset of PDAC. It has been shown how the main pathogenic bacteria present in periodontal disease and representing an increased risk of PDAC onset are Porphyromonas gingivalis (P. gingivalis), Neisseria elongate (N. elongata), Fusobacterium and Streptococcus mitis (S. mitis).(7)

It has been first hypothesized that this could represent an epiphenomenon, while more recently new hypotheses focus on the possibility of oral microbiome having a direct pro-tumorigenic action on the pancreatic gland, or an indirect one through induction of a pro-inflammatory state with immune system alteration.(7)

Furthermore, it was once believed that the pancreas was a germ-free organ, as not being in direct connection to the gut microbiota. Nonetheless, a few recent studies have proved that PDAC lesions can present bacteria, especially in terms of intracellular microbiome, both for tumoral cells as also in immune cells, and that this microbiome is more abundant than paired healthy pancreatic tissue. This microbiome, as for other solid tumor types, might be relevant also for encoding specific metabolic functions of tumors.(8,9)

Also, PDAC can show the same bacterial profile of the duodenum, supporting the idea that the duodenal microbiota could migrate in the pancreas through the pancreatic duct, via the orifice of the major papilla and possibly, before this, from the mouth to the duodenum.(7-12) Pushalkar et al. showed, in fact, that orally administrated fluorescently labeled bacteria in mice could reach the pancreas via the gastro-intestinal tract. (9) In humans, however, this route of dissemination has not yet been confirmed as also the occurrence of bacteria in the pancreas has not been shown to contribute to carcinogenesis. It cannot be excluded that in humans, modifications of the duodenal microbiome could be due to the alteration in exocrine pancreatic secretion and therefore be a result of the tumor rather than a cause. This is not the case for animal models, where a clearer cause-effect has been proved. In fact in germ-free mice, the lack of bacteria appears to act as a protective factor, leading to reduced pancreatic dysplasia and diminished intra-tumoral fibrosis in a spontaneous PDAC mouse model. (10) In addition, bacterial ablation in non-germ free mice also led to reduced tumor burden. (10) A recent study by Riquelme et al. (9) showed that tumor microbial diversity is associated with better outcomes in resected PDAC patients, and that microbiome can shape the immune response promoting T cell activation. Furthermore, the transplantation of feces from long term survivor patients to KPC mice showed a significant reduction of tumor growth compared to mice with transplantation from healthy controls or short term survivors.(9)

Microbiome is known to vary widely between individuals, and is known to be affected by age, gender and can undergo modification due to the type of diet, the use of certain drugs such as proton pump inhibitors (PPI), antibiotics and probiotics, history of smoking or alcohol use, comorbidities such as diabetes and gastric atrophy. (7) Also, the onset of pancreatic exocrine insufficiency due to chronic pancreatitis or PDAC, especially of the head with pancreatic duct obstruction, can lead to microbiome alteration. (7) In this view,

it is of note that the few studies conducted so far on the duodenal microbiome included patients with lesions of the head, therefore with likely modification of the microbiome because of the ducts obstruction rather than related to the disease itself.(12)

The world of the microbiome, therefore, represents a novel topic and road further to be explored in the context of PDAC, both for its probable role as a relevant risk factor but also for its possibility to co-operate in the therapeutic approach for PDAC patients.(7)

1.4 Transcriptomic subtypes for PDAC

Latest guidelines for the diagnosis and treatment of PDAC have started to acknowledge the relevance of identifying germline mutations, such as those affecting genes BRCA1 and 2, that would confer a better survival with the use of platinum-based therapy, as also with the use of the recently approved PARP-inhibitors (13).

Beside the aspect of germline mutation, also the identification of somatic molecular profiling has started to raise the attention in order to guide chemotherapy (5,6,13). There are, in fact, very encouraging data describing a significantly improved OS for patients affected by PDAC, receiving therapies matched to their tumor molecular profiling (14).

In the past ten years, beside germline or somatic mutations, there has been a growing interest in the evaluation of transcriptomic signatures, as these have shown, mostly in surgical cohorts of patients, a potential for the prediction of patient survival and chemotherapy response (15,16). The most recent classification of PDAC on transcriptomic subtypes relying on the evaluation of tumoral cells includes two main subtypes, the classical one and the basal-like (15-19). The methodology through which these subtypes have been identified is RNA sequencing which was performed on PDAC tissue derived mostly from surgical specimens. Nonetheless, only 20-30% of patients affected with PDAC are amenable of surgical resection at diagnosis (20), whereas the great majority presents with unresectable disease, needing to be addressed to chemotherapeutic treatments. It is, therefore, unclear whether these two subtypes are represented only in resectable disease or are present in the majority of PDAC.

One of the main issues in detecting the molecular subtype is the need to identify biomarkers that could possibly discriminate between these two. GATA6 is one of the main biomarkers that has raised interest and has been suggested in many studies as

correlated to the classical subtype of PDAC (21,22). Another possible, less investigated, marker described is ZEB1, a transcription factor regulator of epithelial-to-mesenchymal transition, that has been related to chemotherapy drug resistance (to gemcitabine specifically) and with poor prognosis (23-25). The inclusion of the PolyC exone in HAP17 splicing event was also reported recently as correlated to P53 missense hotspot mutations, discovered in 35-40% of PDAC patients and associated to an aggressive course of disease (26).

1.5 Endoscopic Ultrasound tissue acquisition

Endoscopic Ultrasound (EUS) is a technique that combines a gastroscope with partial side view with an ultrasound probe to explore the thorax and abdominal organs from within the gastrointestinal tube. This technique allows also tissue acquisition (EUS-TA), either with Fine Needle Aspiration (FNA) or Biopsy (FNB), representing the gold standard technique for the diagnosis of the majority of PDAC patients., having a rate of complication < to 1% (27).

EUS can therefore be the best method for the acquisition or tissue also aiming at the evaluation of somatic mutations/alterations. This has already been performed with DNA extracted from samples acquired through EUS-FNA and FNB, with very good results (28-30). On the other hand, the evaluation of RNA from EUS-acquired samples has been rarely performed with studies retrieving poor quantity and quality RNA (31-36).

2. Aim of the work

The aims of our project were:

1. To assess oral and duodenal microbiome in a cohort of PDAC patients with tumoral lesions not obstructing (PDAC-NO) the bile or pancreatic duct, and compare it to matched healthy controls (HC).
2. To evaluate whether, among the cohort of PDAC-NO patients, there could be any association of the microbiome to survival
3. to identify the best methodology, in terms of tissue handling and conservation and also choice of EUS needle, to obtain a good quantity and quality RNA from samples of PDAC patients acquired through EUS-TA.
4. to evaluate whether the RNA derived from EUS-TA could be used for transcriptomic in order to measure tissue contamination and investigate tumor classification and aggressiveness.

3. Results

3.1 Microbiome and PDAC

3.1.1 Comparison between PDAC-NO and HC

In total, 114 samples were collected from 33 PDAC and 24 healthy controls (HC). Among the 33 PDAC, 9 were excluded as found to be obstructing the ducts, with a total of 24 PDAC without pancreatic/bile ducts obstruction (not obstructed, NO).

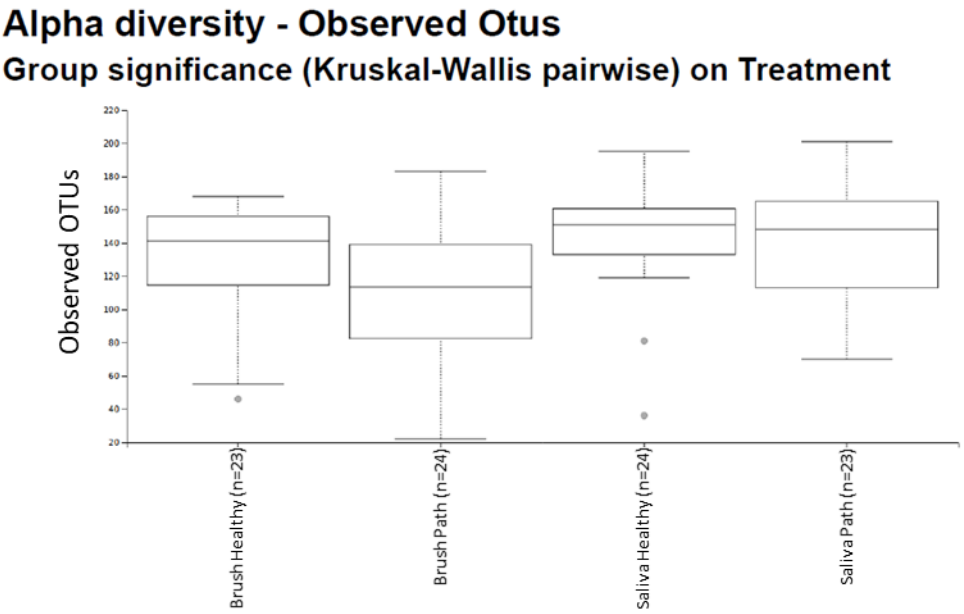
Demographic data and environmental factors associated to microbiome modifications of the two subgroups are represented in Table 2. As expected, there was a higher rate of diabetics and periodontitis among PDAC patients. On the other hand, factors that may influence microbial composition, such as use of proton pump inhibitors, antibiotics, probiotics and mouthwash were not differently distributed among PDAC-NO and HC.

	PDAC-NO (24)	**HC (24)**	**Univariable analysis OR (95% CI)**	**p-value**	**Multivariable analysis OR (95% CI)***	**p-value**
Age, mean (±st dev)	68,5 (± 9,9)	67,8 (± 15,5)	1 (0.96 – 1.05)	0,86		
Gender, male	11 (45,8%)	11 (45,8%)	1 (0.32 – 3.11)	1		
BMI, mean (±st dev)	24,6 (± 4,6)	25,1 (± 3,6)	0.97 (0.84 – 1.12)	0,64		
BMI≥25	8 (33,3%)	10 (41.6%)	0.65 (0.20 – 2.12)	0.47		
Ever smoker	14 (58,3%)	11 (55%)	1.15 (0.35 – 3-79)	0.82		
Active smoker	6 (26,1%)	2 (10%)	3.18 (0.56 – 17.9)	0,19		
Ever alcohol use	5 (20,8%)	10 (55,6%)	0.21 (0.05 – 0.81)	0,024	0.32 (0.06 – 1.65)	0.17
Diabetes	7 (29,2%)	0	126E+006	0,0094	75,2E+006	0.09
Metformin	5 (20,8%)	0	112E+006	0,0496		
Periodontitis	10 (45,5%)	3 (15%)	4.72 (1.07 – 20.89)	0,0407	2.14 (0.37 – 12.28)	0.39
Allergies	2 (8,3%)	4 (17,4%)	0.43 (0.60 – 2-42)	0,36		
Mouthwash	7 (31,8%)	8 (38,1%)	0.76 (0.21 – 2.66)	0,66		
Mouthwash in the morning	0	4 (19%)	/	0,11		
PPI in the last month	12 (54,5%)	15 (68,2%)	0.56 (0.16 – 1.91)	0,35		
Antibiotics in the last month	5 (20,8%)	2 (9,1%)	2.63 (0.45 – 15.23)	0,28		
Probiotics in the last month	8 (38,1%)	3 (15%)	3.49 (0.77 – 15.79)	0,10		

Table 2. Demographic data and environmental factors associated to microbiome modifications of the two subgroups, PDAC-NO and HC, with p-value of their comparison. *Age and sex adjusted.

Bacterial 16S DNA gene was extracted, amplified and sequenced, with rarefaction analysis excluding 2 samples (technical success 97.9%).

There was a significantly reduced Alpha-diversity as measured by Operational Taxonomic Units (OTUs), also called Observed Features, in PDAC-NO duodenal brushing compared to PDAC-NO saliva (p=0.007, q=0.02), as also to HC brushing, despite this significance was lost after correction (p=0.048, q=0.09) (see Figure 1).



		H	p-value	q-value
Group 1	Group 2			
brushHealthy (n=23)	brushPath (n=24)	3.878252	0.048915	0.097831
	salivaHealthy (n=24)	2.584923	0.107886	0.161828
	salivaPath (n=23)	1.044243	0.306837	0.368204
brushPath (n=24)	salivaHealthy (n=24)	11.648676	0.000642	0.003855
	salivaPath (n=23)	7.249492	0.007092	0.021276
salivaHealthy (n=24)	salivaPath (n=23)	0.199832	0.654856	0.654856

Figure 1. Alpha-diversity of the different subgroups, as measured by Operational Taxonomic Units (OTUs), and their comparison.

When comparing healthy and pathological samples in terms of pairwise difference, a significant difference between brushing and saliva was seen, with saliva always having a

higher ASV compared to duodenal brushing. Nonetheless, these differences were not different between healthy and pathological samples (see **Figure 2**).

Longitudinal pairwise difference

Pairwise difference tests

	W (wilcoxon signed-rank test)	P-value	FDR P-value
Group			
Healthy	32.0	0.002137	0.003172
Path	41.0	0.003172	0.003172

Pairwise group comparison tests

		Mann-Whitney U	P-value	FDR P-value
Group A	**Group B**			
Healthy	**Path**	308.0	0.344542	0.344542

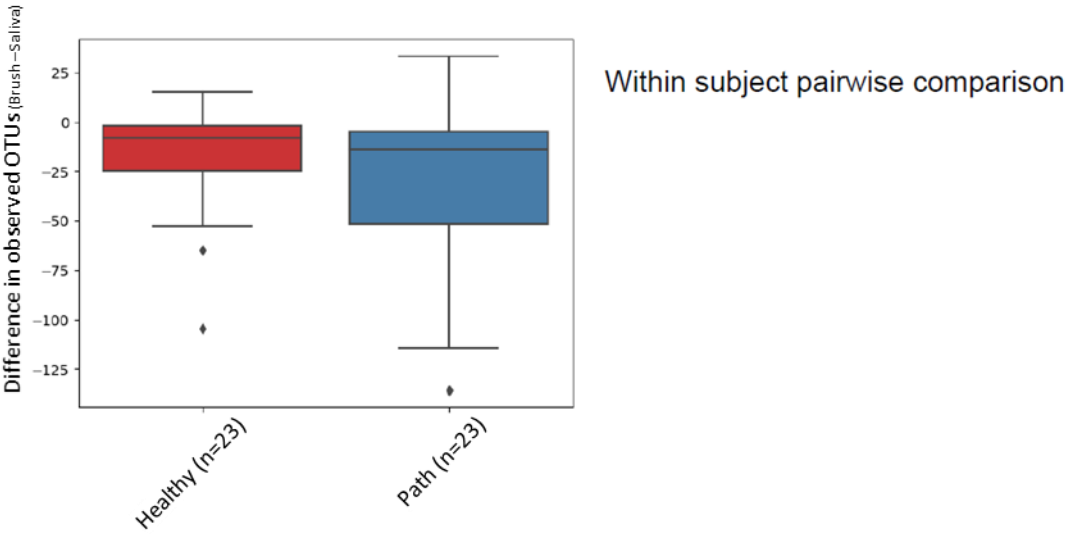
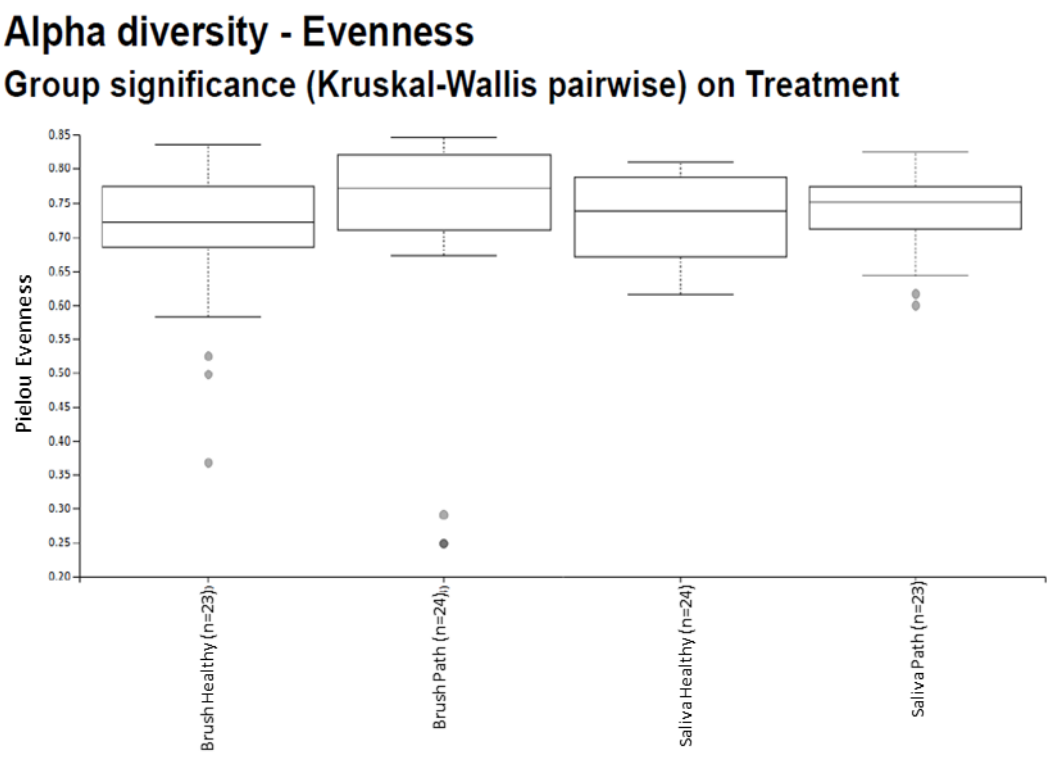


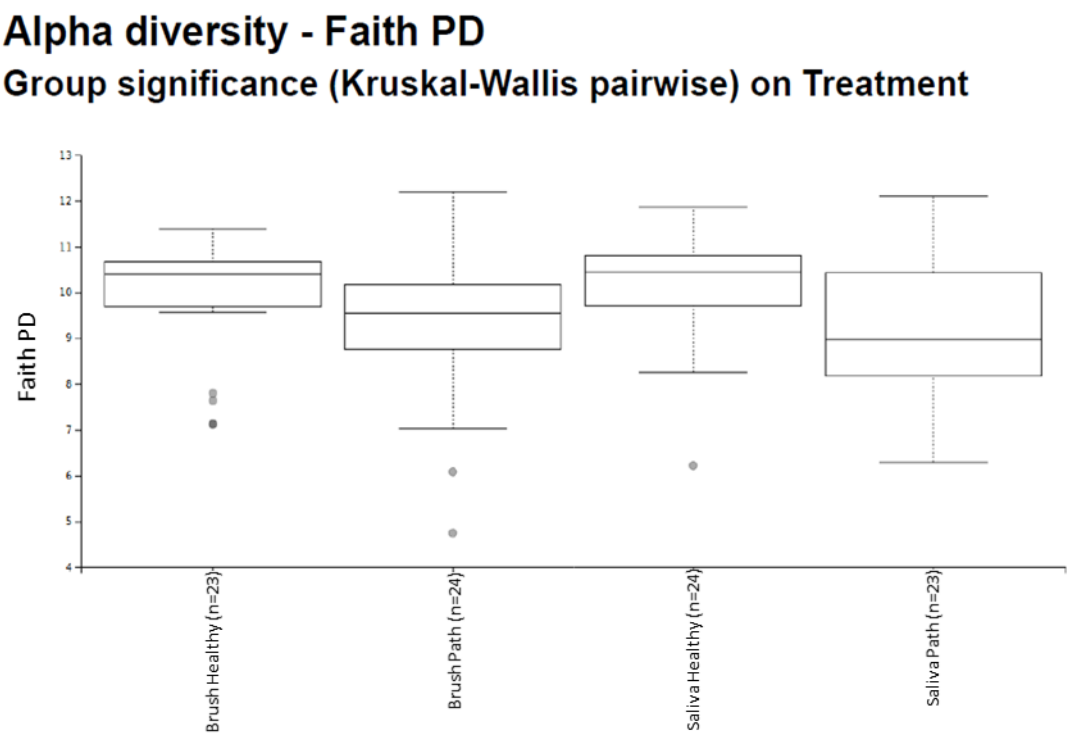
Figure 2. Longitudinal pairwise difference between HC and PDAC-NO samples

No significant difference was instead seen, in terms of alpha-diversity in terms of Evenness and Faith PD between the different groups of samples (see **Figure 3** and 4).



		H	p-value	q-value
Group 1	**Group 2**			
brushHealthy (n=23)	**brushPath (n=24)**	2.283062	0.130793	0.392380
	salivaHealthy (n=24)	0.239583	0.624507	0.749409
	salivaPath (n=23)	0.715400	0.397657	0.596485
brushPath (n=24)	**salivaHealthy (n=24)**	2.328231	0.127046	0.392380
	salivaPath (n=23)	1.087409	0.297046	0.594092
salivaHealthy (n=24)	**salivaPath (n=23)**	0.036685	0.848108	0.848108

Figure 3. Alpha-diversity of the different subgroups, as measured by Evenness, and their comparison.



		H	p-value	q-value
Group 1	**Group 2**			
brushHealthy (n=23)	**brushPath (n=24)**	4.993207	0.025447	0.076341
	salivaHealthy (n=24)	0.586957	0.443598	0.532318
	salivaPath (n=23)	2.199051	0.138096	0.207144
brushPath (n=24)	**salivaHealthy (n=24)**	6.122449	0.013348	0.076341
	salivaPath (n=23)	0.130888	0.717513	0.717513
salivaHealthy (n=24)	**salivaPath (n=23)**	3.120018	0.077336	0.154672

Figure 4. Alpha-diversity of the different subgroups, as measured by Faith PD, and their comparison.

In terms of Beta-diversity, there was a significant difference in terms of Unweighted Unifrac, between duodenal brushing and saliva both for HC (p=0.002, q=0.003), and for

for PDAC-NO (p=0.001, q=0.002). Also, saliva between HC and PDAC-NO was significantly different (p=0.02, q=0.02) (Figure 5).

Beta diversity - Unweighted Unifrac

Pairwise permanova results on Treatment

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	**Group 2**					
brushHealthy	**brushPath**	47	999	1.228691	0.225	0.2250
	salivaHealthy	47	999	4.402322	0.002	0.0030
	salivaPath	46	999	9.563886	0.001	0.0020
brushPath	**salivaHealthy**	48	999	6.936229	0.001	0.0020
	salivaPath	47	999	11.277544	0.001	0.0020
salivaHealthy	**salivaPath**	47	999	2.685832	0.022	0.0264

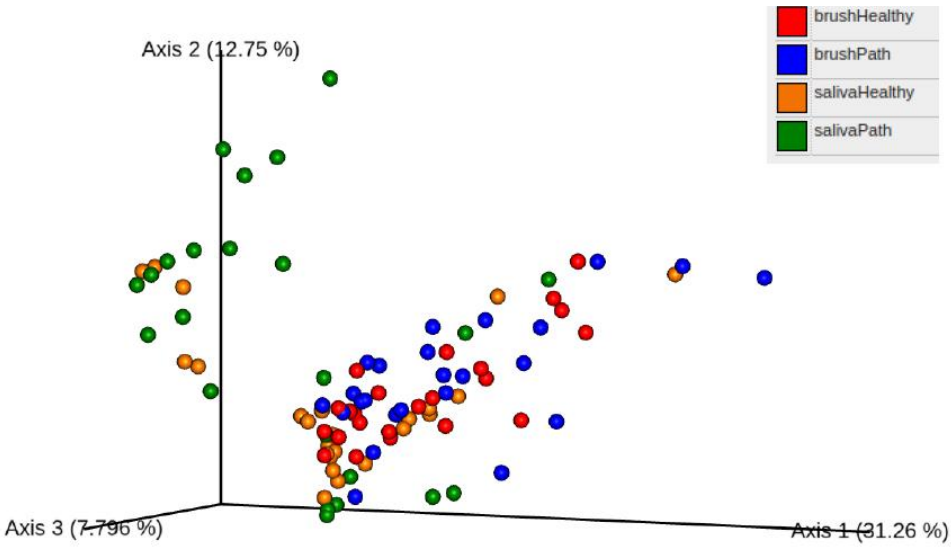


Figure 5. Beta-diversity of the different subgroups, as measured by Unweighted Unifrac, and their comparison.

Pairwise differences did not retrieve any significant difference (data not shown).

In terms of Weighted Unifrac, a significant difference was seen in terms of saliva and duodenal brushing both in HC (p=0.005; q=0.01), and in PDAC-NO (p=0.001; q=0.003)(see **Figure 6**).

Beta diversity - Weighted Unifrac

Pairwise permanova results on Treatment

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	**Group 2**					
brushHealthy	**brushPath**	47	999	1.261315	0.278	0.3336
	salivaHealthy	47	999	4.108042	0.005	0.0100
	salivaPath	46	999	4.970595	0.007	0.0105
brushPath	**salivaHealthy**	48	999	7.100668	0.001	0.0030
	salivaPath	47	999	7.549495	0.001	0.0030
salivaHealthy	**salivaPath**	47	999	0.953497	0.395	0.3950

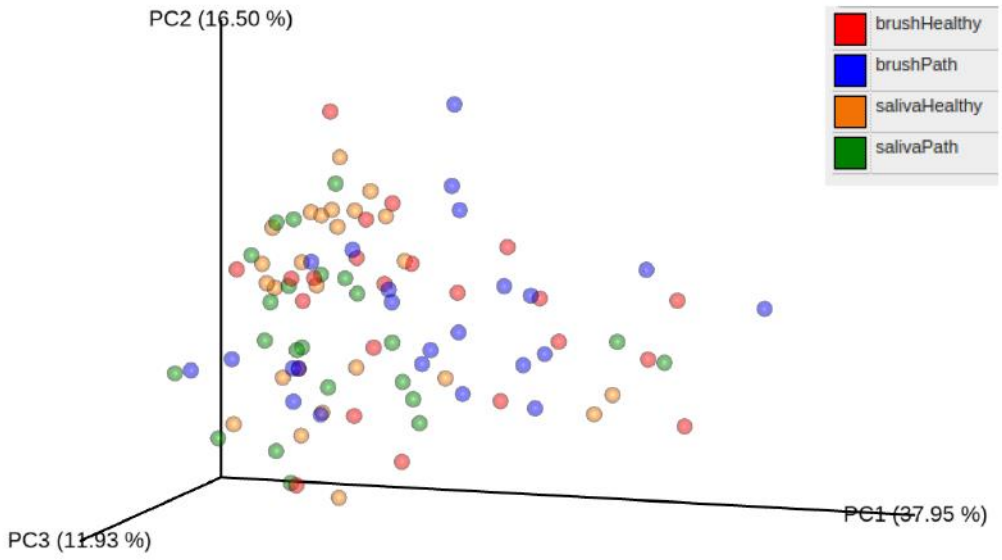


Figure 6. Beta-diversity of the different subgroups, as measured by Weighted Unifrac, and their comparison.

Beta-diversity evaluated with Bray Curtis retrieved a significant difference between brushing and saliva of HC (p=0.004, q=0.006), and of PDAC-NO (p=0.001; q=0.002) (see **Figure 7**).

Beta diversity - Bray Curtis

Pairwise permanova results on Treatment

		Sample size	Permutations	pseudo-F	p-value	q-value
Group 1	**Group 2**					
brushHealthy	**brushPath**	47	999	1.386286	0.059	0.0708
	salivaHealthy	47	999	2.233279	0.004	0.0060
	salivaPath	46	999	2.842599	0.001	0.0020
brushPath	**salivaHealthy**	48	999	4.285316	0.001	0.0020
	salivaPath	47	999	3.464866	0.001	0.0020
salivaHealthy	**salivaPath**	47	999	1.063105	0.335	0.3350

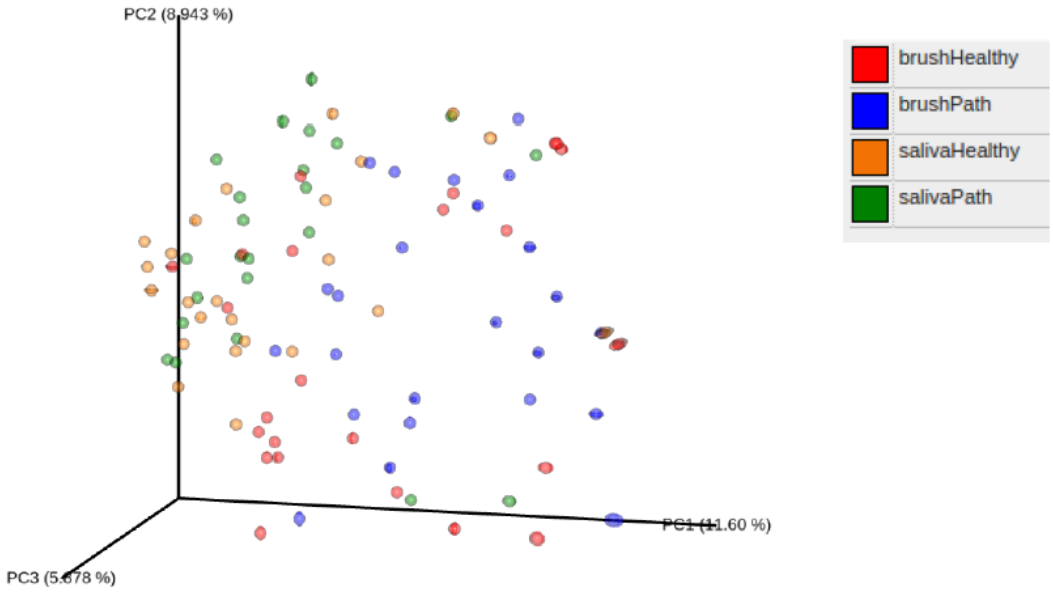


Figure 7. Beta-diversity of the different subgroups, as measured by Bray Curtis, and their comparison.

Concerning differential abundances, both ANCOM and MixOmics were employed. Principal Component 1 alone explained 20% of differences. Overall differences are shown in **Figure 8**.

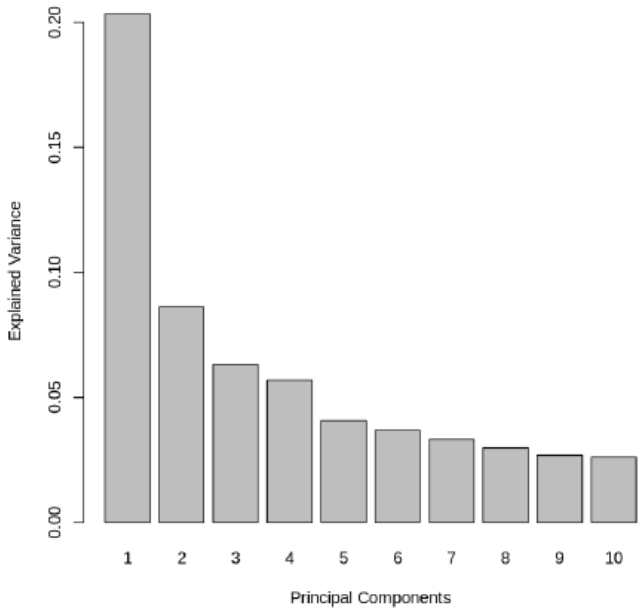
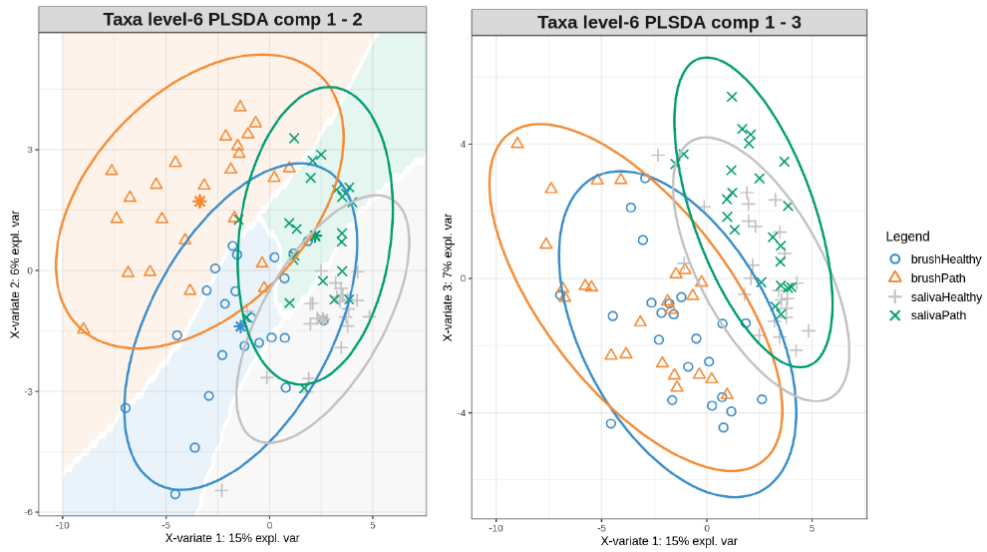


Figure 8. Principal Components for Beta-diversity and their relative explained variance, with PC1 explaining more than 20% of variance.

Partial Least Squares Discriminant Analysis (PLSDA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) where then applied for the four different subgroups, as shown in Figure 9. As shown in the figure, they mostly overlap based on the site of the sample rather than on the type of patient (whether PDAC-NO or HC).

PLSDA



sPLSDA

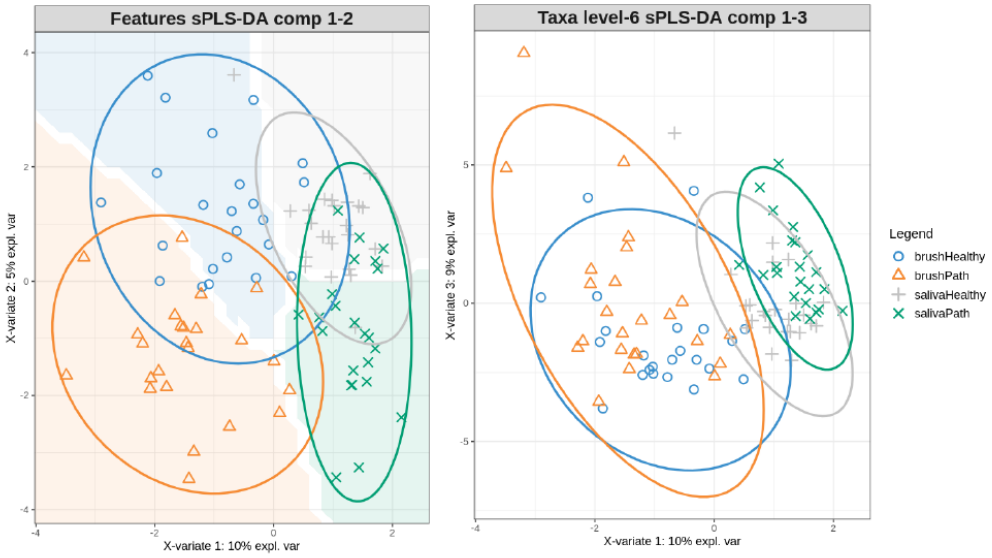


Figure 9. Partial Least Squares Discriminant Analysis (PLSDA) and Sparse Partial Least Squares Discriminant Analysis (sPLSDA) for the four different subgroups and their relative overlap determined more on the site of the sample than on the type of patient.

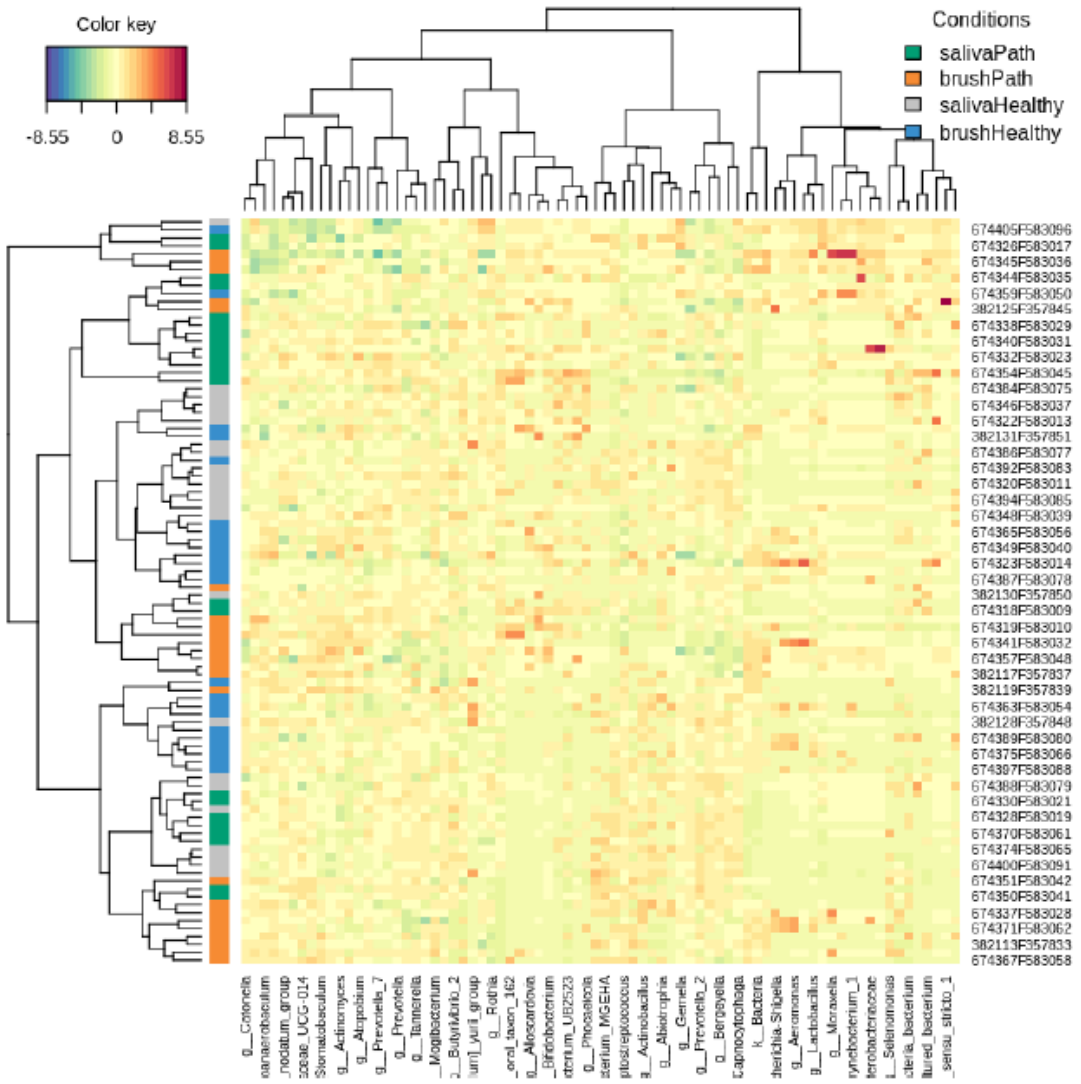
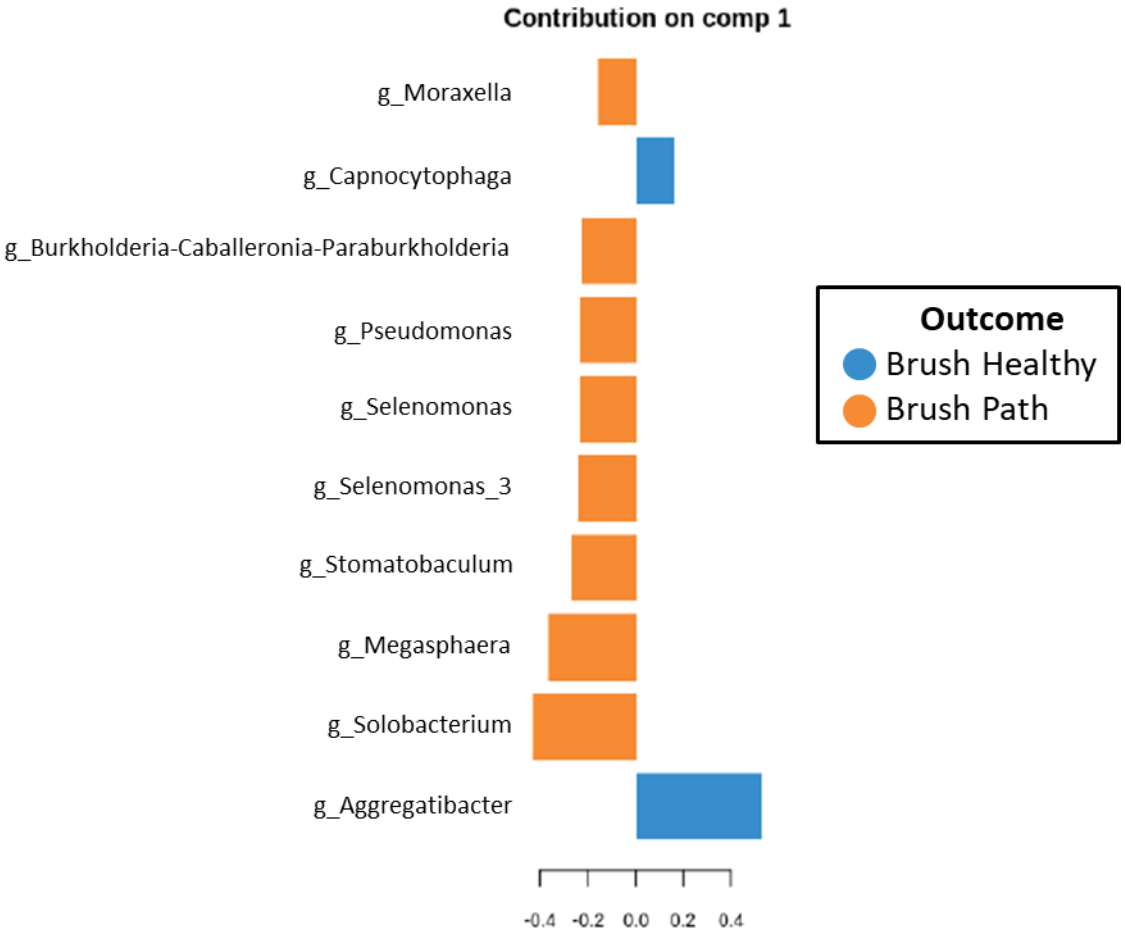


Figure 10. Heatmap of the whole cohort of samples, with abundance of different genus

In terms of evaluation of specific genus contributing to the PLSDA, as we can see in Figure 11, genus *Aggregatibacter* is able to discriminate brushing in HC compared to brushing in PDAC-NO. Furthermore, genus *Solobacterium* is more abundant in brushing of PDAC-NO. Other relevant genus are shown in Figure 11.


Contribution on comp 1
g_Moraxella
g_Capnocytophaga
g_Burkholderia-Caballeronia-Paraburkholderia
g_Pseudomonas
g_Selenomonas
g_Selenomonas_3
g_Stomatobaculum
g_Megasphaera
g_Solobacterium
g_Aggregatibacter
-0.4
-0.2
0.0
0.2
0.4
Outcome
Brush Healthy
Brush Path

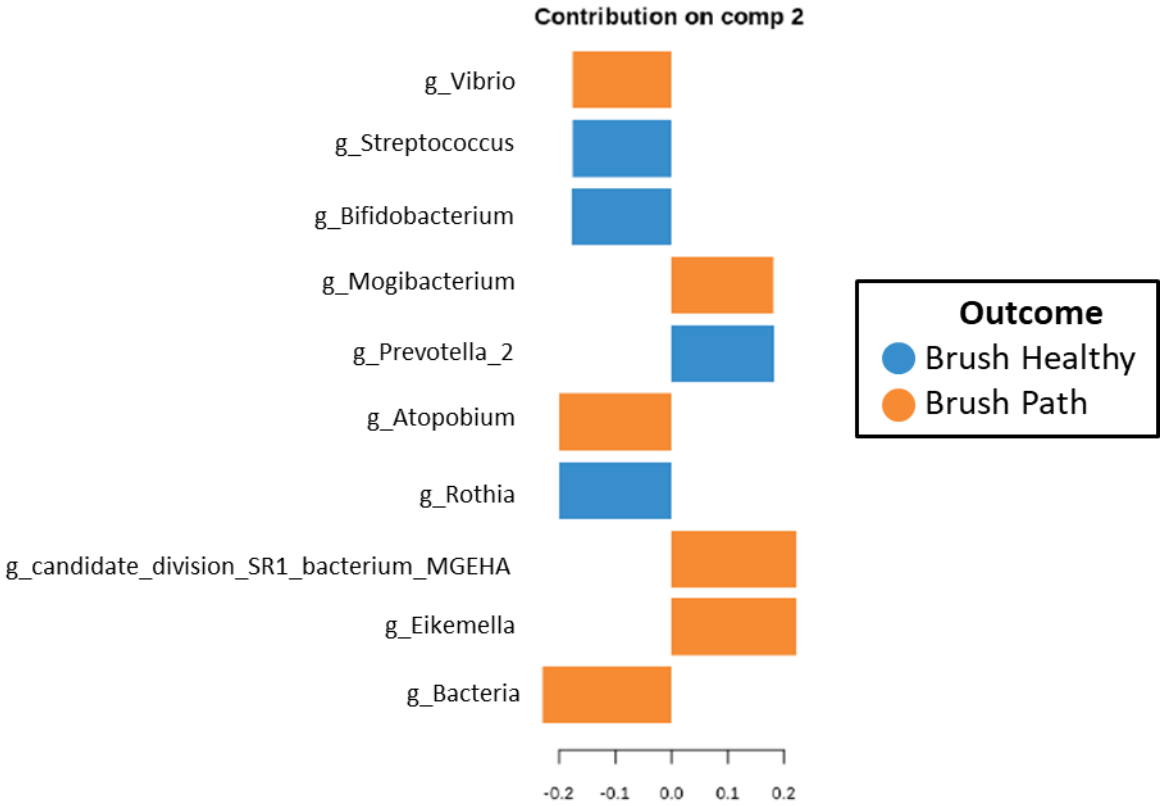


Figure 11. Taxa discriminating, based on Component 1 and 2, the brushing of HC and those of PDAC-NO

3.1.2 PDAC-NO survival analysis

Among PDAC-NO patients, an analysis of the possible association between microbiome signatures and survival was carried out. A ROC curve was first developed to identify the best cut-off for the alpha-diversity to correlate with survival.

Stratifying PDAC-NO patients based on the alpha-diversity of samples, a higher alpha-diversity was associated with a longer survival of patients, both according to OTUs, with an HR of 0.98 (CI 95% 0.97-1; p=0.05) as also Faith PD, with an HR of 0.68 (CI 95% 0.59-0.92; p=0.02) (see Figure 12 and 13). Nonetheless, these data should be interpreted with caution as the limited sample size might have led to a type II error.

Alpha diversity

	HR	UPPER	LOWER	PVALUE
ALPHA OBSERVED	0.98	1	0,97	0.05

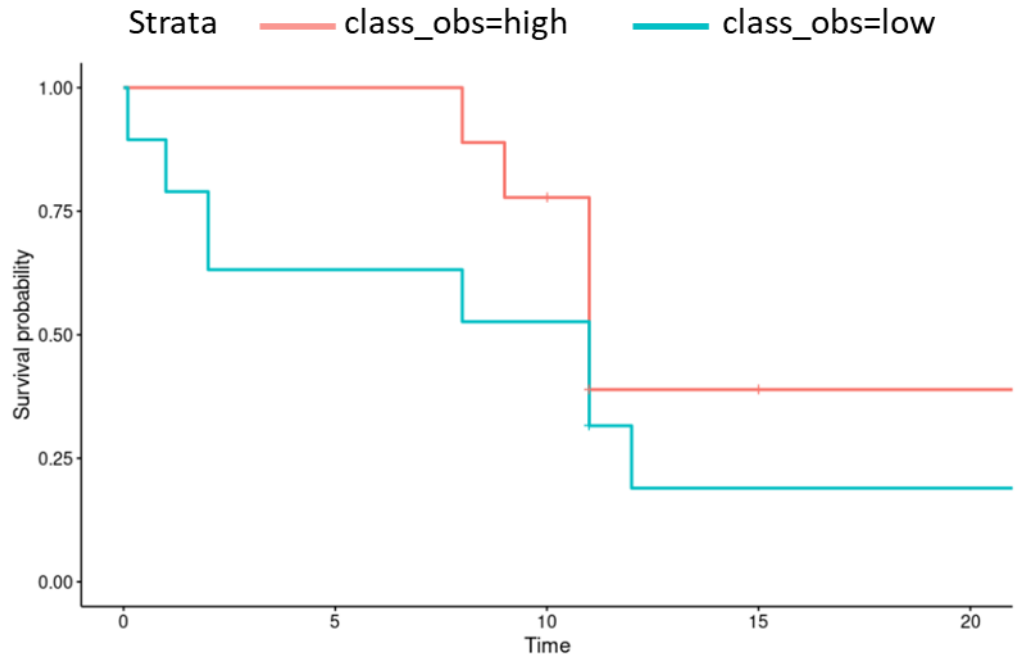


Figure 12. Hazard Ratio and Kaplan-Meier curves with survival probability of PDAC-NO patients based on their alpha-diversity, calculated as observed OTUs.

	HR	UPPER	LOWER	PVALUE
ALPHA FAITH	0,68	0,92	0,59	0,02

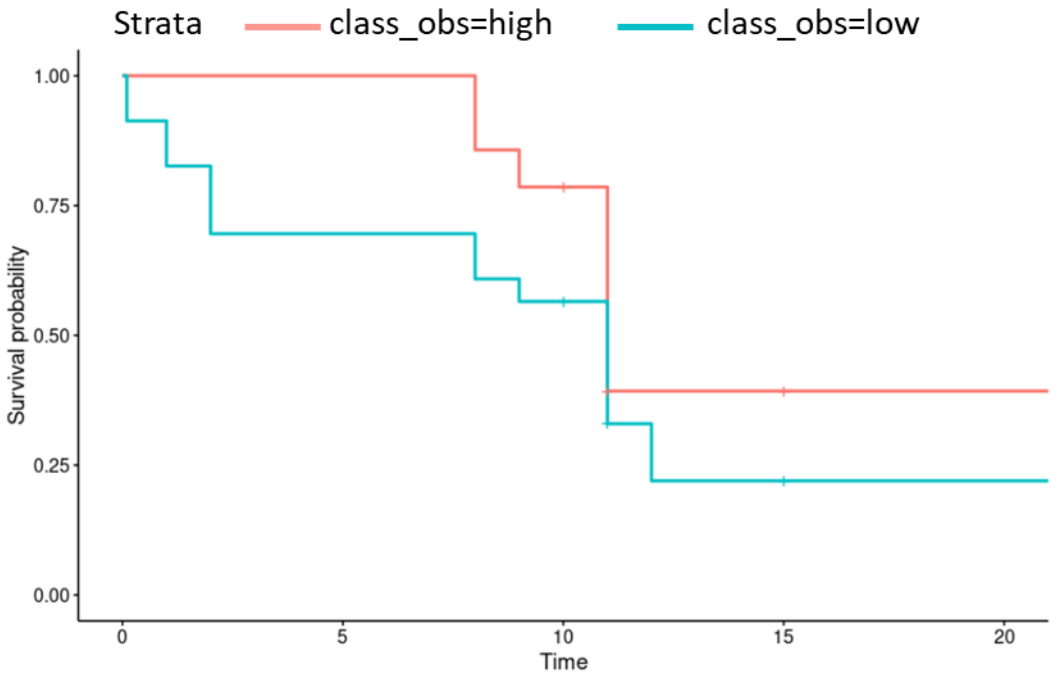


Figure 13. Hazard Ratio and Kaplan-Meier curves with survival probability of PDAC-NO patients based on their alpha-diversity, calculated as Faith PD.

Furthermore, a list of specific genus were associated to an increased or reduced survival of the patients, as shown in Figure 14 and 15.

In fact, *Firmicutes Catonella* was associated to a significantly reduced risk of death, as also *Fusobacterium* and *Filifactor* and *Tannerella* in the saliva, while *Streptococcus* and *Actinobacillus* were associated to an increased risk of death. For the brush samples, the presence in the duodenum of *Catonella* again and *Filifactor* and *Granulicatella* were associated to a reduced risk of death and prolonged survival, while *Bifidobacterium* was associated to an increased risk.

Saliva

Genus	pvalue	HR	lower	upper
Firmicutes_Clostridiales_Lachnospiraceae_Catonella_NA	0	0,65	0,48	0,87
Firmicutes_Lactobacillales_Streptococcaceae_Streptococcus_NA	0,02	1,7	1,08	2,68
Fusobacteria_Fusobacteriales_Fusobacteriaceae_Fusobacterium_NA	0,03	0,55	0,33	0,93
Firmicutes_Clostridiales_Peptostreptococcaceae_Filifactor_NA	0,03	0,77	0,6	0,97
Proteobacteria_Pasteurellales_Pasteurellaceae_Actinobacillus_NA	0,05	1,23	1	1,52
Bacteroidetes_Bacteroidales_Tannerellaceae_Tannerella_NA	0,05	0,77	0,6	1
Firmicutes_Bacillales_Staphylococcaceae_Staphylococcus_NA	0,08	1,4	0,96	2,05
Actinobacteria_Bifidobacteriales_Bifidobacteriaceae_Bifidobacterium_NA	0,1	1,21	0,96	1,52
Spirochaetes_Spirochaetales_Spirochaetaceae_Treponema_2_NA	0,1	0,79	0,59	1,05

Figure 14. List of bacteria retrieved in the saliva, with Hazard Ratio relating their presence in the sample to OS.

Brush

Genus	pvalue	HR	lower	upper
Firmicutes_Clostridiales_Lachnospiraceae_Catonella_NA	0,01	0,63	0,45	0,88
Fusobacteria_Fusobacteriales_Fusobacteriaceae_Fusobacterium_NA	0,02	0,66	0,47	0,93
Firmicutes_Clostridiales_Peptostreptococcaceae_Filifactor_NA	0,03	0,72	0,55	0,96
Actinobacteria_Bifidobacteriales_Bifidobacteriaceae_Bifidobacterium_NA	0,04	1,2	1,01	1,42
Firmicutes_Lactobacillales_Carnobacteriaceae_Granulicatella_NA	0,04	0,73	0,54	0,98
Proteobacteria_Vibrionales_Vibrionaceae_Vibrio_NA	0,08	1,92	0,93	3,95
Firmicutes_Lactobacillales_Lactobacillaceae_Lactobacillus_NA	0,08	1,15	0,99	1,33
Actinobacteria_Corynebacteriales_Corynebacteriaceae_Corynebacterium_1_NA	0,09	1,2	0,97	1,47
Firmicutes_Bacillales_Staphylococcaceae_Staphylococcus_NA	0,09	1,18	0,97	1,44
Proteobacteria_Pseudomonadales_Moraxellaceae_Moraxella_NA	0,1	1,12	0,98	1,28

Figure 15. List of bacteria retrieved in the brushing sample of the duodenum, with Hazard Ratio relating their presence in the sample to OS.

3.2 Transcriptomic analysis of pancreatic cancer

3.2.1 Patients enrolled, RNA quality and quantity

Between November 2018 and October 2019, 1,954 EUS were performed. A total of 37 patients were enrolled having a mean age of 68.3 years ± 10.9 standard deviation (SD), and with a quote of male population of 48.6%. Concerning the location of the lesions, twenty-four (64.9%) were situated in the head of the pancreas, having a mean diameter at EUS of 32.8±10.6 mm, 51.4% were defined as borderline resectable lesions, and 35.1% were locally advanced diseases (Table 3).

	Total patients enrolled (n= 37)
Age, years (mean ±SD)	68.3 (±10.9)
Sex, male, n (%)	18 (48.6%)
Location of the lesion	
Head	24 (64.9%)
Body-tail	13 (35.1%)
Resectable	4 (10.8%)
Borderline Resectable	19 (51.4%)
Locally Advanced	13 (35.1%)
Metastatic	1 (2.7%)
Dimension of lesion at EUS, mm (mean ±SD)	32.8 (±10.6)

SD: Standard Deviation

Table 3. Patient and tumor characteristics of the PDAC patients enrolled for the RNA extraction project.(34)

Concerning the quantity of RNA in terms of median concentration, samples conserved in Trizol resulted having a significantly higher RNA concentration (10.33 ng/uL) than those conserved in RNALater (0.19 ng/uL; $p < 0.0001$) or snap frozen (0.64 ng/uL; $p < 0.0001$)(Figure 17a). Concerning the RNA quality, there was no significant difference for Trizol (median RIN 5.15) respect to snap frozen (median RIN 5.85; $p = 0.8$), while

both these two conservation methods resulted in a non-significantly higher RIN compared to RNALater (2.7; p= 0.07 compared to snap frozen; p= 0.08 compared to Trizol; see Figure 16b). Interestingly, the needles employed did not affect the RNA quantity or quality (see Figure 16a and b).

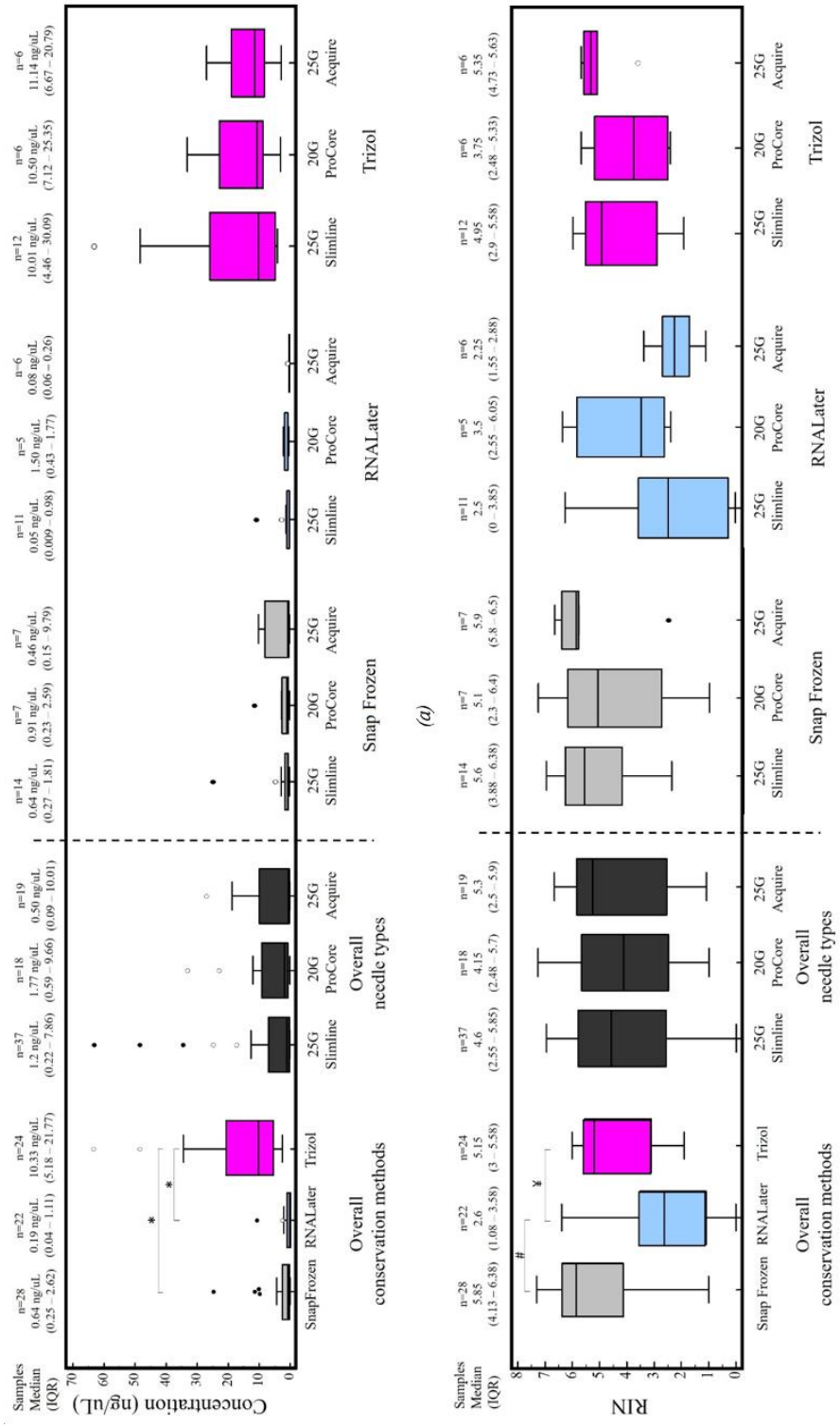
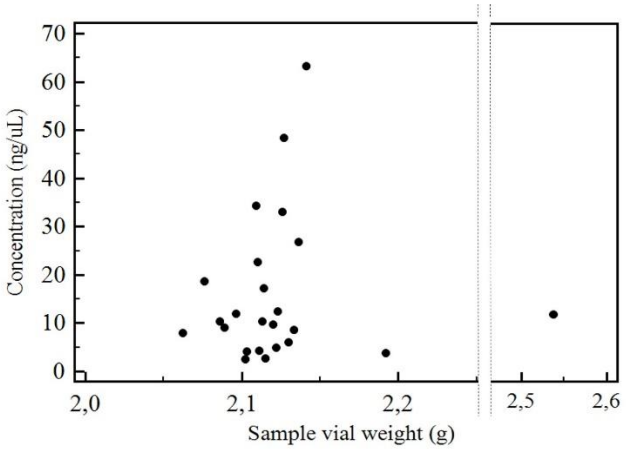
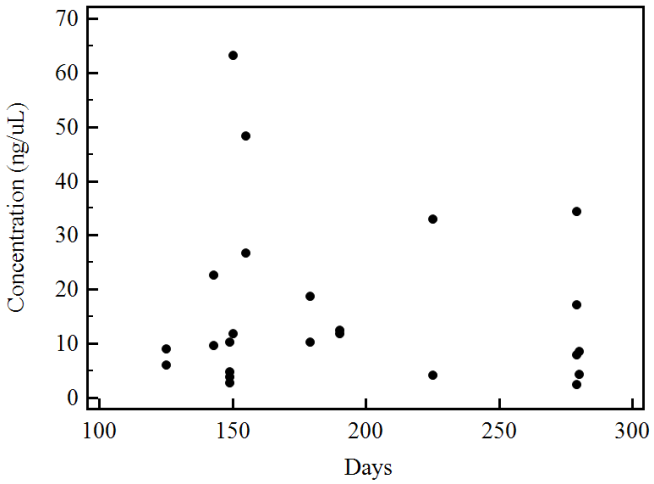


Figure 16. Panel A. RNA concentration in relation to conservation method and needle type. Panel B. RNA Integrity Index (RIN) in relation to conservation method and needle type. *: $p<0.0001$ #: $p=0.07$ ¥: $p=0.08$ (36)

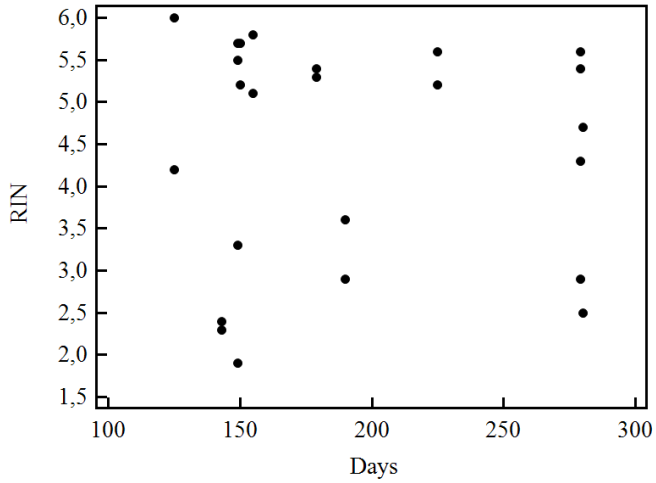
In order to evaluate whether initial sample (vial) weight or days between tissue acquisition and RNA extraction could have an impact on RNA concentration or RIN, we carried out correlation on Trizol samples. No significant correlation was found between weight of the sample and RNA concentration, nor its RIN (Figure 17a and b), or time between sampling to extraction and its RIN (see Figure 17 c).



(a)



(b)



(c)

Figure 17. Analysis of correlation.

Panel A. RNA concentration and weight of the initial sample vial (3A; Kendall's Tau = 0.13, p=0.39)

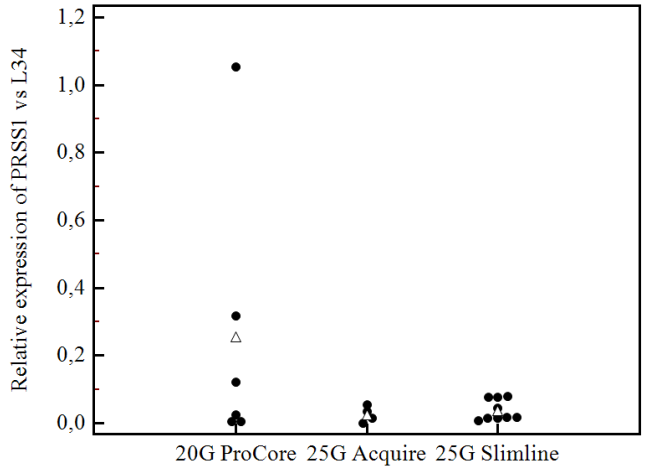
Panel B. Time between sampling and RNA extraction and RNA concentration (3B; Kendall's Tau = 0.03, p=0.84)

Panel C. Time between sampling and RNA extraction and RIN (3C; Kendall's Tau = 0.04, p=0.78). (36)

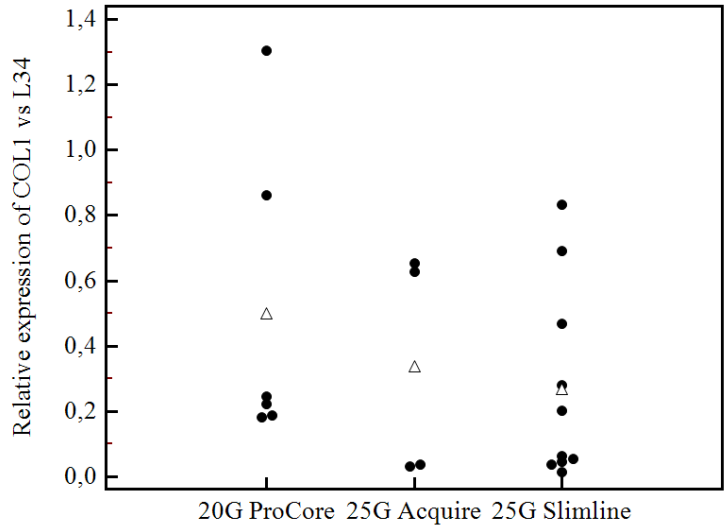
3.2.2 Contamination analysis with stromal or acinar tissue

In order to evaluate whether one needle was superior to the others in terms of reducing stromal or acinar contamination, real-time PCR (qPCR) analysis was performed. The expression of PRSS1, as marker of acinar cells, and of COL1, the gene of collagen 1 as marker of stromal cells were evaluated.

Considering a threshold of 60 ng for total RNA quantity and 3 for RIN, among the totality of 74 samples acquired and collected, 28 were adequate for qPCR: 25% of the snap frozen samples were adequate, 13.6% of those conserved in RNALater and 75% of the Trizol samples. Of the 28 adequate samples, 20 were FNA/FNB matched samples from 10 patients and were therefore employed for qPCR. The 20G FNB needle resulted in a non significantly higher mean contamination from acinar (20G ProCore®: 0.25, 25G Acquire®: 0.04, 25G Slimline®: 0.03; p=0.16), and stromal cells (20G ProCore®: 0.50, 25G Acquire®: 0.33, 25G Slimline®: 0.26; p=0.48) (Figure 18).



(a)



(b)

Figure 18. Mean contamination from stromal and acinar cells based on PRSS1 (a) and COL1 (b) gene expression. (36)

3.2.3 Evaluation of prognostic markers

GATA6 is an endodermal transcription factor that has been recently suggested as marker of classical subtype for PDAC, and also associated with a prolonged survival and favourable prognosis (16, 21, 22). On the other hand, the expression of ZEB1, a transcriptional factor, regulator of epithelial-to-mesenchymal transition, has been correlated with worse prognosis and also resistance to gemcitabine treatment (23-25). qPCR was performed on the 20 selected samples obtained from 10 PDAC patients with both FNA and FNB needles to investigate gene expression of GATA6 and ZEB1. Significant expression of both of these two genes was identified in all samples, suggesting suitability for gene expression analysis of both needles as prognostic markers in PDAC patients. We then hypothesized that the GATA6/ZEB1 ratio could be associated with a worse outcome and increased aggressiveness (21-25), and therefore investigated its correlation with overall survival (OS). Surprisingly, GATA6/ZEB1 ratio showed a trend toward significant correlation with OS (Kendall correlation coefficient t(tau)=0.442; p=0.08)(see Figure 19). As confirmation, the additional EMT marker SLUG (SNAI2)(37-39) was evaluated and correlated to ZEB1 expression, with positive and significant correlation (Kendall's Tau = 0.78; p<0.0001).

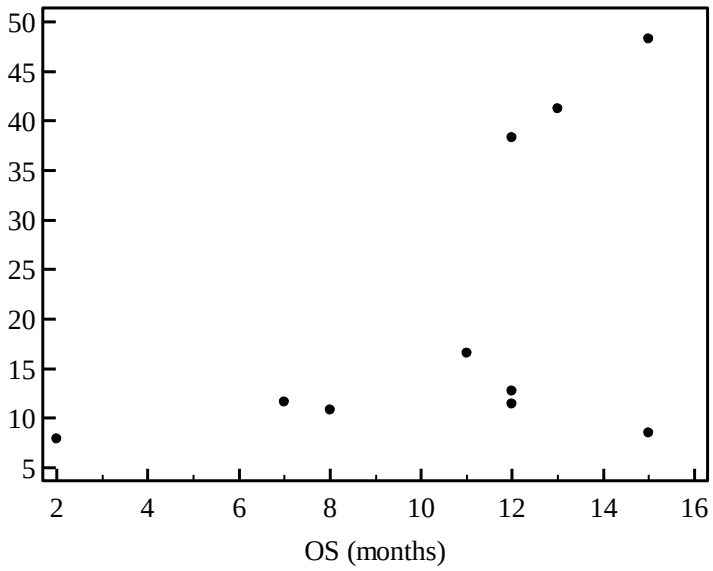
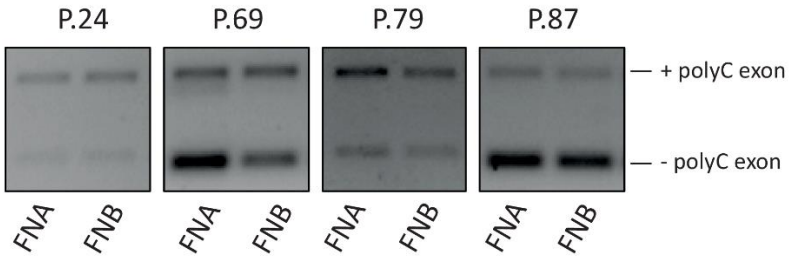
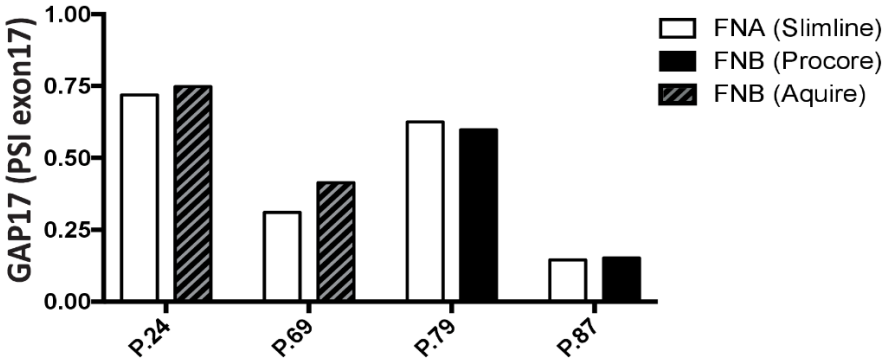


Figure 19. GATA6/ZEB1 ratio showing a trend towards correlation with overall survival (Kendall's Tau = 0.44, p=0.08). (36)

The inclusion of a polyC-rich exon in the GTPase GAP17 (ARHGAP17 gene) was recently shown to cooperate with mutant KRAS protein in activation of the downstream pathway, leading to widespread changes in alternative splicing in PDAC (24). To evaluate whether splice variants of the same gene are detectable in EUS-TA samples, conventional PCR (sq-PCR) analyses were performed using primers in exon that flank the polyC exon of ARHGAP17. In all 20 samples the two splice variants of the gene (+ the polyC exon) were detectable (Figure 20A), irrspective to the needle employed. Also, densitometric evaluation of the percentage of Spliced-In Index (PSI/ψ) of the polyC exon inclusion (Figure 20B) was feasible in all samples, ranging between 25-75%. This suggests that RNA deriving from EUS-TA is suitable also for analysis of splicing events associated with diagnosis.



(a)



(b)

Figure 20. Examples of polyC-rich exon inclusion evaluation in the GTPase GAP17 (ARHGAP17 gene). (36)

RNAsequencing was then performed on the 15 samples with adequate quantity and quality, having a mean RIN of 4.7 (range 3-6). These PDAC patients beared a final OS of 11 months in median. RNAsequencing was successful in 100% patients. Unsupervised clustering employing relevant genes was applied and correlated to OS, with PURIST score identifying 1 patient as basal-like, who had an OS of 3 months (see figure 21), while the mean survival of other patients was of 12.4 months.

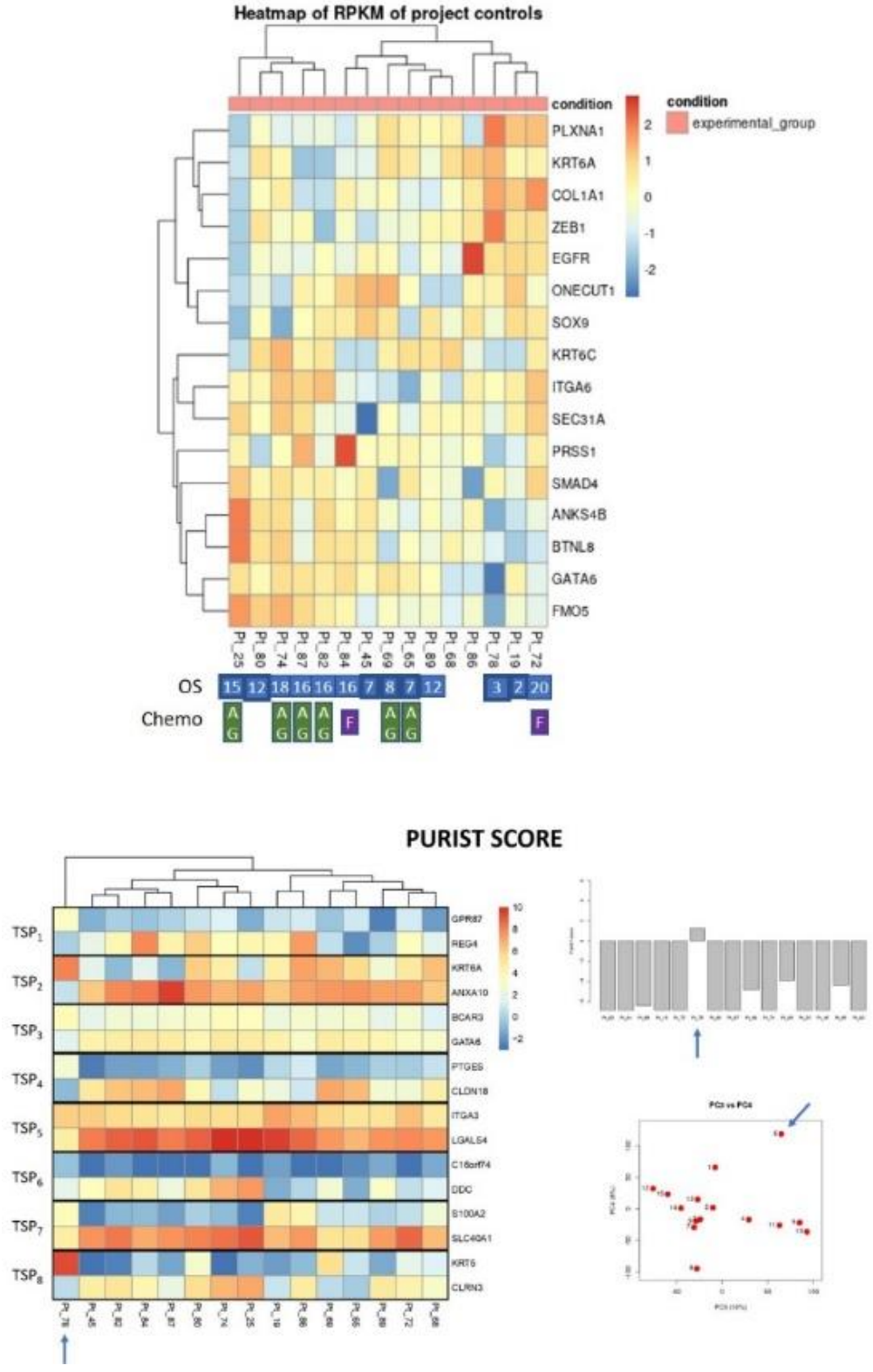


Figure 21. Heatmap of RNAsequencing performed on 15 samples. Unsupervised clustering employing relevant genes was applied and correlated to OS, with PURIST score identifying 1 patient as basal-like, who had an OS of 3 months

4. Discussion

Despite the incredible efforts in terms of clinical and translational research in the last decade, the overall survival and life expectancy of patients affected by PDAC remains extremely poor and has not changed in the recent decades (4). Personalized medicine for PDAC is slowly seeing the light as acknowledged by the most recently published guidelines, underlining the relevance of germline mutations identification and somatic molecular profiling (5,6,13). Furthermore, insights on the relevant role of microbiome as associated to PDAC onset, but also having an impact on treatment response, are slowly being uncovered, and could further play a role in lowering the risk of high risk individuals or work as game changers as additional treatments to current chemotherapeutic standards, as it is being seen for other tumor types.(7) It is in fact recognized that the microbiome or microbiome-derived metabolites might work as potential new therapeutic approaches in the treatment of cancer (7), as these can be important modulators of the tumor microenvironment contributing to regulate inflammation, proliferation, and cell death. (40)

4.1 Microbiome and PDAC

The relevance of microbiome for solid tumors has been slowly discovered in the latest years, especially for tumors like melanoma or lung cancer, showing its impact in the response to chemotherapy or immunotherapy. Nonetheless, when it comes to PDAC, the literature presents mostly studies on the association of specific oral microbiome patterns with the onset of the tumor, or also some data on duodenal microbiome related to the presence of tumors, specifically of the head of the pancreas (7). The evaluation of both the oral and the duodenal microbiome together has rarely been performed, therefore these studies lacked on the investigation of the hypothesis of the migration from the oral environment, through the GI tube, to the duodenum, and backward into the major papilla and the pancreatic duct to colonize the pancreas with a pro-tumorigenic effect (7-12).

In the present project, bacterial 16S DNA gene was successfully extracted and sequenced in all patients, with rarefaction analysis excluding only 2 samples. The technical success of 97.9% shows therefore the feasibility of performing a microbiome evaluation of the saliva and duodenum with the methods illustrated in our project.

In terms of results, as hypothesized in our sample size calculation, a lower alpha-diversity was retrieved in the duodenum of PDAC-NO samples compared to HC, as measured by Operational Taxonomic Units (OTUs), but not in terms of Evenness and Faith PD, in PDAC-NO brushing compared to PDAC-NO saliva, as also to HC brushing, despite this significance was lost after correction.

Furthermore, when the pairwise difference comparing healthy and pathological samples retrieved a difference significant between brushing and saliva, with saliva always having a higher ASV compared to brushing, but these differences were not retrieved as relevant when comparing healthy and pathological samples.

In terms of Beta-diversity, especially in terms of Unweighted Unifrac, a significant difference was seen between brushing and saliva for HC, and between brushing and saliva for PDAC-NO. Also, saliva between HC and PDAC-NO was significantly different.

When trying to identify the most abundant genus, some specific bacteria were found to discriminate the samples of HC and PDAC-NO. For example, genus Aggregatibacter was the most abundant bacterium able to discriminate brushing in HC compared to brushing in PDAC-NO. Also, Solobacterium was retrieved as more abundant in brushing of PDAC-NO.

Interestingly, there were not only differences between HC and PDAC-NO, but also when analyzing PDAC-NO patients, an association of microbiome characteristics and survival was retrieved. In fact, stratifying PDAC-NO patients based on the alpha-diversity of samples, a higher alpha-diversity was associated with a prolonged overall survival of patients, both according to OTUs as also Faith PD. Nonetheless, these data should be interpreted with caution as the limited sample size might have led to a type II error.

Furthermore, a list of specific genus were associated to an increased or reduced survival of the patients: Firmicutes Catonella was associated to a significantly reduced risk of death, as also Fusobacterium, Filifactor and Tannerella in the saliva, while Streptococcus and Actinobacillus were associated to an increased risk of death. For the brush samples, the presence in the duodenum of Catonella again, Filifactor and Granulicatella were associated to a reduced risk of death and prolonged survival, while Bifidobacterium was associated to an increased risk.

In terms of strengths, this is the first study evaluating both the oral and duodenal microbiome of patients with PDAC and not obstructing the ducts; a sample size calculation was also performed after the enrollment of the first 10 patients, and the majority of confounding factors related to microbiome alteration have been recorder; lastly our results do get along well with the hypothesis of a high variety of bacterial species being a sign of good gastrointestinal health, compared to other microbiological nice.

In terms of limits, samples were not frozen but instead stored at room temperature for months, but this was done accordingly to manufacturer instructions, the sample size is low, and therefore results must be interpreted with caution; nonetheless the number of the cohort was suggested by a rigorous sample size calculation; also some papers underline the limitations of the use of marker gene sequencing, as this can only provide information on bacteria, while excluding the other microbes, and also this identifies only microbes included in reference database, highlighting the possibility of applying novel techniques such the whole metagenomic analysis. In addition, regional differences among patients were not taken into account, while in terms of ethnicity all patients were Caucasian. Finally, considering that the duodenal brush is extracted through the operating channel of the endoscope that has passed through the mouth and stomach and possibly suctioning secretions, we cannot exclude contamination of the sample from other upper GI regions.

Further investigations in the field might possibly relate tumor molecular subtypes to specific microbiome patterns, as a cross-talk and bidirectional shaping between host genetics/transcriptomics and microbiome might be hypothesized. It has been in fact recently shown (41) that basal-like tumors demonstrate activated antimicrobial immunity and inflammatory response, and differentially abundant microbial genera in basal-like versus classical tumors. For example, Pseudomonas has been demonstrated to be associated with short-term survival in PDAC patients as also with increasing abundance in basal-like tumors.(41)

Also, in case future developments will suggest the use of the oro-intestinal microbiome as diagnostic marker, additional studies comprehending a different type of control group should be carried out, possibly including diseases that might mimic a PDAC, where the employment of the microbiome could play a role in the differential diagnosis (such as

other types of pancreatic tumors, or patients with autoimmune pancreatitis or chronic pancreatitis).

In conclusion, a lower alpha-diversity especially in the duodenum is associated with PDAC, and this is not related to the presence of a pancreatic duct obstruction. Also, a lower alpha diversity is able to discriminate short-term survival and the presence of a specific microbiome signature both in the saliva and in the duodenum is able to correlate with the survival of PDAC patients. Further studies are needed in this context to evaluate also its association with response to specific chemotherapeutic regimens.

4.2 Transcriptomic analysis of pancreatic cancer

In the latter decades, the evaluation of germline and somatic mutations and transcriptional features have been recognized as bearing a potential for patients stratification and identification of the proper treatment in several human cancers. When it comes to PDAC, nonetheless, the pathway towards personalized medicine is still under construction and has started seeing the light only in the most recent years and it is still not appliable under most aspects. Concerning transcriptomics, two main subtypes of PDAC have been described when taking into consideration tumoral cells. This was based on differential gene expression signatures: the classical and the basal-like. The classical subtype of PDAC is described as bearing a better prognosis and seems to be responding better to Folfirinox, while the basal-like is characterized by a worse prognosis and studies show its possible response to gemcitabine-based therapy or even targeted-therapies (16). These molecular subtypes have been first identified through a clusterization based on RNA-sequencing, but recent reports considered the possibility of identifying these subtypes based on single markers (22). Furthermore, a possible limit of the classical and basal-like classification is due to employment of surgical samples, therefore representative of less than 20% of PDAC patients, as only this small quote is amenable of surgical resection at diagnosis (20). On the contrary, the majority of patients bearing a PDAC undergo EUS-TA. Hence our aim to investigate the best sampling technique and conservation method to extract a quantity and quality of RNA from EUS-TA that is

adequate for molecular investigations, and to determine its actual usability for q-PCR and RNAsequencing for the evaluation of relevant markers for prognostic purposes.

Nonetheless, RNA extraction from pancreatic tissue is extremely difficult, especially when compared to DNA extraction and evaluation, mostly for the abundance of RNAase in the pancreatic gland, causing a rapid degradation of the RNA, starting as soon as the sample is acquired. Also, PDAC bears a low tumoral cells-to-stroma ratio, as also the use of EUS, with needles passing through the gut wall, could bring contamination of the sample with gastric or duodenal wall cells or with healthy pancreatic tissue (42). So far, there have been only few studies evaluating RNA extraction from EUS-acquired samples, with poor quantity and quality of RNA. In a first study, only 5-10 ng of RNA were extracted from each sample of EUS-FNA, and also there was no mention of the quality of the extracted RNA(28). A paper by Berry et al. reported an amount around to 12.9 ug of RNA of relatively low quality (mean RIN of 3) retrieved with one pass of FNA, kept snap frozen in liquid nitrogen (32). Another example found in the literature is the employment of archival formalin-fixed paraffin-embedded (FFPE) EUS-FNB samples with its RNA extraction with novel 22G Forktip needle (33), reporting only 45 samples (28.8% of the total number) resulting adequate for Nanostring analysis, and again with RNA integrity index not reported. Another example of RNA extraction, fourty snap frozen samples were acquired through a 22G needle with a standard Meneghini tip, with a good quantity of RNA being extracted (1 μg), and with integrity of RNA evaluated through agarose gel electrophoresis and spectrophotometry but not quantitatively reported (34). From this study, the evaluation of qPCR analysis for VEGFR genes was deemed feasible. Nonetheless, none of these studies investigated the variety of methods to conserve the sample, and the different needles in a side-by-side way, to determine how to best acquire RNA from EUS. Therefore, our study is the first to compare 3 different needles and conservation methods, reporting how the use of Trizol resulted in higher quantity of RNA, while no difference was reported based on the choice of the needle to be employed.

In fact, in our study the use of Trizol allowed to reach a quantity of RNA with one single FNA or FNB pass of 300 ng in median, and a range of 60-1,890 ng. In terms of quality of RNA, the integrity index in median was of 5. This resulted in 75% of Trizol

samples resulting adequate to be employed for qPCR also for delicate analyses such as the evaluation of splicing events.

Among the three types of needles, in terms of contamination, we observed a non-significant higher amount of both stromal and acinar contamination with the 20G ProCore FNB needle, and therefore we can imagine that in case of studies wanting to evaluate in particular the stromal component, this needle or other larger FNB could be preferred. Also, as reported in the correlation analyses, we can suggest that relative flexibility can be tolerated in the procedure especially in terms of days between sampling and extraction, that resulted not affecting the final RNA concentration or RIN.

A wide number of reports proved, in the latest years, the superiority of needles with a Franseen tip in terms of adequacy of the tumor sample for diagnostic purposes (39,42). Nonetheless, it is not clear whether this could also mean a higher quantity or better quality of RNA, as reported in our results.

Another concept is the dimension of the needle in terms of Gauge. Park et al (30) recently reported how larger needles do retrieve a higher quantity of DNA from EUS-TA. Surprisingly, in our study no benefit was seen with the use of the 20G needle compared to other needles, possibly due to the differences in terms of stability between DNA and RNA.

The relevance of the employment of GATA6 as biomarker has been underlined in silico and also in clinical studies on surgical specimens. GATA6 works as a modulator of pancreatic development, acting as activator of epithelial differentiation (21, 44).GATA6 is known to be highly expressed in the classical subtype and it has also been proposed as being a sufficient marker used alone to determine PDAC molecular subtype (22). Nonetheless, it has been reported how a possible coexistence of the basal and classical subtype might be present (17). A well-known transcription factor that promotes ZEB1 is a that promotes epithelial-mesenchimal transition and therefore tumor invasion and metastatic spread is ZEB1; this factor has also been implicated in the resistance to gemcitabine-based therapies (23-25). In our project, two biomarkers known to have an association with the prognosis were evaluated on 20 samples from 10 PDAC patients. None of these two genes resulted being associated with OS. Nonetheless, a correlation between the GATA6/ZEB1 ratio and OS resulted reaching a statistical significance.

Another relevant topic for PDAC biomarkers are splicing factors It has been in fact proved that splicing is often de-regulated in PDAC, and this could also contribute to the classification of PDAC patients more than gene expression (45,46).

We therefore decided to explore whether our samples could also be employed for the evaluation and detection of prognostic splicing events. Escobar-Hoyos et al. recently proved how TP53 missense hotspot mutations, usually associated to an aggressive behavior for PDAC, would cause the inclusion in the ARHGAP17 gene of polyC exon (26). As shown in our results, we evaluated this specific splicing event in our samples, showing its feasibility in 100% of cases. Furthermore, 15 of these samples from 15 different PDAC subjects were employed for RNAsequencing, with a 100% feasibility and also a possible correlation with survival, and application of the PURIST score.

This current part of the project has some limits: this study was mostly a feasibility study with the aim of determining the best experimental conditions but still bearing a very small sample size, as also missing a head-to-head comparison of the three needles and conservation methods, as also the carry out of qPCR not on the totality of samples but only on a minority. Furthermore, the present study is lacking a comparison with the PRSS1 and COL1 gene expression evaluation from normal pancreatic tissue, as considered not ethic. Also, the GATA6/ZEB1 ratio and its correlation with survival can only bear a speculative significance but it is not validated. Lastly, the patients undergoing GAP17 splice event evaluation did not have any evaluation of the mutational status of P53.

The present project has nonetheless some strengths as this is the first study that evaluates specifically and also in a prospective manner three different needles currently employed clinically for EUS-TA in PDAC patients, and three conservation methods for the evaluation of RNA for transcriptomic evaluations. The results observed in terms of quantity of RNA extracted showed a high amount of RNA, but also a good quality, adequate to perform in 100% of cases both qPCR for contamination and prognostic markers, but also to investigate splicing events.

To conclude, in this part of the project we proved that EUS-acquired samples from patients with PDAC, conserved in Trizol allowed to extract a significantly higher quantity and bore a sufficient RIN that permitted the evaluation of biomarkers through qPCR, also for splicing events, as also RNAsequencing.

5. Materials and methods

5.1 Microbiome and PDAC

5.1.1 Study design and population

We conducted a prospective observational study upon approval of Internal Review Board (IRB BIOGASTRO/2011 updated on 06/11/2017). Patients with clinical and radiological suspect of non-metastatic PDAC, not obstructing the pancreatic and bile duct (PDAC-NO), naïve to any treatment, were considered for enrollment as cases, upon informed consent.

Healthy controls (HC) consisted in patients undergoing upper Gastrointestinal endoscopy for any reason, with the exclusion of patients with esophageal or gastric resection, patients with known pancreatic diseases (ongoing or previous), patients with atrophic gastritis or celiac disease or suspect for neoplastic conditions. These patients were enrolled based on their matching (on gender and age) with a ratio 1:1 to PDAC-NO.

Data registered consisted in demographic and clinical variables with also factors associated to microbiome alteration as described in literature, as also data on reasons to perform upper GI endoscopy, in case of HC, and tumor characteristics, in case of PDAC. All collected variables are reported in the CRF in Figure 22.

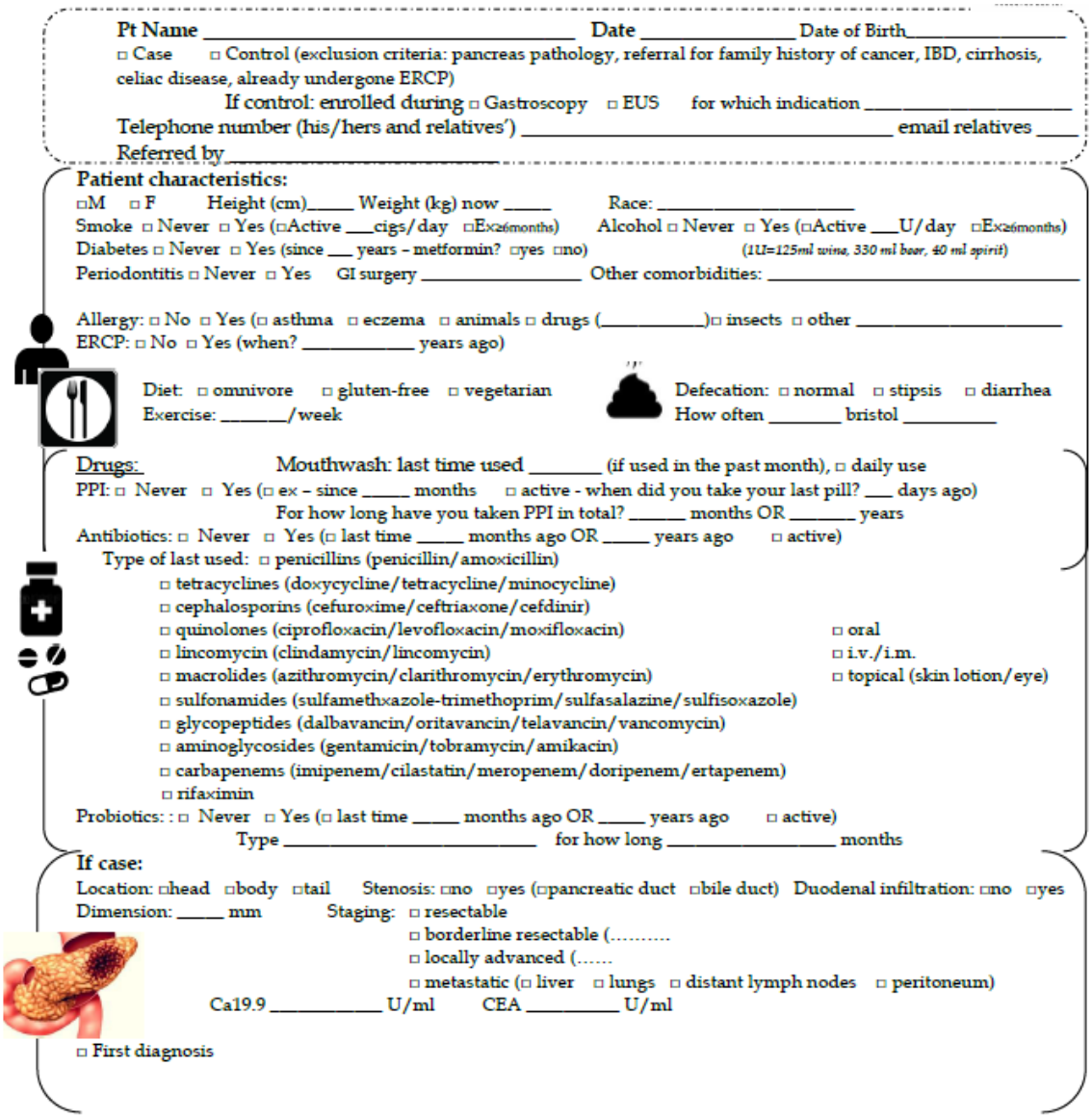


Figure 22. CRF of the data collected for patients undergoing saliva and duodenal sampling for microbiome evaluation.

5.1.2 EUS, Gastroscopy and specimen processing

EUS procedures were performed under deep sedation with intravenous infusion of Propofol (Diprivan®, Zeneca, Germany), using a Pentax therapeutic linear echoendoscope (EG3870UTK, EG38J10UT) and Hitachi ultrasound platforms (Arietta 850, Arietta V70) by expert endoscopists performing over 500 EUS procedures/year. Gastroscopies were performed under moderate sedation with low dose intravenous

infusion of Propofol (Diprivan®, Zeneca, Germany), using a Pentax gastroscope (EG-2901).

Before the procedure, patients that, based on the clinical history, seemed to be eligible for enrollment, were given informed consent and then the sputum (4 ml) was collected in the stabilizing solution Omnigen-Oral DNA – Microbial DNA (Voden Medical, Italy).

The duodenal microbiota was collected during the procedure, with the use of a gastrointestinal cytology brush (Cook Medical ECB-5-180-3-S), used during the EUS or upper GI endoscopy procedure, with the duodenal wall within 3 cm from the major papilla being brushed to collect microbiome. The brushing was then inserted in the specific vial with stabilizing solution OMNIgene Gut (Voden Medical, Italy). Both vials were stored at room temperature for at most 3 months, following manufacturer instructions.

Bacterial 16S DNA gene was then extracted from each paired sample, retrotranscribed with reverse transcriptase and random primers and 16S rRNA PCR was subsequently performed. Next Generation Sequencing was then performed and results analyzed and compared through Qiime2 software.

5.1.3 Statistical analysis

A sample size calculation was performed after enrollment of the first 10 patients (7 PDAC-NO and 3 HC). The method that was employed was based on the paper by Casals-Pascual et al. (47). In order to obtain a power of 90% to identify a significant Alpha-diversity, 24 PDAC-NO patients and 24 HC were needed.

Reads preprocessing was performed with cutadapt, and denoising with DADA2 (for 260, rev 245). Targeting at least 10,000 reads per sample, input raw reads varied between 25,227 and 117,432. Amplicon sequence variant (ASV) was filtered with a threshold of 0.01% of frequency. Taxonomic association was performed through feature-classifier against clusterized OTUs, also called Observed Features, at 99% of GreenGenes 13-8 and Silva 132 database.

Rarefaction analysis was performed at 16037 reads. Alpha-diversity was evaluated in terms of Observed OTUs, Evenness and Faith PD, with group significance calculated with Kruskal-Wallis pairwise test.

Beta-diversity was then analyzed with Unweighted Unifrac, Weighted Unifrac and Bray Curtis, with Pairwise permanova test applied.

Subsequently, longitudinal analysis against alpha and beta-diversities were performed. Differential abundances analyses were performed with ANCOM e mixOmics, while multivariate analysis was performed with Maaslin2.

Survival analysis was performed plotting Kaplan-Meier curves based on alpha-diversity with LogRank test used to calculate Hazard Ratio (HR) and alpha-diversity cut-off determined after a ROC curve calculation.

5.2 Transcriptomic analysis of pancreatic cancer

5.2.1 Study design and population

We conducted a prospective observational study upon approval of Internal Review Board (IRB BIOGASTRO/2011 updated on 06/11/2017). Patients with clinical and radiological suspect of non-metastatic PDAC, naïve to any treatment, were considered for enrollment upon informed consent. An explanatory representation of the study design is reported in Figure 23.

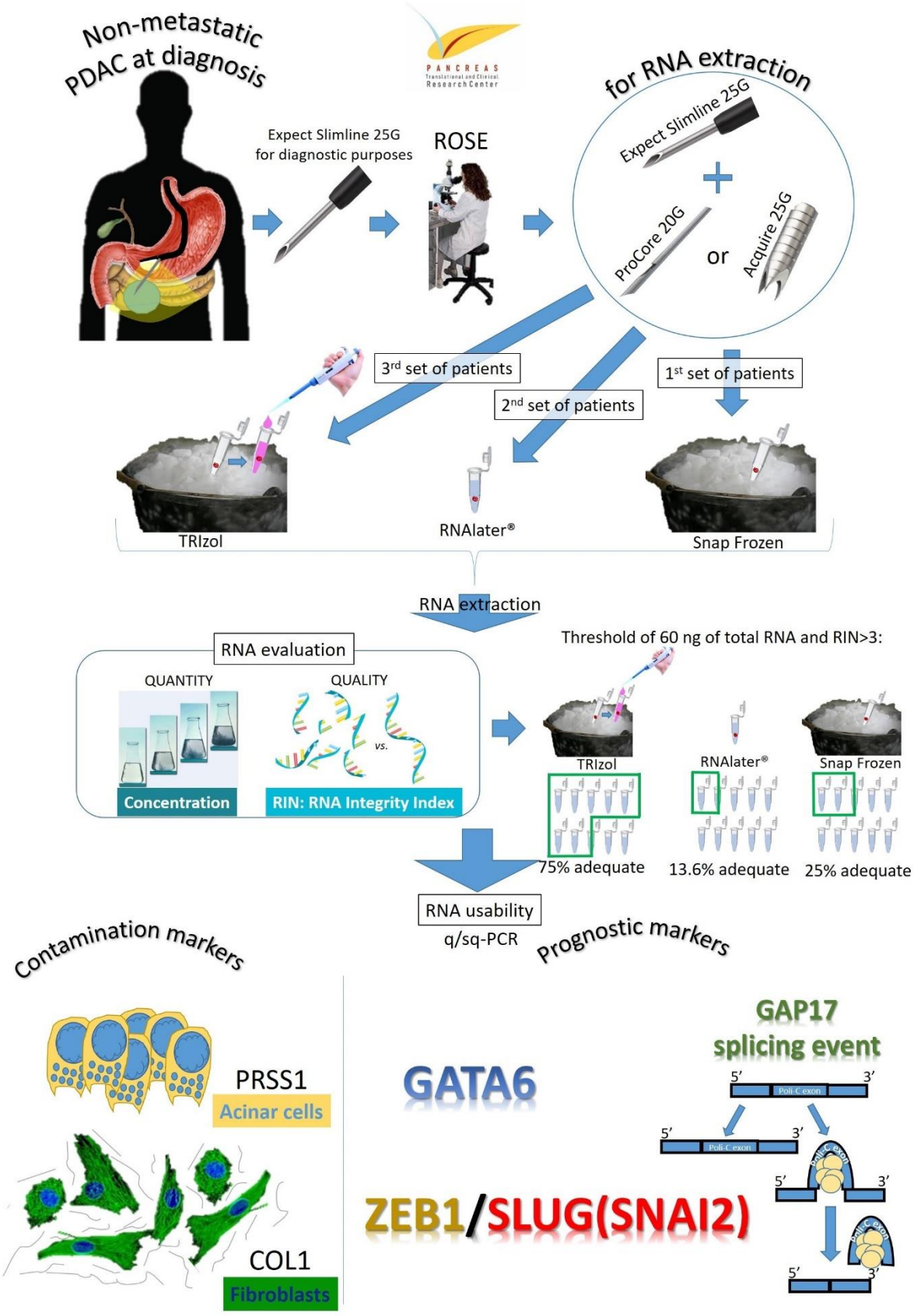


Figure 23. Explanatory representation of the transcriptomic study design. (34)

5.2.2 Description of EUS and specimen processing

EUS procedures were performed under deep sedation with intravenous infusion of Propofol (Diprivan®, Zeneca, Germany), using a Pentax therapeutic linear echoendoscope (EG3870UTK, EG38J10UT) and Hitachi ultrasound platforms (Arietta 850, Arietta V70) by expert endoscopists performing over 500 EUS procedures/year. Lesion size and features evaluation was performed either from the stomach or from the duodenal bulb and the second portion based on lesion location. The EUS-TA was performed from the most stable position, the closest of the lesion to the probe and the best window for puncture in terms of absence of blood vessel interposition, evaluated with Color Doppler.

Three different needles were employed and evaluated, and for all of them the slow-pull technique was employed. The first EUS-TA was always performed with a 25G FNA needle (Expect Slimline®, Boston Scientific) for diagnostic purposes, with the sample being expressed on a glass slide. At least 2 slide smears were prepared in each case, and then fixed in 100% alcohol solution and stained with hematoxylin and eosin with standard techniques. An onsite cytopathologist would then examine the slides in order provide a real-time impression on the adequacy and nature of the lesion, whether benign (when the cytologic specimen did not reveal malignancy), suspicious, or malignant nature of the cells. Pathologic criteria were employed to define the diagnosis, considering tissue architecture, hyperchromatic nuclei, pleomorphism, presence of giant cells and high nuclear-to-cytoplasmic ratio. In case of no adequacy, another pass with the same 25G FNA needle was performed, with subsequent reevaluation. If also the second pass would not result in adequacy, the patient was excluded from the study.

In case of adequacy and suspect of malignancy, another pass with the 25G FNA needle (Expect Slimline®, Boston Scientific) would be performed, and then an additional pass with a different needle (either a 20G needle (ProCore, Cook Medical) or a 25G needle (Acquire®, Boston Scientific)) was performed, with these FNB needles alternated for each consecutive patient. These two samples for each patient were expressed in two different vials. Three types of conservative conditions were then established:

The first 14 consecutive patients had the samples stored in empty vials positioned in dry ice and then immediately stored at -80°C.

The second set of 11 consecutive patients had the samples positioned in vials containing 600 uL of RNALater (Invitrogen®, ThermoFisher) at room temperature for at least 1 hour and then stored at -80°. The third set of 12 consecutive patients had the samples positioned in empty vials positioned in dry ice, with a subsequent addition of 1000 uL of Trizol (Invitrogen®, ThermoFisher), and then stored at -80°C.

There were therefore a total of nine experimental conditions, with 3 different needle types and 3 different conservation methods. No sample size calculation was performed.

RNA was extracted with the use of the RNeasy Plus Mini Kit (Qiagen®, Germany) following the standard protocol. A hand-held battery operated homogenizer VWR Pellet Mixer (VWR International, United States) was adopted for the homogenization of samples on ice, with the aim of having no visible floating fragment. Final RNA was diluted in 30 uL of RNAse-free water. 2100 Bioanalyzer with the RNA 6000 Pico kit (Agilent Technologies, Germany) was used for the RNA quantification and evaluation of quality (integrity).

5.2.3 Analysis of gene expression and splicing assay

For the evaluation of the feasibility to perform transcriptomic analyses on the samples obtained, 20 samples acquired from 10 patients were employed. These samples were selected as having adequate RIN (>3) and quantity (>60 ng) to perform real-time quantitative PCR (qPCR). PRRS1 and COL1 were employed as markers respectively of acinar and stromal contamination, and GATA6 and ZEB1 (as also SLUG) as markers respectively of classical subtype and epithelial-to-mesenchymal transition (EMT) and semi-quantitative PCR (sqPCR) for splicing analysis of ARHGAP17(26). For each sample, 50 ng of RNA was retrotranscribed with M–MLV reverse transcriptase (Promega) and random primers (Roche). Semi-quantitative (sqPCR) and qPCR analyses were employed for gene expression and splicing pattern analysis, with the use of 2 ng of cDNA template. We then calculated the percentage Spliced-In Index (PSI/ψ) from PCR products densitometric analysis as ψ = exon inclusion/ (exon inclusion + exon skipping) band intensities. We further carried out qPCR analysis using LightCycler 480 SYBR

Green I Master (Roche) and LightCycler® 96 Real-Time PCR System (Roche) system (Applied Biosystems), following manufacturer's instructions. The 2- ΔΔCq method was employed to quantify the relative changes in gene expression, and the reactions were normalized on endogenous control gene L34.

Concerning RNAsequencing, 15 patients with non-metastatic PDAC and having adequate quantity of RNA (70 ng) and Integrity Index (RIN) ≥ 3 underwent RNAseq with Illumina Nova-Seq 6000 system to yield 100M paired-end reads of 100bp for sample. Unsupervised clustering according to markers associated to molecular subtypes and evaluated through in silico analysis of TCGA, as also the PURIST score were applied and correlated to overall survival (OS) and chemotherapy used.

5.2.4 Statistical analysis

Descriptive statistics were calculated as means and standard deviation (SD) for normally distributed continuous variables, as means and SD and median and interquartile range (IQR) for continuous variables with skewed distribution, and as numbers and percentages for categorical variables.

Unpaired T-test was employed for the comparison of normally distributed continuous variables, Mann-Whitney U test for continuous variables with skewed distribution. One-way analysis of variance (ANOVA) was used to test the difference between the means of gene expression among the different needle types. Correlation analyses were performed calculating the Kendall rank correlation coefficient.

All calculations were performed using MedCalc version 13 (MedCalc Software, Belgium). A P value < 0.05 was considered statistically significant.

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