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STUDIES ON MOLECULAR AND IMMUNE SIGNATURES FOR DETECTION OF PANCREATIC CANCER AND COVID-19

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Studies on Molecular and Immune Signatures for Detection of Pancreatic Cancer and COVID-19

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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Dedicated to my beloved parents, brothers and sisters,

and most of all, my wife and soulmate Mashael,

and our son Mohammad…

ABSTRACT

Background and objectives

Etiological factors and accurate diagnostic biomarkers have remained elusive to pancreatic cancer, a disease with > 90% five-years mortality rate. The recent understanding of microbiome interaction with host organs has opened new research avenues on the potential role of microbiota in the tumor microenvironment. The objectives in **Study I and II** aim to investigate molecular and microbiome related biomarkers in plasma, saliva, pancreatic fluid and tissues from patient groups diagnosed with pancreatic cystic neoplasms, and their correlation with pancreatic neoplastic grade.

The COVID-19 outbreak occurred in an unprecedented transmission rate which necessitate diagnostic biomarkers to manage the pandemic. The objective in **Study III** aims to investigate the potential use of saliva as a non-invasive approach for assessment of immune exposure to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2).

Results

Study I shows that elevated bacterial 16S DNA copies and IL-1β levels in pancreas cysts correlate with high neoplastic grade. Analysis of the intracystic pancreas microbiome shows co-occurrence and enrichment of oral bacterial species. Bacteria DNA level in pancreas is also associated to prior exposure to invasive endoscopic procedures.

Study II shows that elevated plasma and salivary antibody reactivity to oral pathogens (particularly *F. nucleatum* or Fap2 of *F. nucleatum*) is associated with intraductal papillary mucinous neoplasm (IPMN) diagnosis showing high-grade dysplasia or invasive cancer.

Study III shows that salivary antibody reactivity to SARS-CoV-2 spike and capsid antigens persist up to 9 months after mild COVID-19 with a new multiplex antibody assay. Presence of specific salivary antibodies also correlates to COVID-19 like symptoms in a second undiagnosed cohort. The virus-specific IgG in saliva appears stable and tolerates temperature and detergent pre-treatments.

Conclusion

Collectively, the results indicate that oral microbes have a role in disease progression of pancreatic cystic neoplasms. Reducing the inflammatory microbiome may be a potential therapeutic strategy. The salivary antibody testing against oral pathogens holds interesting promise for early identification of high-risk pancreatic tumors. Furthermore, salivary antibody testings on a multiplex platform perform well and can support immune diagnostics of COVID-19. By combining at-home sample collection and the multiplex strategy, salivary diagnostics can be a sensitive and non-invasive alternative to conventional tests currently available.

LIST OF SCIENTIFIC PAPERS

I. Gaiser RA, Halimi A, Alkharaan H, Lu L, Davanian H, Healy K, Hugerth LW, Ateeb Z, Valente R, Fernández Moro C, Del Chiaro M, Sällberg Chen M. Enrichment of oral microbiota in early cystic precursors to invasive pancreatic cancer.
Gut. 2019 Dec;68(12):2186-2194.

II. Alkharaan H, Lu L, Gabarrini G, Halimi A, Ateeb Z, Sobkowiak MJ, Davanian H, Fernández Moro C, Jansson L, Del Chiaro M, Özenci V, Sällberg Chen M. Circulating and Salivary Antibodies to Fusobacterium nucleatum Are Associated With Cystic Pancreatic Neoplasm Malignancy.
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III. Alkharaan H#, Bayati S#, Hellström C, Aleman S, Olsson A, Lindahl K, Bogdanovic G, Healy K, Tsilingaridis G, Palma PD, Hober S, Månberg A, Nilsson P, Pin E*, Sällberg Chen M*. Persisting Salivary IgG against SARS-CoV-2 at 9 Months After Mild COVID-19 – A Complementary Approach to Population Surveys. #,* Both authors contributed equally.
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CONTENTS

LIST OF ABBREVIATIONS

PC	Pancreatic cancer
PCN	Pancreatic cystic neoplasms
IPMN	Intraductal papillary mucinous neoplasm
MCN	Mucinous cyst neoplasms
SCA	Serous cystadenomas
LGD	Low-grade dysplasia
HGD	High-grade dysplasia
CEA	Carcinoembryonic antigen
CA 19-9	Carbohydrate antigen 19-9
Fap2	Fusobacterium autotransporter protein 2
FadA	Fusobacterium adhesin A
TIGIT	T-cell immunoglobulin and ITIM domain
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecules 1
Gal-GalNAc	Galactose-β (1–3)-N-acetyl-D-galactosamine
NK	Natural killer
CRC	Colorectal cancer
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
MERS- CoV	Middle East respiratory syndrome coronavirus
COVID-19	Coronavirus disease 2019
ACE2	Angiotensin-converting enzyme 2
S-protein	Spike protein
RBD	Receptor-binding domain
N-Protein	Nucleocapsid protein
EUS	Endoscopic ultrasonography
RT-PCR	Reverse transcription polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
PBS	Phosphate-buffered saline
BSA	Bovine serum albumin

1 INTRODUCTION

1.1 Pancreatic cancer

1.1.1 *Global incidence and mortality*

Pancreatic cancer (PC) is highly lethal, with the incidence rate closely matching the mortality rate [1]. It is the fourth leading cause of cancer mortality in the United States [1]. In Sweden, it is estimated that over 1400 patients died of pancreatic cancer in 2017 [2], and approximately 330,400 people die of PC each year worldwide [3]. The yearly incidence rates of PC are on the rise, and it is expected to be the second cause of cancer mortality before 2030 [1,4]. Up to 91% of PC patients die within five years of diagnosis, and more than 50% of them die in the first six months after the diagnosis [1,5,6]. A substantial reason behind the high lethality of PC may be due to the lack of apparent symptoms and accurate diagnostic methods to detect the cancer, especially at an early stage, which results in mostly diagnosing PC in an advanced stage [7]. Consequently, patients with late-stage PC were reported to have a high recurrence rate even after potentially curative resection of the pancreas [1]. Biological characteristics and heterogeneity of the pancreatic tumor cells have contributed to PC´s resistance to radiotherapy and chemotherapy [8]. Postmortem examinations showed that about 90% of PC cases were compromised by distant invasion and metastasis [9].

Several risk factors are associated with increased risk of pancreatic cancer, such as cigarette smoking [10], obesity [11], diabetes mellitus [12], chronic pancreatitis [13], family history of pancreatic cancer [14], as well as periodontal disease [15]. Pancreatic cancer is an aging-associated disease that develops mostly in adults between 60 and 80 years old [1,5] with a 1.5 fold higher incidence rate in men than in women [5]. Yet, there is no standard screening program for patients at high risk nor a clinically significant biomarker for PC. Early detection of PC is an instrumental step to reduce PC mortality rate. Much remains to be explored about the pathogenesis, progression, diagnostic and therapeutic approaches for this cancer.

The pancreas consists of both an exocrine and an endocrine part. The exocrine component contributes to the digestion of proteins, fats, and carbohydrates, thereby enabling absorption; the pancreas's endocrine component produces and secretes different hormones such as insulin and glucagon. Pancreatic cancer mainly develops in the exocrine duct and originates from the epithelial cellular lining of pancreatic ducts. Pancreatic ductal adenocarcinoma (PDAC) is the most common form of malignant pancreatic tumors, and it is thought to arise from benign precursor lesions that are classified under pancreatic cystic neoplasms (PCN) [16].

1.1.2 Precursors to pancreatic cancer

1.1.2.1 *Pancreatic cystic neoplasm (PCN)*

Pancreatic cystic neoplasms (PCN) have become an increasingly common clinical finding as the cross-sectional imaging diagnostic modalities are becoming more sensitive [17–19]. Estimates from several studies show that the prevalence can be as high as 40% in the general population [18,20,21]. There are over 30 entities of cystic lesions in the pancreas known today, Kloppel and colleagues [22] have classified these into four groups. These groups are classified by the cystic lesion biology and morphology as: neoplastic epithelial, neoplastic non-epithelial, non-neoplastic epithelial, and non-neoplastic non-epithelial cystic lesions. Examples of common/uncommon PCNs are shown in **Figure 1**. The neoplastic epithelial lesions constitute around 90% of PCN [23] and include the intraductal papillary mucinous neoplasm (IPMN), mucinous cyst neoplasms (MCNs), serous cystadenomas (SCA), and others. These three major subgroups (IPMN, MCN, and SCA) represent about 85% of all PCN, of which IPMN constitutes up to 45% [24–26].

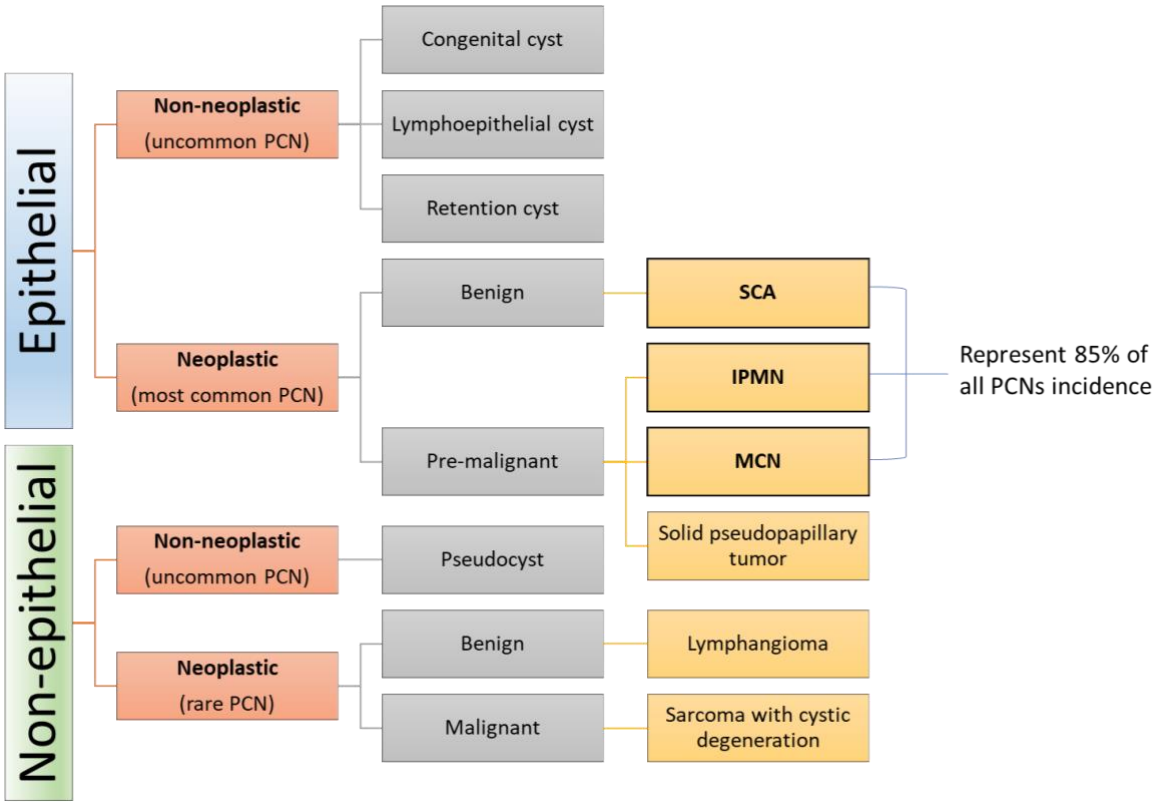

Fig. 1. Classification of pancreatic cyst neoplasms (Adapted from Chandwani, R et al,. 2016 [24])

1.1.2.2 *Intraductal papillary mucinous neoplasm (IPMN)*

One of the most common PCNs is the intraductal papillary mucinous neoplasm (IPMN) [23,27]. IPMN is epithelial neoplastic cyst in the pancreas's ductal systems distinguished by the papillary projection of proliferated epithelial cells and mucin secretion that leads to dilated pancreatic duct [28]. IPMN can arise in the main and side branches of pancreatic ducts, in the head, body, and tails of the pancreas [23]. They may be localized, diffused, or multifocal distributed in the pancreatic ducts [23].

IPMNs are known by their association with pancreatic malignancies, and it is believed that the dysplastic pattern of IPMN can progress from low-grade dysplasia (LGD) to high-grade dysplasia (HGD) and then transform to invasive carcinoma [27,29,30]. The growing incidence of IPMN and its clear tendency to progress to invasive cancer led to great attention and efforts to detect it in its benign stage (LGD), which fortunately constitute most cases and does not require surgical intervention [17,23,28]. Preoperative diagnostic accuracy is still not entirely reliable, and the guidelines of whether to resect the pancreas surgically as a prophylactic procedure or follow up the case conservatively are still debated [17]. Thus, the surgical resection and routine follow-up are indicated for most IPMNs patients as the final diagnosis can only be obtained histologically after operation [17,28].

1.1.2.3 *Mucinous cyst neoplasm (MCN)*

MCNs are notably less frequent than IPMNs, with a majority of the incidence occurring in women [23]. In two extensive studies, the average of patients' age at diagnosis was 45 and 48 years, with a predominant incidence in women in 95% of the cases [31,32]. As with IPMNs, MCNs produce mucin within the cysts and are classified according to the degree of dysplasia of the epithelial lining into low-grade dysplasia and high-grade dysplasia. MCNs also can potentially develop invasive carcinoma, which is shown histologically in about a third of surgically resected cases [33,34]. MCNs can be histologically distinguished from IPMN and SCA by ovarian-like stroma underlying the epithelial layer [32].

1.1.2.4 *Serous cyst adenoma (SCA)*

SCAs are benign microcysts, which are characterized histologically by their cuboidal epithelium and thick walls [24]. The fluid within the cyst is clear and thin and featured by the absence of carcinoembryonic antigen (CEA), a marker found to be elevated in mucinous cysts (e.g., IPMN and MCN) [35,36]. SCA constitutes approximately 16% of all PCNs, and they possess very diminished malignancy transformation risk or invasive behaviour [37]. Thus, surgical

intervention is not recommended, as the risk of mortality of pancreatic resection (2%) exceeds the risk of malignant transformation (>1%) [17,38].

1.1.3 *Oral bacteria and pancreatic cancer*

Several studies over the past decades suggested that microbiota play oncogenic roles and influence therapeutic responses in different tumours [39–43]. In a recent study by Pushalkar et al., the increased relative abundance of bacteria in the pancreas and microbial dysbiosis in the gut were found to promote pancreatic tumorigenesis and induce immune suppression both in mice and humans [44].

Distinct strains of oral commensal bacteria were found to be associated with distal tumor microenvironments in colorectal cancer [45,46]. Table 1 presents several epidemiological studies that have investigated the association between oral microbiota and pancreatic cancer. In a study conducted in 2017, oral microbial dysbiosis was noted to occur prior to the development of pancreatic cancer [47]. Furthermore, the link between pancreatic cancer and a history of periodontal disease or periodontal disease pathogens has also been reported in case-controlled studies [15,48–50]. Consistent with these observations, periodontal disease pathogens and an increased level of plasma antibodies against distinct oral pathogens were related to an increased risk of pancreatic cancer [49,50]. Fan, X. et al., reported in a large American study that the relative abundance of *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in saliva was positively associated with the risk of PC. In contrast, the salivary richness in phylum Fusobacteria and its genus *Leptotrichia* appeared to decrease the risk of developing PC [50]. The latter was found at the phylum and genus level without information shown at the species or strain level.

1.1.4 *Fusobacterium nucleatum and cancer immunity*

Despite the vast global microbial diversity, most microbiological studies have focused for decades on a few important taxa because of the challenges in cultivation and the high cost of genetic sequencing. This limited information makes the unstudied taxa all the more important [51]. Advanced sequencing techniques, such as metagenomics, revealed a vast diversity of microorganisms and helped to better understand many of these understudied microbes and their contribution to human diseases [52]. Among them, Fusobacteria phylum remains a prime example of understudied microorganisms [52].

Table 1. Epidemiological or cohort studies investigating the association between oral microbiota and pancreatic

Authors, Publication year	Study country	Sample type, Detection method	Comparison, Sample size	Outcome
Farrell et al., 2012 [53]	USA	Saliva, 16S gene qPCR	Pancreatic cancer (n= 38) Chronic pancreatitis (n= 27) Healthy control (n= 38)	Significant variation of salivary microbiota between pancreatic cancer and healthy controls. *G. adiacens* and *Streptococcus mitis* showed significant variation between chronic pancreatitis and controls
Michaud et al., 2013 [49]	Europe	Blood antibodies, immunoblot array	Pancreatic cancer (n= 405) Healthy control (n= 416)	High antibodies level against *Porphyromonas gingivalis* associated with increased PC risk
Torres et al., 2015 [54]	USA	Saliva, 16S rRNA gene sequencing (V3-V4)	Pancreatic cancer (n= 8) Non-pancreatic disease/cancer (n= 78) Healthy control (n= 22)	Higher ratio of Leptotrichia to Porphyromonas in pancreatic cancer compared to healthy and non-pancreatic diseases groups
Fan et al., 2016 [50]	USA	Oral wash, 16S rRNA gene sequencing (V3-V4)	Pancreatic cancer (n= 361) Healthy controls (n= 317)	Relative abundance of *P. gingivalis* and *Aggregatibacter actinomycetemcomitans* was associated with high risk of PC. Fusobacteria and its genus *Leptotrichia* appeared to decrease the risk of developing PC.
Olson et al., 2017 [55]	USA	Saliva, 16S rRNA gene sequencing (V4-V5)	Pancreatic cancer (n= 40) IPMN (n= 39) Healthy controls (n= 58)	No significant difference in microbial diversity between PC and IPMN or controls. Higher relative proportions of Firmicutes in PC, and Proteobacteria in healthy controls
Wei et al., 2020 [56]	China	Saliva, 16S rRNA gene sequencing (V3-V4)	Pancreatic cancer (n= 41) Healthy controls (n= 69)	Carriage of *Streptococcus* and *Leptotrichia* was associated with a higher risk of PC

Fusobacteria phylum is currently classified into two families: the *Fusobacteriaceae* family and the *Leptotrichiaceae* family [52]. They are anaerobic, rod-shaped bacteria with unique metabolic capabilities; for example, *Psychrilyobacter atlanticus* possess metabolic capability that can break down nitramine explosives [57].

Fusobacterium nucleatum (*F. nucleatum*) is an emerging pathogen species under the *Fusobacteriaceae* family, and one of the most abundant bacteria in the oral cavity [58–60]. *F. nucleatum* is a highly heterogeneous species with four recognized subspecies; *ss nucleatum, ss animalis, ss vincentii* (*fusiforme*) and *ss polymorphum*. Among those four subspecies, *ss nucleatum* appears more frequently associated with disease [61,62]. *F. nucleatum* is non-motile, gram-negative, opportunistic oral anaerobe and known to play an integral part in biofilm formation of dental plaque [63,64]. With its unique elongated rod shape and multifunctional cell membranes proteins, *F. nucleatum* can adhere and coaggregate with many other microbial cells and build infrastructure bridge between early and late colonizers to create multispecies colonies [63–65]. However, *F. nucleatum* is normally found in the mouth and other mucosal sites and it has been isolated from healthy tissues, indicating that they are a natural commensal member of oral microbiome [59,60,66,67]. Under diseased conditions, *F. nucleatum* is referred to as an opportunistic pathogen as it has been frequently isolated from diseased tissues. In fact, *F. nucleatum* is one of the most commonly isolated oral bacteria in oral and extra-oral diseases [68–71]. This association of *F. nucleatum* with many extra-oral diseases is accumulative, but the pathogenesis remains unclear [71].

F. nucleatum is gaining attention from the scientific community recently due to its over-representation in many cancer types, such as oral, gastric, colon and pancreatic tumors [45,46,72–75]. Although *F. nucleatum´*s etiopathological role remains unclear, several studies identified specific proteins as the driving virulence factors in the pathogenesis and tumorigenesis of *F. nucleatum* in colorectal cancer [76–78]. *F. nucleatum* Fap2 protein plays a critical immunomodulating role by binding to TIGIT receptors and inhibiting NK cell cytotoxicity and other T cells [77]. The FadA is another active virulence factor that has been identified as a mediator for *F. nucleatum* adhesion and invasion through binding to E-cadherin and activation of the β-catenin signaling pathway, leading to elevated expression of inflammatory and proliferation factors in epithelia cells, which promotes colorectal carcinogenesis (**Figure 2**) [78].

Consequently, further studies are needed to investigate the role of oral bacteria in IPMN and the immune responses against these bacteria, which may provide a potential opportunity for early diagnostic biomarkers for invasive pancreatic tumors.

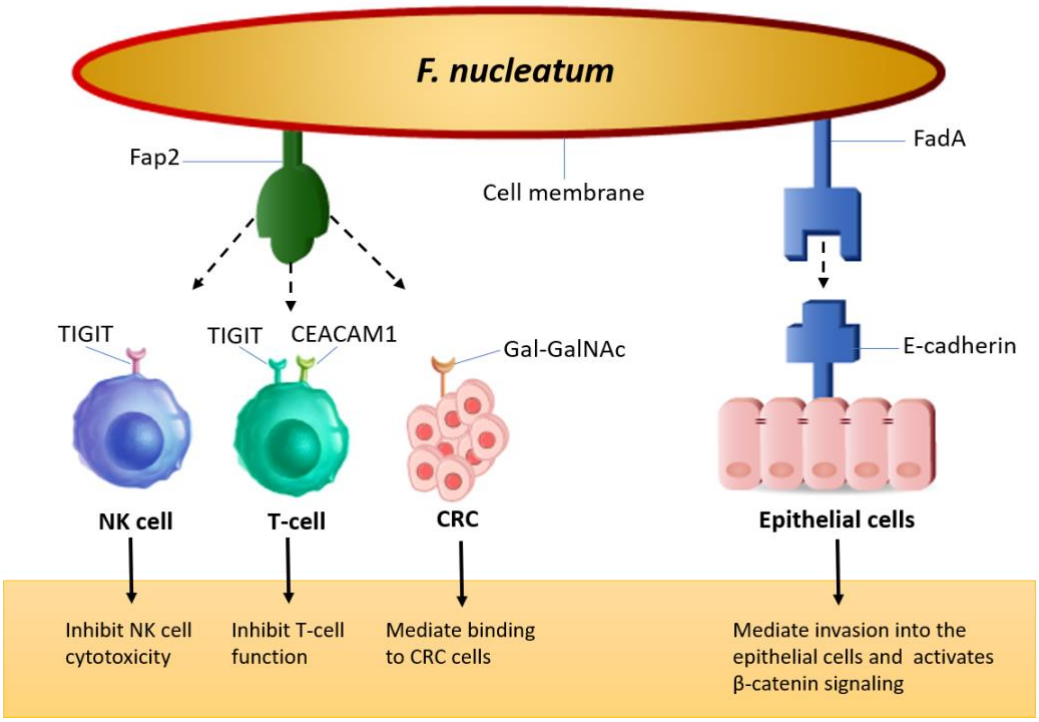

Fig. 2. An overview of *F. nucleatum* virulence mechanisms. Fap2: fusobacterium autotransporter protein 2; FadA: Fusobacterium adhesin A; TIGIT: T-cell immunoglobulin and ITIM domain; CEACAM1: carcinoembryonic antigen-related cell adhesion molecules 1; Gal-GalNAc: D-galactose-β (1–3)-N-acetyl-D-galactosamine; NK: natural killer; CRC: Colorectal cancer (REF [76-78]).

1.2 COVID-19

1.2.1 *Global incidence and mortality*

In the 21st century, three emerging strains of the diverse coronaviruses group outbroke in humans from assumed zoonotic origin, causing mild to severe respiratory infections. The first outbreak was in 2002 with severe acute respiratory syndrome coronavirus (SARS- CoV), the second was in 2012 with the Middle East respiratory syndrome coronavirus (MERS- CoV), and the third was in 2019 with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the cause of coronavirus disease 2019 (COVID-19) [79].

The SARS-CoV-2 outbreak occurred in an unprecedented transmission rate since it was first reported in Wuhan, China in late December 2019 [80] and was declared a pandemic by WHO on 11 March 2020 [81]. As of April 2021, COVID-19´s global morbidity reached over 128 million confirmed cases and a mortality of up to 2.8 million from all six continents [82].

1.2.2 *SARS-CoV-2 infection and transmission*

Although genetic analysis showed that SARS-CoV-2 is a natural virus of animal origin and clustered with betacoronavirus found in bats and pangolin [83,84], there is no solid evidence yet about the intermediate host and when and where this novel virus transmitted to humans. As in SARS-CoV and MERS-CoV, SARS-CoV-2 targets the angiotensin-converting enzyme 2 (ACE2) receptor and utilizes it to enter host cells [85–87]. The virus envelope spike (S) glycoprotein mediates SARS-CoV-2 entry into cells and is divided into two functional proteins: S1 protein (which includes the receptor-binding domain (RBD) mediates the binding to ACE2 receptor and S2 protein mediate the adhesion of SARS-CoV-2 to host cell membrane as shown in **Figure 3** [86,88]. Biochemical analysis found that the spike protein of SARS-CoV-2 evolved and gained a higher binding affinity from its previous ancestor SARS-CoV, which can explain the higher transmission ability of this later virus [89,90]. However, another study reported a similar binding affinity of SARS-CoV-2 compared to the previous SARS- CoV [91]. The oro-nasal route is considered the main entry gates for SARS-CoV-2, and the ACE2 receptors have also been found to be highly expressed in the mucosa of nasal, pulmonary, as well as oral epithelial cells [92–95].

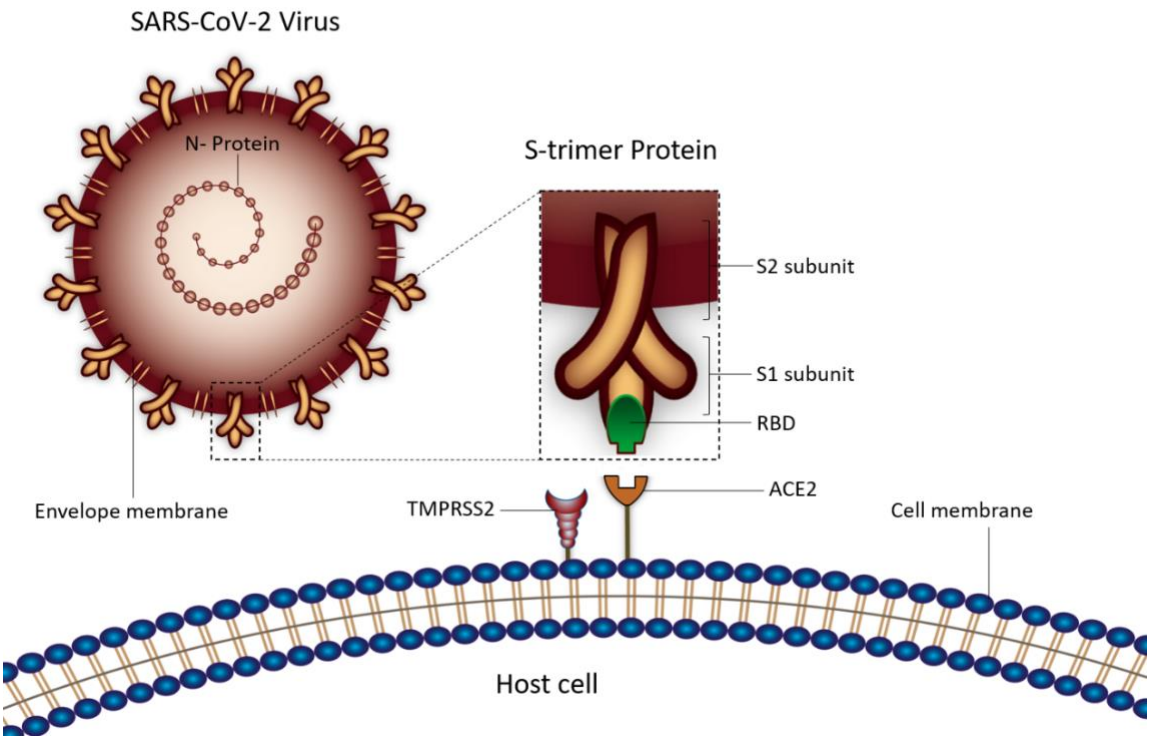

Fig. 3. Schematic overview of SARS-CoV-2 spherical structure showing the S-trimer protein in pre-fusion conformation and N-protein (complexed with the viral RNA genome). TMPRSS2 prime the S-protein to allow virus-cell fusion and cell entry. The S1 protein contains the RBD protein, which mediates binding to the ACE2 host cellular receptor. SARS-CoV-2: severe acute respiratory syndrome coronavirus 2; S-Protein: spike protein: N-Protein: nucleocapsid protein; RBD: receptor-binding domain; ACE2: angiotensin-converting; TMPRSS2: transmembrane serine protease 2 (adapted from REF [86-89]).

1.2.3 *Oral manifestations in COVID-19*

The SARS-CoV-2 transmission can occur through the oral cavity, and ACE2 receptors are expressed in the mucosal of oral epithelial cells [94,95]. Recently, Huang et al. reported that even acellular and cellular salivary fractions from asymptomatic individuals may transmit SARS-CoV-2 ex vivo [95]. This finding proposes a potential infection-related inflammatory response in the oral cavity organs and tissues, such as the salivary glands, tongue, and other oral sensory nerve endings. In fact, quantitative and qualitative impairment of taste bud sensitivity and dysfunctional gustatory responses are unique primary symptoms of SARS-CoV-2 infection, and is reported in 60% of patients with COVID-19. This impairment of taste bud sensitivity and dysfunctional gustatory responses were not reported in SARS and MERS infections [96–98]. Other case report studies observed various oral signs and symptoms related to SARS-CoV-2 infection, such as non-specific oral ulcerations, desquamative gingivitis, and fungal candidiasis [98–100]. However, there is no clear evidence whether these oral manifestations are directly related to SARS-CoV-2 infection or only as consequences of the compromised immune system or adverse reactions of pharmacotherapy [101].

1.2.4 *Mucosal immunity to SARS-CoV-2*

Mucosal immunity forms the largest part of the entire immune system and refers to lymphocytes and antigen presenting cells in the epithelia of gastrointestinal and respiratory tracts [102]. Antigen-activated B-lymphocytes, called plasma cells, secrete the antibodies into the lumen of the respiratory or gastrointestinal tract [102]. Antibodies bind to microbes and toxins to prevent them from infecting cells through several effector mechanisms such as neutralization of microbes and toxins, coating (opsonize) microbes and transporting them for phagocytosis, and activation of the complement system that promote phagocytosis and destruction of microbes [102]. The predominant antibody isotype produced in mucosal tissues is IgA (SIgA), and because of the large surface area of the mucosal organs, SIgA constitutes approximately 2 out of 3 grams of total antibody produced daily by healthy individuals [103]. SIgA is produced by plasma cells in the lamina propria and migrated through the epithelial layer to bind to and neutralizes microbes in the lumen of mucosal organs [104].

In general, the first protective barrier against microbial invasion is the mucosal surface of the respiratory and digestive systems [105]. As SARS-CoV-2 primarily infects the upper respiratory tract, mucosal immune responses are expected to be induced via nasopharynx-associated lymphoid tissues, which refers to the adenoids, tonsils, and lining epithelium in the nasopharynx area [102]. Other possible mucosal inductive sites were also reported in the bronchial lymphoid tissue in children [106], lacrimal duct [102], and

the oral cavity [107]. Bronchial lymphoid tissue is an effective inductive site of mucosal and systemic immune responses. However, it is normally involuted in the lungs of healthy adults [106], which raises a very important question about their potential instrumental contribution in the reported resistance to SARS-CoV-2 infection in children and adolescents patients [108].

1.3 Molecular signature and disease biomarker

1.3.1 *Biomarker definition*

Biomarker is not a concept that is exclusive to modern medicine. The need for biomarkers to diagnose illness and diseases has been recognized in the history of medicine for centuries. Before 400 BC, ancients physicians used body fluids to diagnose diseases. Urine that attract insects was used as a way to diagnose patients with boils [109].

Empirical sophisticated diagnostic techniques have come a long way and installed biomarkers as an imperative cornerstone of the modern healthcare system. Biomarkers are a vital part of healthcare development, offering comprehensive application tools for disease diagnosis, prediction, and prognosis. The term of biomarker is defined at the joint leadership conference of the U.S. Food and Drug Administration (FDA) and the National Institutes of Health (NIH) [110] as "A defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes, or biological responses to an exposure or intervention, including therapeutic interventions. Biomarkers may include molecular, histologic, radiographic, or physiologic characteristics. A biomarker is not a measure of how an individual feels, functions, or survives." and categorized into

- risk biomarker
- diagnostic biomarker
- monitoring biomarker
- prognostic biomarker
- predictive biomarker
- pharmacodynamic/response biomarker
- safety biomarker.

There are many types of biomarkers. Molecular biomarkers (e.g., genes, proteins, metabolites), imaging-based biomarkers (e.g., magnetic resonance images (MRI), X-ray), and other measurable biomarkers such as body weight, temperature, and heart rate. Molecular biomarkers play an integral role in the diagnosis and therapy guidelines in the modern healthcare system and even more so with increasing advancement in laboratory technologies.

1.3.2 *Molecular biomarkers*

From nucleic acid to protein, molecular biomarkers have been routinely used to diagnose health conditions in the current healthcare system. The molecular biomarker is defined as the candidate biomarker for a disease that can be detected based on a platform such as genomics and proteomics technologies [111]. Although the importance of molecular biomarkers has been increasingly recognized in recent years, their validation is a lengthy process, so that only a few biomarkers have so far been routinely used in clinical practice [112,113]. As a general rule, the most sensitive and specific biomarker is of limited clinical use if it cannot be approached in the least invasive manner possible.

1.3.3 *Current biomarkers for pancreatic cystic neoplasms*

The absence of a predictive biomarker and validated screening program for patients at high risk of developing PC limited the ability for early detection of PC. However, there is emerging data on biomarkers in the past two decades with the advancement of cross-sectional imaging modalities and molecular diagnostic techniques. Identification of the cystic precursor to PC is of primary importance for preventive intervention that would improve the poor prognosis of PC.

1.3.3.1 *Imaging-based biomarkers*

The advancement in cross-sectional imaging provided tremendous help in the detection of pancreatic neoplasms. The most commonly used imaging modalities are computed tomography (CT) and magnetic resonance imaging (MRI). MRI is the preferred method for fellow-up of PCN [114]. Endoscopic ultrasonography (EUS) is commonly employed as an adjunct diagnostic tool to improve the accuracy of the initial cross-sectional imaging diagnosis or to obtain a cyst fluid sample for cytology and biochemical analysis [17]. Despite advancements in cross-sectional imaging, radiographic imaging is not yet able to provide a definitive diagnosis[24]

1.3.3.2 *Cystic fluid analysis*

To improve the diagnostic accuracy of PCNs, EUS with fine-needle aspiration is commonly indicated as an adjunctive procedure for cytopathological examination [17]. Multiple markers have been studied, including the carcinoembryonic antigen (CEA) [115], carbohydrate antigen (CA) 19-9 [115], K-ras, and amylase. For example, CEA level is used for the differentiation between mucinous and non-mucinous PCN [36], an increased level of CA 19-9 is associated with the increased risk of malignancy [17] and the presence of amylase in the cystic fluid is a sign to exclude pseudocyst (as it has no connection with pancreatic ducts) [116]. However, these tumor markers suffer from low sensitivity and specificity and are not considered as screening tools for PC [117]. Similarly, cytological studies based on cyst fluid also appeared unreliable as the fluid may have low cellularity and extracellular contamination [116].

1.3.3.3 *Cancer mutation*

Cancers are driven by an accumulation of activated oncogenes and tumor suppressors by mutations or overexpression. Several gene mutations have been implicated in pancreatic neoplastic cyst formation. These include KRAS, p53, GNAS and SMAD4 [118]. KRAS and GNAS mutations showed high

sensitivity in detecting IPMN in highly sensitive techniques, such as next-generation sequencing (NGS) [119], yet appeared suboptimal in their specificity for differentiating invasive pancreatic tumors [120].

1.3.4 *Current biomarkers for COVID-19*

The use of COVID-19 biomarkers in the current pandemic state is not only important for pandemic management but also are necessary for developing therapeutics and preventive measures including vaccination. Detection and quantification of the virus or the virus-specific antibodies are presently standard analytical methods used in COVID-19 diagnostics. There are mainly PCR-based assays [121] or immunological assays [122,123]. The current gold standard for COVID-19 diagnosis is real-time RT-PCR detection of SARS-CoV-2 RNA using samples taken from upper respiratory (naso-pharyngeal swab with or without saliva)[121,124]. Methods based on saliva specimens were found as a sensitive alternative for diagnosis of asymptomatic and mild SARS-CoV-2 infection [125,126]. Other tests based on the detection of viral antigens also exist as rapid screening alternatives [127] and the available tests today are both laboratory-based or self-performed using lateral-flow devices.

1.3.4.1 *Biomarkers of COVID-19 severity*

Routine laboratory tests applied in clinical settings have been analyzed to assess their association with COVID-19 severity. Elevated concentrations of serum biomarkers such as urea, creatinine, cystatin C were associated with severe cases of SARS-CoV-2 infection [128]. Inflammatory biomarkers such as C-reactive protein, procalcitonin, IL-6 were also reported to be significantly increased in severely infected patients compared with non-severe cases [93,129]. Critical risk factors that could be used as biomarkers for COVID-19 severity include the patient's age, as higher ACE2 density was found to be positively correlated with age [93]. However, all of the previous findings were based on retrospective and cross-sectional studies that are vulnerable to confounding factors and need validation.

2 RESEARCH AIMS

2.1 General aim

The general aims for studies I and II of this thesis were to investigate molecular and immunological biomarkers (in plasma, cyst fluid, pancreatic tissue, and saliva) in patients diagnosed with PCN, and the microbiome association with a particular interest in IPMN.

The aim for Study III was to investigate the potential use of salivary antibodies as an immunological marker for exposure to SARS-CoV-2.

2.2 Specific aims

Study I

To investigate pancreatic cyst fluid and plasma in patients diagnosed with PCN, with regard to potential pancreatic microbiome, inflammation, and their relation to disease severity.

Study II

To investigate humoral responses to oral microbes in the plasma and saliva of patients diagnosed with PCN, including response to *F. nucleatum* Fap2.

Study III

To investigate salivary antibody immunity against viral proteins of SARS-CoV-2 in individuals with or without COVID-19 diagnosis.

3 MATERIALS AND METHODS

3.1 Ethical considerations

The studies performed here were approved by the Swedish Ethical Review Authority and complied with the declaration of Helsinki. All studies participants signed an informed consent form prior to study participation.

3.2 Patients recruitment

In study I, a total of 105 patients undergoing surgical pancreatectomy for suspicion of high-risk pancreatic cyst tumors were recruited from 2017-2019 at Karolinska University Hospital, Stockholm, Sweden. In study II, a total of 109 plasma and 65 paired saliva samples (among them 8 saliva samples from healthy individuals) were collected from patients undergoing surgical pancreatectomy for suspicion of high-risk pancreatic cyst tumors were recruited from 2017-2019 at Karolinska University Hospital, Stockholm, Sweden. The patients in study I and II were stratified according to post-operative histopathological diagnosis into non-IPMN, LGD-IPMN, or HGD-IPMN and cancer.

In study III, a total of 256 saliva samples were collected and stratified into three cohorts. Cohort 1: included 74 samples from convalescent COVID-19 patients collected from June to December 2020; cohort 2: included 147 samples from undiagnosed individuals who donated their saliva between May-Nov 2020; cohort 3: included 35 pre-pandemic saliva samples collected from 2018 before the COVID-19 outbreak. Samples in study III were collected from participants at Karolinska University Hospital, Dental Clinics of Karolinska Institutet and Eastman Institute, Stockholm, Sweden.

3.3 Self-reported questionnaire and examination

In study II, a self-reported questionnaire was used for all participants who donated saliva (n=57). This questionnaire was composed of eight closed-ended questions to assess the oral health condition. The questions were formulated based on the CDC-AAP case definitions for surveillance of periodontitis [130]. Clinical dental health examination was performed for healthy participants (n= 8) without known PCN diagnosis to provide better control baseline data for subsequent analysis. The clinical dental examination was performed by recording full-mouth bleeding on probing (BOP), periodontal pocket depth (PPD), plaque index (PI) and number of missing teeth.

In study III, self-reported questionnaire was used to collect COVID-19-related information from participants. Data related to potential exposures, including self-experienced symptoms in the past three months, and various risk factors such as travel abroad, exposure to COVID-19, clinical work were collected.

3.4 Blood collection

In study I and II, peripheral venous blood sample were collected in 10 ml K2 EDTA tubes (BD Vacutainer) and centrifuged for plasma (20 min at 2000 g at room temperature) in Ficoll Paque PLUS (GE Life Sciences) density gradient according to manufacturers' instructions, then immediately stored at −80°C.

3.5 Saliva collection

In study II, stimulated saliva was collected in the evening before pancreas surgery. The participants were asked to refrain from eating, drinking, smoking, or using oral hygiene products for at least 1 h prior to collection. Stimulated whole saliva was collected in sterile 50 mL polyethylene tubes and stored immediately stored at −80◦C in 1.5 mL aliquots.

In study III, unstimulated saliva samples were self-collected in 2 ml tubes using standardized instructions given by this study, processed and stored at -80°C within 24 h.

3.6 Microbial DNA extraction

Microbial DNA was isolated in study I from intracystic fluid and plasma; and in study II from bacterial culture or saliva using ZymoBIOMICSTM DNA Mini Kit (Zymo Research, Irvine, California, United States) according to the manufacturer's instructions in a biological class II flow cabinet. In study I, DNA was also extracted from formalin-fixed paraffin-embedded (FFPE) pancreatic tissue slices using the AllPrep DNA/RNA FFPE Kit (Qiagen, Sollentuna, Sweden). All isolated DNA were stored at −20°C until use.

3.7 Sequencing and quantification of bacterial DNA

In studies I and II, universal bacterial 16S DNA gene copy number was amplified by TaqMan qPCR using forward primer: 5'- TGGAGCATGTGGTTTAATTCGA-3, and reverse primer: 5'-TGC GGGACTTAACCCAACA-3', as 16S probe was 5'-FAM-CACGAGCTGACGACA[A/G]CCATGCA-TAMRA-3' [131]. In study I, cyst fluid microbial composition and diversity was assessed by full-length 16S rRNA gene sequencing PacBio Single Molecule, (GATC Biotech, Konstanz, Germany). For gDNA quantification of *F. nucleatum* (studies I, II), and *Granulicatella adiacens* (*G. adiacens)* (study I), primers used for *F. nucleatum* 16S gene were: Forward 5'-AGGGTGAACGGCCACAAG-3', Reverse: 5'-TCTCGGTCCATTGTCCAATATTCC-3' and probe 5'-FAM- ACACGGCCCTTACTCC -TAMRA-3' [132,133]. Primers for *G. adiacens* were: Forward 5'- CAAGCTTCTGCTGATGGATGGA-3', Reverse 5'-CTC AGGTCGGCTATGCATCAC-3', and 5'-FAM- GCTAGTTGGTGAGGTAACGGCTCA-TAMRA-3' was used as probe [53].

3.8 Bacterial cell isolation and inactivation methods

In study II, microbial strains of *Streptococcus gordonii* (*S. gordonii*), *Streptococcus anginosus* (*S. anginosus*), *G. adiacens*, and *Escherichia coli* (*E. coli*) were isolated from pancreatic cyst fluid samples by cultivation and identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Bremen, Germany) profiling (Study I), thereafter cultured on blood and hematin agar plates. *F. nucleatum* and *P. gingivalis* strains were isolated from clinical blood cultures, identified in standard clinical routine by MALDI-TOF MS, then cultured on blood agar in anaerobic milieu 48 h at 37°C. The plates were examined after 24 h. Liquid cultures were prepared with glycerol (20% final concentration) prior to storage at −80°C. For enzyme-linked immunosorbent assay (ELISA) analysis, the bacteria preparations were pre-treated by heat-inactivation. Briefly, the bacteria pelleted at 1×10^9 CFU were washed twice in PBS and heat-inactivated at 85°C for 1 h. The *F. nucleatum* Fap2 (Fap2) mimotope TELAYKHYFGT as described earlier by prediction analysis (The Immune Epitope Database (IEDB) Analysis Resource) [134], was synthesized to 98% purity (Genscript, New York, United States). Of note, an ELISA optimization was first performed with either heat- or formalin-inactivated bacteria preparations of *F. nucleatum* (1×10^8 CFU/ml and 1×10^7 CFU/ml) and plasma samples in serial dilutions at 1:300, 1:1500, 1:7500. The heat inactivation method using *F. nucleatum* concentration of 5×10^7 CFU/mL, and 1:300 plasma dilution were selected for the subsequent analysis**.**

3.9 ELISA assays

In study II, the heat-killed bacterial preparations were prepared for antibody analysis. The *F. nucleatum* and Fap2 mimotope were the antigens of interest. *P. gingivalis*, *S. gordonii*, *S. anginosus*, and *G. adiacens* were included as oral bacterial controls and *E. coli* as a non-oral bacterial control. Indicated bacteria were diluted to the concentrations of 5×10^7 CFU/mL and the mimotope at 10 µg/mL in coating buffer (sodium carbonate buffer 50 mM, pH 9.6). Thereafter, 100 µL of the antigen were added to each well of a Nunc MaxiSorp™ 96-well ELISA plate (Sigma-Aldrich Sweden AB, Stockholm, Sweden) for overnight incubation at 4°C. The antigen-coated wells were washed three times with washing buffer (0.05% Tween-20 [VWR Chemicals, Spånga, Sweden] in PBS), and blocked by blocking/dilution buffer (1% BSA and 2% goat serum [Sigma-Aldrich, G6767] in PBS) and incubated for another 1 h at 37°C and washed before addition of sample diluents. Diluents of plasma (1:300) and saliva (1:16) respectively, were added in duplicate wells and incubated for 1 h at 37°C. After washing, the wells were incubated with a secondary antibody the goat anti-human IgG/IgA conjugated with peroxidase diluted 1:10000 (Sigma-Aldrich Sweden AB) for 1 h at 37°C. Thereafter the substrate tetramethylbenzidine (R&D Systems, Minneapolis, Minnesota, United States) was added for 20 min and the reaction was stopped with the addition of 0.16 M sulfuric acid. The optical density was read at 450 nm (Multiskan MS, Thermo Labsystems, Vantaa, Finland). The total IgG and IgA detection was also performed, in which samples were diluted to 1:125 000 and 1:5000 for plasma and saliva, respectively, and detected as above as for specific antibodies. Internal controls consisting of high-reactive and low-reactive

patients' plasma or saliva samples were included in each plate. The OD measurements of internal controls were used to calculate the inter/intra-assay coefficient of variability for all runs. Inter-assay coefficient of variability was 8.5 ± 4.5% and 10.4 ± 2.3%, respectively, for plasma and saliva assays, while the intra-assay coefficients of variability were 8 ± 2.3% and 8.7 ± 1%, respectively.

The competitive ELISA assay was performed by first coating plates with Fap2 peptide overnight. On the following day, saliva samples with or without pre-incubation with *F. nucleatum*, Fap2, or the *E. coli* control (2 h at 37◦C) were added to the Fap2 peptide-coated plates and analyzed as above (**Figure 4**). Total salivary and plasma IgA and IgG antibodies were determined using Human IgA/IgG ELISA Kit (Novus Biologicals, Colorado, United States) according to the manufacturer's instructions.

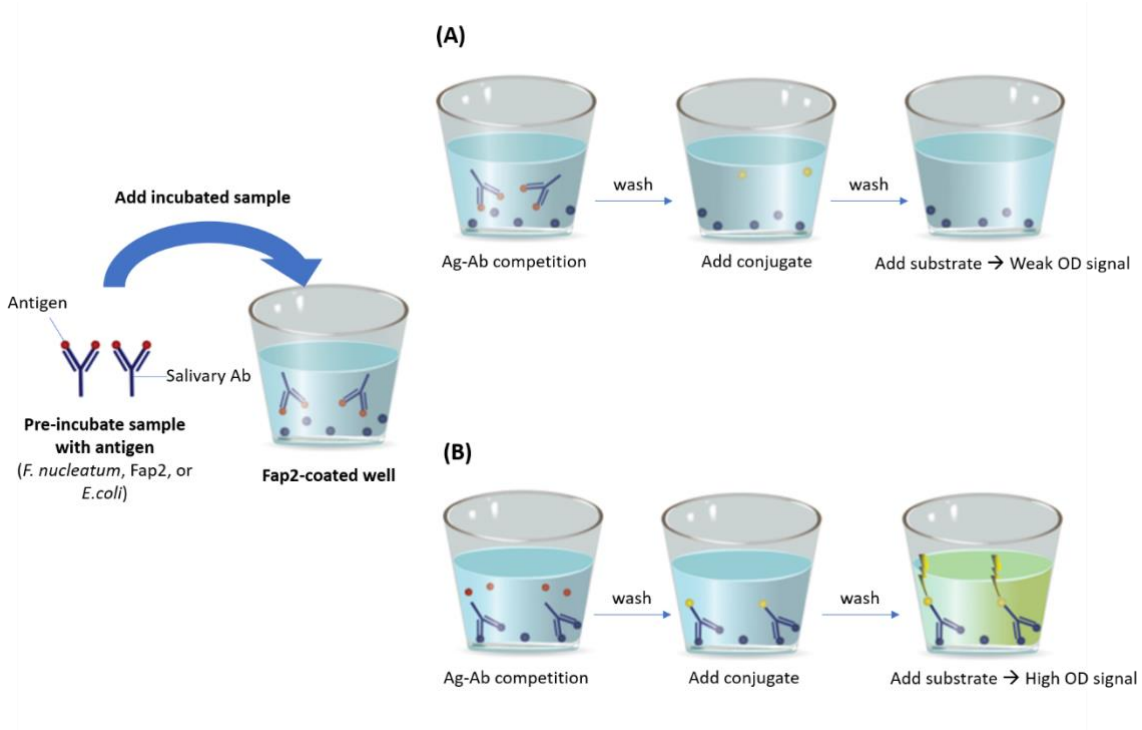

Fig. 4. Principle for the competitive ELISA used in this thesis. Pre-incubated saliva sample with indicated antigen was added to the Fap2-coated wells and allowed for Ag-Ab competition. When the pre-incubated antigen outcompetes the Fap2-coated antigen in binding to the salivary antibodies, the concentration of the salivary antibodies will be low after wash **(A)**. When the Fap2-coated antigen outcompetes the pre-incubated antigen in binding to the salivary antibodies, the concentration of the bound salivary antibodies will be high after wash **(B).** Fap2: fusobacterium autotransporter protein 2; Ag: antigen; Ab: antibody; OD: optical density.

3.10 Multiplex bead-based immunoassay

In study III, salivary antibodies against SARS-CoV-2 were analyzed by a multiplex bead-based immunoassay (**Figure 5**). The assay was optimized first using a panel of five SARS-CoV-2 protein antigens: 1) spike trimers comprising the prefusion-stabilized spike glycoprotein ectodomain

(expressed in human embryonic kidney (HEK) cells and purified using a C-terminal Strep II tag); 2) spike S1 domain (Sino Biological, expressed in HEK and purified using a C-terminal His-tag); 3) spike RBD domain (expressed in HEK cells and purified using the mFc C tag); 4) full -length nucleocapsid protein; 5) nucleocapsid C-terminal chain. Both nucleocapsid were expressed in *E.coli* and purified using a C-terminal His-tag [135,136]. All antigens were diluted to 80 µg/ml (in 2-(N-morpholino) ethane sulfonic acid buffer, pH 4.5 [SigmaAldrich]) and coated on specific color-coded bead type (bead ID) (MagPlex-C, Luminex corp.). The antigen-suspended beads were pooled to form the bead array. Anti-human IgG (309-005-082, Jackson Immunoresearch) and anti-human IgA (800-338-9579, Bethyl) labeled on the surface of microspheres, and the EBV EBNA1 protein (ab138345, Abcam) were added as a sample loading control. Saliva samples were diluted 1:5 in assay buffer (3% bovine serum albumin [w/v], 5% non-fat milk [w/v], 1×PBS supplemented with 0.05 % [v/v], and Tween20 [VWR, 437082Q]) and incubated with the bead array for 1 h at room temperature and 650 RPM rotation. Afterward, the antigen-antibody complexes were cross-linked by adding 0.2% paraformaldehyde (AlfaAesar, 30525-89-4) in PBS 0.05% Tween 20 (PBS-T) for 10 min at room temperature. Salivary IgG or IgA that were captured on the antigen-coated beads were detected by the fluorescent R-phycoerythrin-conjugated anti-human IgG (H10104, Invitrogen) diluted 0.4 µg/mL, or R-phycoerythrin-conjugated anti-human IgA (800-338-9579, Bethyl) diluted 0.2 µg/mL in PBS-T, respectively, after 30 minutes incubation at room temperature. The read-out consisted of the bead-based median fluorescent intensity (MFI) using a FlexMap3D system and the xPONENT software (Luminex Corp.).

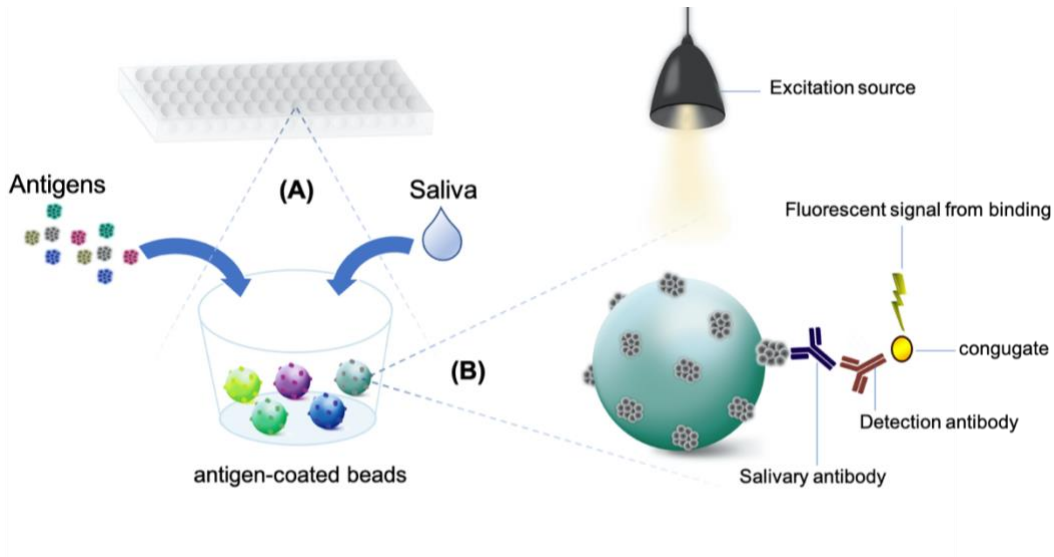

Fig. 5. Illustration principle for the multiplex bead-based immunoassay used in this thesis. The saliva sample is added to a mixture of color-indexed beads pre-coated with indicated antigens **(A)** to form antigen-antibody complexes **(B)**. The captured salivary antibody is detected by the secondary antibody conjugated to transmit a reporter fluorescent signal. The emitted light from the reporter signal is measured and the antigen specificity is determined by the color of the bead.

3.11 Statistical analysis

Statistical analyses were mainly performed with GraphPad Prism (version 8.00 for Windows, GraphPad Software, La Jolla, CA, United States). In study I, pairwise statistical comparisons between each group were performed using Fisher's exact test (for qualitative parameters), and Kruskal–Wallis test with Dunn's multiple comparisons correction (for quantitative parameters). Non-parametric Kruskal-Wallis test with Dunn's multiple comparison test were performed to determine the quantitative difference of bacterial 16S DNA copies, IL-1β or LPS between the diagnosed groups. Spearman's analysis was used to correlate 16S DNA and IL-1β quantities. Mann-Whitney unpaired non-parametric test were used to compare 16S DNA or IL-1β levels between IPMN LGD and IPMN HGD and also to compare the levels of 16S DNA or IL-1β in relation to previous endoscopic procedures or use of proton-pump inhibitors. To analyse bacterial compositional, Chao1 richness, Shannon diversity or inverted Simpson's indices were analyzed and compared using Kruskal-Wallis test with Dunn's test. Bar plots were shown also for bacterial relative abundance at phylum and genus levels. Beta diversity between samples visualised by cluster heatmap, PCoA spider plots and bacterial co-occurrence network. Linear discriminant analysis effect size was used to identify the relative abundant bacterial genera between the three diagnosed groups (IPMN LGD, IPMN HGD and cancer).

In study II, for quantitative and qualitative parameters Kruskal–Wallis test with Dunn's multiple comparisons and Fisher's exact test were used, respectively. To analyse the difference in antibody responses between the diagnosed groups, Kolmogorov–Smirnov test was used. The correlation between plasma IgG and salivary IgA reactivity against *F. nucleatum* was examined with Pearson analysis. Pearson analysis was used to correlate plasma IgG and salivary IgA reactivity against *F. nucleatum*. Pairwise Wilcoxon test was used for competitive ELISA. The ELISA cut-off values were computed based on the formula described by Frey et al. on healthy controls [137], values above the cut-off were assigned as reactive while those that were lower as non-reactive.

In study III, visualizations of the multiplex bead array data were performed using R (version 3.6.1) with RStudio (version1.2.1335) and the additional packages heatmap (1.0.10), reshape2 (1.4.3). GraphPad Prism Version 9.0.0 was used for the non-parametric comparisons Mann-Whitney test and Spearman correlation analysis. For comparisons of binomial datasets, N1 Chi-squared test was used. A cutoff for positivity was calculated per antigen as the mean + 6X SD of 12 negative pre-pandemic reference samples carefully selected based on their signal intensity distribution. Descriptive analyses were made on clinical characteristics and the number of observations, presented as numbers and percentages.

4 RESULTS AND DISCUSSION

4.1 Study I

IPMN is a common type of PCN and has significant potential to malignant transformation. A better pre-operative identification of high risk IPMN patients will not only improve pancreatic cancer management but also reduce the burden of unnecessary surgical interventions and life-long follow-ups.

4.1.1 Study population characteristics

The clinical characteristics of all study subjects were grouped on diagnosis-basis and analyzed. In comparison, the cancer group is on average older than non-IPMN group and represented with higher serum concentrations of Ca 19-9, hemoglobin A1c, albumin, and bilirubin than non-IPMN. In general, Ca 19-9 is the only tumor biomarker approved by the Food and Drug Administration (FDA) and offers a sensitivity of 79% and specificity of 82% to detect tumor [138]; but is not used to predict tumors in asymptomatic patients [139]. There were more incidences of high levels of Ca 19-9 (>34 U/mL) in the cancer group (70.4%) compared to non-IPMN group (23.8%) in our study.

4.1.2 Bacterial 16S DNA and IL-1β quantification

Intracystic bacterial 16S DNA copies and IL-1β were found in this study to be associated with pancreatic cystic lesions severity. Compared to non-IPMN, higher bacterial 16S DNA copies and IL-1β were found in IPMN (p=0.0042 and p=0.029, respectively) and cancer (p=0.0008, and p=0.0024, respectively). The elevated bacterial 16S DNA were also positively correlated with IL-1β levels. Post-IPMN stratification into IPMN-LGD and IPMN-HGD showed higher IPMN-HGD intracystic bacterial 16S DNA (p=0.02), and IL-1β (p=0.0032) compared to IPMN-LGD. This finding of elevated proinflammatory IL-1β agrees with other studies that reported IL-1β levels in the cyst fluid as a discriminative marker to predict IPMN dysplastic grade [140,141]. The inflammatory mediator IL-1β is known to play a crucial role in cancer: promoting invasiveness, and metastasis via inflammation in the cancer microenvironment [142]; alternatively, inflammation can be a result of ductal obstruction and release of damage-associated molecular pattern (DAMP) molecules. To investigate potential microbial involvement in this intracystic inflammatory response, bacterial 16S DNA and pathogen-specific molecules (LPS) were quantified in our study and correlated to IL-1β levels. The results of elevated cyst fluid IL-1β levels in high-risk IPMN group and their positive correlation with bacterial load and LPS levels suggest a local pancreatic microbiome role in the malignant progression of IPMN.

4.1.3 Microbial compositional analysis

To investigate bacterial composition in the IPMN cyst fluid and whether it is associated with disease progression, full-length bacterial 16S rRNA genes were measured using PacBio technology. This was performed on cyst fluid that passed quantity and quality control for content of 16S DNA. It was found that IPMN LGD (n=14) or IPMN HGD (n=8) and cancer (n=14) group revealed a highly diverse microbiome and showed no significant differences at operational taxonomic unit (OTU) level for Chao1

richness, Shannon diversity, or inverted Simpson's indices. At the phylum level, IPMN LGD was predominated by Proteobacteria, while IPMN HGD and cancer were either dominated by Firmicutes or Proteobacteria. At the genus level, the microbial composition was found to be heterogeneous with no distinctive profile according to the group diagnosis.

4.1.4 Co-occurrence of oral pathogens in IPMN

Visualization of bacterial species with network analysis revealed a co-occurrence of *F. nucleatum* with several other species, including *Serratia marcescens, Parvimonas micra, Prevotella melaninogenica, Haemophilus parahaemolyticus, Streptococcus anginosus, Bergeyella* sp. HMT322, *Kluyvera ascorbata, Eikenella corrodens, Campylobacte concisus* and *Campylobacter showae,* some of which appear to be members of the oral microbiome. Other known oral members also showed high relative abundances, such as *G. adiacens* and *S. anginosus*.

4.1.5 Relative abundant bacterial genera based on group diagnosis

Result determined by LEfSe (Linear discriminant analysis Effect Size) that determines the features most likely to explain the differences between classes by coupling standard tests for statistical significance with additional tests encoding biological consistency and effect relevance [143] showed 15 relatively abundant bacterial genera in the three diagnosed IPMN groups. Among them, *Granulicatella, Serratia,* and *Fusobacterium* were found to be highly abundant in IPMN HGD compared to IPMN LGD and cancer. Subsequent validation analysis for *F. nucleatum* genome in IPMN cyst fluid and formalin-fixed tissue samples by a targeted qPCR assay showed increased *F. nucleatum* DNA copy number in the IPMN HGD and cancer group.

The association of *F. nucleatum* in tumor tissue has been demonstrated previously in colorectal cancer, not only by PCR [73,144], but also via non-amplification-based techniques such as 16S ribosomal RNA (rRNA) fluorescent in situ hybridization (FISH) [145]. The biology of tumor mechanisms - angiogenesis, permeability, immunosuppression, and the hypoxic condition may attract microbiome to inhabit the tumor microenvironment [146]. Earlier studies indicate that *F. nucleatum* is an example of overrepresented microbiota in the tumor microenvironment with its anaerobic nature, and specific adhesion capabilities to Gal-GalNAc lectin expressed on the surface of host cells and many microbe cells [147]. Abed and colleagues also demonstrated that *F. nucleatum* Fap2 protein can attach to tumor-overexpressed Gal-GalNAc in colorectal cancer tissues in an experimental mouse model, suggesting that targeting Fap2 may reduce *F. nucleatum* potentiation of cancer and offer diagnostic opportunity as well [76].

4.1.6 Association of intracystic bacterial load and clinical parameters

To investigate potential bacterial translocating factors, we correlated bacterial DNA quantity with routine clinical procedures. Prior invasive endoscopic procedures (IEP), use of proton-pump inhibitors (PPI) or antibiotics were examined. Our results showed an association between the intracystic bacterial quantity and the history of IEP ($p=0.0017$), which goes in line with another study that reported a higher bacterial amount in the pancreas of patients who required endoscopic biliary stenting to decompress

biliary obstruction compared with those who did not require the procedure [148]. However, more IEP exposures did not increase the bacterial load, and the history of IEP was also not associated with the severity of IPMN. In fact, some IPMN HGD and cancer patients with no history of IEP were still having higher intracystic bacterial load than SCN group (p=0.0268), suggesting other bacterial translocation routes into the pancreas could exist. Furthermore, IL-1β or *F. nucleatum* increase was independent of the history of IEP and use of PPI. The routes of bacterial translocation are debated, but accesses via gastrointestinal tract, circulation, and lymphoid system have been proposed in the literature [149]. As reported by Geller et al, although the presence of microbiota can be detected in 76% (86/113) of pancreatic cancer samples, it is also seen in 15% (3/20) of normal, non-pathologic pancreatic tissues[150]. Our result is interesting, giving a plausible scenario to the potential reflux phenomenon of bacteria from the duodenum to the pancreas, especially with the reported similarity of abundant bacteria in PC to bacterial flora of duodenum [151]. Taken together, these findings suggest that the pancreas, a previously regarded sterile organ, may host microbiome niches, and the abundance of bacteria in pancreas may be a driver to chronic inflammation, generating an inflammatory microenvironment that may further promote tumor progression [152].

4.2 Study II

4.2.1 Characteristics of study subjects

Plasma samples (n = 109) and saliva samples (n = 65) from patients undergoing pancreatic resection surgery and healthy controls were investigated. The samples were stratified into HGD-IPMN + cancer (n = 46), LGD-IPMN (n = 45), and non-IPMN (n = 18) based on the postoperative diagnosis confirmed by histopathology. In these cohorts, the non-IPMN participants were younger than IPMN groups and predominated by females, as they were mainly SCN patients. Diabetes was also found more common in the high-risk IPMN group (IPMN HGD and cancer), which is in agreement with a previous study [153].

4.2.2 Plasma antibody responses to oral microbiota and high-risk IPMN

This study's interest was to evaluate antibody reactivities to the enriched oral bacteria detected in study I. Plasma IgG binding antibodies showed stronger reactivity than IgA when tested against *F. nucleatum*, *G. adiacens*, *S. gordonii*, *S. anginosus*, *E. coli* and *F. nucleatum* Fap2 by indirect ELISA. High-risk IPMN (HGD-IPMN + cancer) group showed higher *F. nucleatum* ($p<0.0006$) and lower *G. adiacens* ($p<0.002$) antibody reactivities compared to non-IPMN group. To rule out potential IgG deficiency bias, total IgG levels were analyzed and showed comparable levels between all groups.

4.2.3 Salivary antibody responses to oral microbiota and high-risk IPMN

Our results showed that the HGD-IPMN + cancer group had higher IgA antibody reactivities to *F. nucleatum*, *G. adiacens, and F. nucleatum* Fap2 as compared to other groups (LGD-IPMN and control). Of note, the oral health periodontal data showed comparable oral health conditions across all groups.

The oral health data served to reduce the potential bias of periodontal disease involvement in the HGD-IPMN + cancer group, as *F. nucleatum* is one of the principal periodontal pathogens [58,154]. Since periodontitis is reported to be prevalent in adults [155], and the IPMN HGD + cancer group were shown in this cohort to be older in age than controls, correlation analysis between age and antibodies levels were performed to determine age as confounding factor. As shown in **Figure 6**, no significant correlations between age and IgG or IgA reactivities was found in both *F. nucleatum* and Fap2. However interestingly, salivary IgA reactivities to *F. nucleatum* Fap2 were shown to be positively correlated to *F. nucleatum* (r= 0.685, p<0.0001), which prompted us to validate salivary IgA bindings to *F. nucleatum* Fap2 and *F. nucleatum* whole cell in subsequent competitive ELISA.

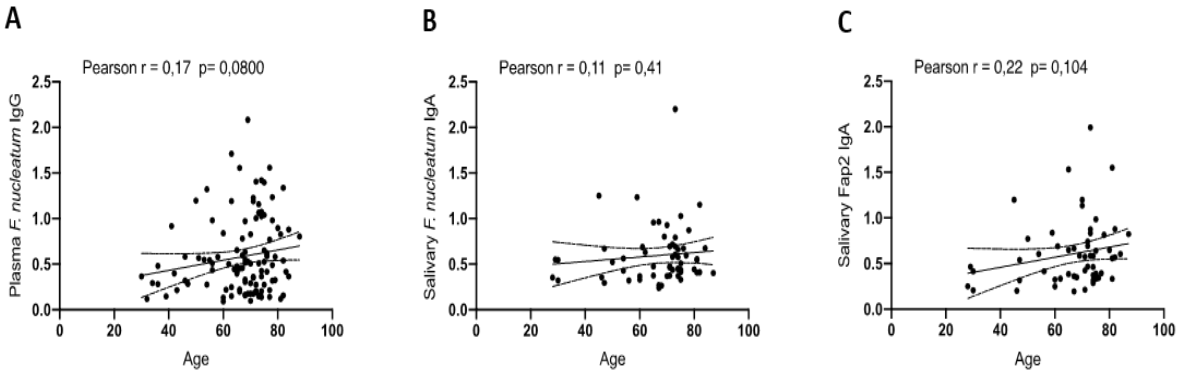

Fig. 6. No correlation between age and the circulating IgG plasma **(A)** or IgA salivary **(B)** antibody reactivity to *F. nucleatum* or Fap2 **(C)** determined by the two-tailed Pearson correlation test.

4.2.4 Confirming salivary IgA specificity to Fap2 mimotope

To confirm the IgA specificity to Fap2, saliva samples pre-incubated with *F. nucleatum* or *E. coli* (control) and added to Fap2-coated wells in a competitive ELISA test. The binding was reduced with *F. nucleatum* pre-incubated samples by 24 ± 31% but not with the control *E. coli* (1.7 ± 25%). Our data also showed that IgA in saliva pre-incubated with Fap2 are also outcompeted for the binding to *F. nucleatum* whole cell (coating antigen), suggesting specific antagonist affinity to Fap2 (**Figure 7**). Our result adds to the current notions on host immune interaction with Fap2, which is considered as a virulence mechanism in the tumor microenvironment. Other Fap2´s virulence mechanisms include the inhibition of the cytotoxicity of NK and other immune cells via activating the TIGIT immune suppressor and invading immune system through targeting CEACAM1 to induce lymphocytic apoptosis [77,156]. Moreover, *F. nucleatum´*s ability to interact with galactose lectin and endothelial cadherin expressed on various mammalian cell surfaces for adhesion and invasion via Fap2 and FadA proteins may explain its transmission ability and overrepresentation in distal organs [157–161].

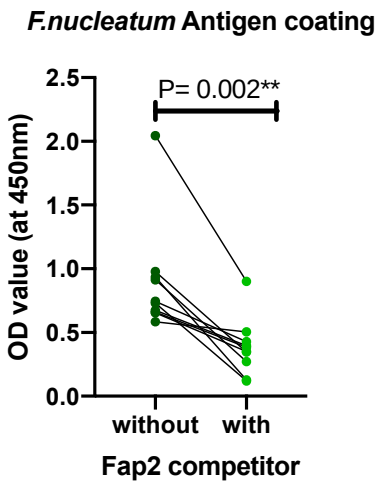

Fig. 7. Competitive ELISA with saliva samples pre-incubated with or without Fap2 competitor, tested in *F. nucleatum*-coated wells. Statistical analysis was performed using Wilcoxon test for two related samples. **p < 0.01

4.2.5 Salivary bacterial 16S DNA and antibody reactivities

Targeted bacterial 16S DNA copy to *F. nucleatum* and *G. adiacens* determined by specific qPCR Taqman assays showed comparable gene copy levels between all groups. Universal 16S gene copy levels were comparable as well across the groups. Moreover, targeted and universal bacterial 16S DNA showed no correlations to the bacteria antibody reactivities.

These findings bring many interesting questions whether mucosal secretory IgA antibodies in saliva has better specific immune recognition than plasma IgG to the virulent factor of oral commensals to create oral microbial symbiosis, or whether these translocated oral strains possess intrinsic feature able to mutate and hide their virulent factor to escape immune recognition when they disseminate to extra-oral sites. A limitation of our study is that we targeted 16S DNA and antibodies to *F. nucleatum* at a species level, considering that the diversity of Fusobacterial community was reported to be significantly reduced when translocating from the oral cavity to gastric or colon sites, suggesting a selective translocation of Fusobacterial strains [162]. However, the reported altered diversity of fusobacterial community between oral cavity and colon were found to be at the strain level rather than species or subspecies levels [163]. Further studies to focus on the virulence factors of the transmissible strains are needed to understand their role in the transmission and exploit them for diagnostic and therapeutic purposes.

4.3 Study III

4.3.1 Salivary antibody responses to SARS-CoV-2 proteins

A multiplexed bead-based immune array was used in this study. First, a panel of recombinant SARS-CoV-2 viral proteins (n = 5) was tested to classify COVID-19 convalescence saliva samples (n = 74) from pre-pandemic saliva samples (n = 35). For salivary IgG, the absolute best accuracy was shown

with the spike trimers foldon (Spike-f, 88% sensitivity, 100% specificity) followed by the nucleocapsid C-terminus protein (NC-C, 66% sensitivity, 100% specificity). Of note, salivary IgA reactivities were weak in general in the convalescent samples, which were collected mainly 3-9 months after post-symptoms-onset, and is consistent with previous studies reporting IgA antibody decay already 1 month post-symptoms-onset [164,165]. In this study, a second cohort of undiagnosed individuals was invited to validate the utility of the salivary antibody assay. Here, the specific salivary antibody responses correlated significantly with recent Covid-19-like symptoms. Moreover, combining symptoms with other risk factors under past three months prior to sampling, such as 1) travel abroad; 2) contact with someone who has been confirmed to be infected with COVID-19; 3) clinical work, further increased IgG positivity frequency in this cohort (**Figure 8A**). This is not noted in those reporting no symptoms (Figure 8B).

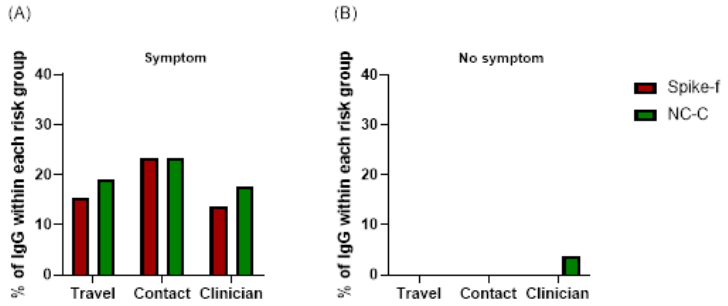

Fig. 8. Frequency of IgG positivity to spike trimer (Spike-f) or nucleocapsid (NC-C) in individuals with self-reported symptoms **(A)**, or no symptoms **(B)** with additional risk factors such as traveling abroad, contact with COVID-19 diagnosed people, or clinical work.

4.3.2 Salivary antibody tolerance to temperature and detergent pre-treatments

The feasibility of using saliva in antibody testing was examined in a series of experimental simulations. Saliva inactivation by heat or detergent treatment for example at 56°C for 1 h or by 1% Triton X-100 (Triton) showed a minor effect on antibody reactivity (the cut-off is based on 10 negative samples). In addition, storing samples at room temperature for one, two, or three days led to slight decay of antibody signals of positive samples.

Unlike conventional ELISA, this study demonstrates that salivary antibodies to more than one antigen can be simultaneously detected by the bead-based multiplex array approach. Taken together, these results showed that the IgG specific to the SARS-CoV-2 spike full-length and nucleocapsid proteins in human saliva can be detected up to 9 months in mild COVID-19 cases by the bead-based array technology. This finding is in line with the SARS-CoV-2-specific IgG antibody duration in serum can be as long as up to 8 months after mild COVID-19, as reported recently in South Korea [166]. However, further longitudinal study is warranted to rule out the possibility of reinfection. Our data also showed that storing samples at room temperature has a minor effect on antibody reactivity, suggesting that a

non-invasive antibody test approach with self-collection at home may serve as a complementary alternative to conventional blood serology testing.

The limitations of our study are the relatively small size and the cross-sectional design. Unobtained biographical information of undiagnosed group (such as age and gender) limited sub-analysis options to evaluate antibody positivity based on these bio-parameters. Another limitation is the different immunoassay methods we used to detect the antibody in serum and saliva.

5 CONCLUSIVE REMARKS

Study I

- Bacterial DNA and inflammation level in pancreas cysts correlate with the cystic neoplastic grade.
- Pancreas microbiome of cyst fluid display co-occurrence and enrichment of oral bacterial species.
- Bacteria DNA level in pancreas is associated to prior exposure to invasive endoscopic procedures.
- Intracystic bacterial DNA testing of pancreas samples may have added values to guide the management of IPMN.
- Reducing the pancreatic inflammatory microbiome may be a potential therapeutic strategy for IPMN patients.

Study II

- Elevated antibodies to oral pathogens (particularly *F. nucleatum* or Fap2 of *F. nucleatum*) in blood or salivary may reflect more severe form of IPMN.
- Salivary IgA against oral pathogens holds promise as a non-invasive biomarker for early identification of high risk cystic tumors in the pancreas.

Study III

- Salivary antibody reactivity to SARS-CoV-2 spike and capsid antigens persist up to 9 months after mild COVID-19.
- The virus-specific IgG in saliva appears stable and tolerates temperature and detergent pre-treatments
- A non-invasive saliva-based SARS-CoV-2 IgG test with home self-collection may serve as a complementary alternative to conventional blood test.

6 POINTS OF PERSPECTIVE

Study I

The findings of the study warrant further studies to investigate the viability and oncogenic potential of the pancreatic microbiome in co-culture or in-vivo experimental models. Experimental studies to assess antibiotic treatment or other antimicrobial strategies as a therapeutic approach to reduce the risk of tumor progression are of great clinical importance. The link between invasive endoscopic procedures and pancreas bacteria contamination needs not only confirmatory studies but also calls for intervention studies, which is important in prevention of bacteria dissemination.

Study II

In order to investigate the role of oral bacteria in IPMN progression, metagenomic and metabolomic profile analysis of paired saliva and cyst fluid samples are an important area to study. In addition, exploring the role of these enriched oral microbiotas, particularly *F. nucleatum,* in the pancreatic tumor microenvironment worth further studies. Moreover, potential *F. nucleatum* modulation to cell-mediated immunity has not been examined in pancreatic tumor patients, which warrants further studies.

Study III

We investigated the persistence of binding antibodies to SARS-CoV-2 in a cross-sectional design. Longitudinal studies to investigate the kinetics of salivary immunity and antibody neutralization efficiency over time are awaited with great interest. Determining the correlation of antibody responses to protection, in natural infection as well as vaccination is highly important. Using saliva and multiplex strategy to monitor the COVID-19 vaccination programs should simplify the investigations vaccine immunity of any given environment. The magnitude and composition of salivary immunity required for protection against SARS-CoV-2 infection should be studied in detail.

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