Bioimpedance Based Biomarker for the Detection of Precancerous and Cancerous Lesions of the Pancreas: Feasibility Animal Study

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Abstract Pancreatic cancer (PC) remains a significant healthcare challenge due to its aggressive nature and poor prognosis. The current gold standard of biopsies has limited diagnostic efficacy due to various shortcomings. We propose a feasibility study for the use of a bioimpedance biomarker to detect PC. The biomarker was evaluated in a double blind study on ex vivo pancreases of mice: 15 K-ras;Trp53;Pdx-1-Cre, 2 K-ras;Pdx-1-Cre, and 9 wild type controls (Study 1); to determine if the biomarker can distinguish between PC and acute pancreatitis (AP), we challenged it with 18 cerulein-induced AP and 6 saline-injected controls (Study 2). The results from Study 1 showed 100% specificity and 94% sensitivity against histopathology outcomes; for Study 2 all AP and saline-injected pancreases were diagnosed as non-cancerous. Regression analysis revealed a positive correlation between biomarker and pathologically analyzed cancer induced fibrosis ($r(15)= 0.82$ ($p < 0.001$)). These findings demonstrate the potential of this bioimpedance biomarker as a diagnostic tool for PC.

Introduction

Pancreatic cancer (PC) accounts for half a million new cases and 4.7% of the world's cancer-related deaths in 2020 ***Globocan*** (***2020***). It is considered one of the most lethal malignancies and a significant healthcare challenge ***Koul et al.*** (***2018***). PC has the lowest survival rate among all known cancers according to the American Cancer Society, due to its aggressive nature and poor prognosis ***CancerStatisticsCenter*** (***2022***); ***Kato and Honda*** (***2020***); ***Young et al.*** (***2020***). This is attributed to the difficulty in early diagnosis and to the lack of standardized guidelines in assessing suspicious pancreatic masses ***Garg and Chari*** (***2020***); ***Yang et al.*** (***2021***). The complex pathophysiology, together with the lack of early diagnostic and prognostic markers are major barriers at the basis of the late and often incurable stage diagnosis of PC. At present, there is no standard screening procedure for early detection of PC as the currently available imaging and endoscopic modalities fail to accurately detect lesions under 3 cm ***Kitano et al.*** (***2019***) and discern malignant from benign lesions. There is demand for an on-site, real-time assessment device that works as a quantitative decision support tool for the endoscopist. A more timely and accurate diagnosis of PC would reduce revisits, expedite treatment, and improve the current prognosis of this disease.

To date, PC diagnosis relies on imaging modalities, including multidetector computed tomography (MDCT), magnetic resonance imaging (MRI), and endoscopic ultrasound (EUS) ***Moradi and Iagaru*** (***2020***); ***Kato and Honda*** (***2020***); ***Michl et al.*** (***2021***); ***Zhang et al.*** (***2018***). The first modality of choice for diagnosing PC is MDCT (Multidetector CT) ***Zhang et al.*** (***2018***). While generally safe and non-invasive, contrast MDCT is accompanied by the risk of nephrotoxicity from the iodine-contrast agent as well as radiation exposure ***Zhang et al.*** (***2018***). MRI is often used as a subsequent test when there is a high suspicion of PC despite a clear CT ***Zhang et al.*** (***2018***). However, both CT and MRI are not very sensitive in detecting the tumor in its initial development while still small ***Kitano et al.*** (***2019***) (typically less than 3 cm) and localized ***Koul et al.*** (***2018***). Endoscopic ultrasound (EUS) guided tissue acquisition is currently the gold standard for sampling pancreatic masses. Confirmation of suspicious lesions is generally obtained via EUS guided needle biopsies, using fine-needle aspiration (FNA) or fine needle biopsy (FNB) ***bio*** (***????***); ***Chang et al.*** (***1997***); ***Varadarajulu and Wallace*** (***2004***); ***Michl et al.*** (***2021***); ***Zhang et al.*** (***2018***). EUS positions an echoendoscope transducer close to the pancreas, allowing for high-resolution visualization of the pancreas and the surrounding structures during the procedure, which increases the chances of obtaining a representative sample of the tumor. Hence, it is ideal for lesions smaller than 2 cm and is relatively safe ***Zhang et al.*** (***2018***); ***Bispo et al.*** (***2021***); ***Shrikhande et al.*** (***2012***); ***Wang et al.*** (***2013***); ***Koul et al.*** (***2018***). Improvements in fine needle biopsy (FNB) technologies and increased availability is further improving the diagnostic yield of EUS guided biopsies.

With all these discussed modalities, confirmation of cancerous lesions is only accomplished when biopsy samples are obtained and screened in an ex vivo setting by a cytopathologist. The challenges to successful biopsies arise from difficulties in physically locating the lesions, inter-observer variability in identifying and grading the lesions, and low diagnostic yield due to insufficient integrity or size of the samples. Additionally, misdiagnosis of tissues may result from pancreatitis, necrosis, or diffusely infiltrating carcinoma ***Chang et al.*** (***1997***); ***Varadarajulu and Wallace*** (***2004***); ***DeWitt et al.*** (***2004***); ***King et al.*** (***2022***); ***Yamashita et al.*** (***2020***); ***Bhutani et al.*** (***2004***). In the pancreatobiliary tract, indeterminate structures often present a diagnostic challenge in differentiating benign from malignant tissues ***Bowlus et al.*** (***2016***), leading to multiple procedures that cause undue stress to patients and additional costs. EUS-FNA or EUS-FNB with cytologic rapid on-site evaluation (ROSE) has been introduced as an efficient diagnostic modality for evaluation of solid pancreatic lesions. ROSE has advantages of providing timely feedback on sample adequacy and optimizing the number of needle passes performed and most of all it may increase the diagnostic yield, since malignant cells that are often detected during later FNA passes would otherwise be missed if tissue sampling stopped prematurely ***Koul et al.*** (***2018***). One study revealed that EUS-FNB alone had a significantly lower diagnostic accuracy than EUS-FNB and ROSE (80.7% vs 93.1%, P = .001), thus suggesting a potential benefit of ROSE during these procedures ***de Moura et al.*** (***2020***). The restricted availability of ROSE and consequently, the limited accuracy of EUS-FNA or EUS-FNB in the absence of ROSE might have constrained widespread utilization of EUS-guided sampling globally. Finally, these modalities are implemented when there is already a high suspicion of PC, by which time cancer tends towards its advanced stages, limiting curative opportunities.

Given the limitations of the current clinical standard for PC diagnosis, multiple research groups are studying advanced methods to improve the diagnostic process for PC. Some newly developed technologies focus on assessing biopsy sample adequacy and cell viability on site right after the samples are collected ***Pritchett et al.*** (***2022***); ***Duke et al.*** (***2022***). These methods need a small amount of specimens for rapid diagnosis and provide indication of the quality of the initial sampling before going for pathology or information to assess if additional samples are needed to be biopsied for a successful pathology, issuing a preliminary diagnosis in a shorter time than traditional approaches ***Pritchett et al.*** (***2022***); ***Duke et al.*** (***2022***). These technologies are based on different principles. Ambient mass spectroscopy enables controlled delivery of a discrete water droplet to a tissue surface for efficient extraction of biomolecules, which is then delivered for analysis ***Zhang et al.*** (***2017***); ***Lu et al.*** (***2020***); optical imaging techniques able to generate images reminiscent of his-

tology without any tissue processing ***Thouvenin et al.*** (***2021***). Finally, there is an automatic method of sample preparation to enhance the evaluation and detection of cancer ***Pritchett et al.*** (***2022***); ***Duke et al.*** (***2022***). Though these techniques are real-time and offer a rapid and nondestructive diagnosis of cancer tissues, they are characterized by some limitations, such as high cost, low resolution ***Sans et al.*** (***2019***); ***Jain et al.*** (***2015***), and the potential of modifying the sample before pathology assessment.Therefore, there is a need for a real-time tool that can evaluate cancer presence in biopsies without affecting the sample, and requires fewer cytology and histology specimens prepared and submitted, decreasing the administrative costs.

In this paper, the authors propose a feasibility study for the use of a novel bioimpedance based biomarker - the Cole Relaxation Frequency (CRF) – to detect PC. We have previously shown The CRF to quantitatively detect cancer in breast, skin, and lung tissues ***Gregory et al.*** (***2012***); ***Svoboda et al.*** (***2018***); ***Bogdanowicz et al.*** (***2022***); ***Guidetti et al.*** (***2022***). The aim of this pilot study is to determine if the CRF based biomarker can detect PC and also discern pancreatitis from PC in the genetically modified KPC and KC mouse model, acute pancreatitis mouse model and wild type controls. These animal models spontaneously and progressively develop PC allowing us to correlate the biomarker values with the lesions as they develop from precancerous to malignancy. The KPC mouse is an established and clinically relevant model of PC which develops many key features observed in human PC ***Hu et al.*** (***2019***); ***Renz et al.*** (***2018***); ***Niknafs et al.*** (***2019***); this work may lay the foundation towards understanding the potential for CRF to inform on cancer stages in humans. Specifically, the biomarker was evaluated in a double blind study on ex vivo pancreases of mice. Two studies were run to determine if the biomarker could discern between K-ras;Trp53;Pdx-1-Cre, 2 K-ras;Pdx-1-Cre, and 9 wild type controls and between acute pancreatitis (AP) and PC by adding to the analysis cerulein-induced AP and saline-injected mice. The device conducted a spectral bioimpedance measurement for CRF biomarker computation. CRF based cancer determinations were compared against histopathology outcomes to calculate specificity and sensitivity. These findings demonstrate the potential of this bioimpedance biomarker as a diagnostic tool for PC. A future implementation of this technology into a digital version of ROSE device would allow the widespread use of ROSE after EUS-FNAs and EUS-FNBs at accessible costs.

Results

In this study, we evaluated the bioimpedance based biomarker to detect PC in genetically modified KPC and KC mouse models, acute pancreatitis mouse model and wild type controls. The KPC mouse model is one of the most used models to evaluate pancreatic ductal adenocarcinoma (PDAC) due to its faithful recapitulation of human pancreatic cancer biology ***Hu et al.*** (***2019***); ***Renz et al.*** (***2018***); ***Niknafs et al.*** (***2019***). Indeed, KPC PDACs provide a unique opportunity to analyze the evolution of cancer in a controlled setting, not otherwise possible in human patients. The study involved two double-blind studies on ex vivo pancreas of mice. In Study 1, the biomarker was tested in n=26 mice (15 KPC, 2KC, and 9 controls), in Study 2 we determined the biomarker ability to differentiate PC from acute pancreatitis (AP), considering n=24 (18 cerulein-induced AP and 6 saline-injected controls) (Figure 1).

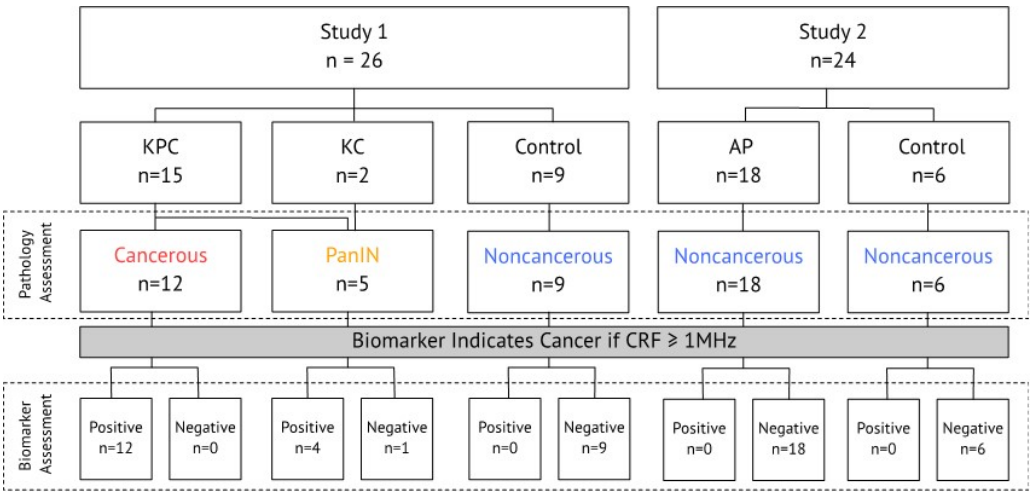

Figure 1. Study design.

The CRF measurements were collected at different locations on the pancreatic sample and CRF determinations allowed to calculate sensitivity and specificity against histopathology outcomes. As far as Study 1 is concerned, based on histopathology, 12 KPC pancreases were confirmed as cancerous, 9 controls were confirmed as noncancerous, while 5 pancreases (3 KPC and 2 KC) presented with pancreatic intraepithelial neoplasia (PanIN), a precancerous condition. Examples of CRF curves for noncancerous, precancerous, and cancerous samples are provided in Figure 2.

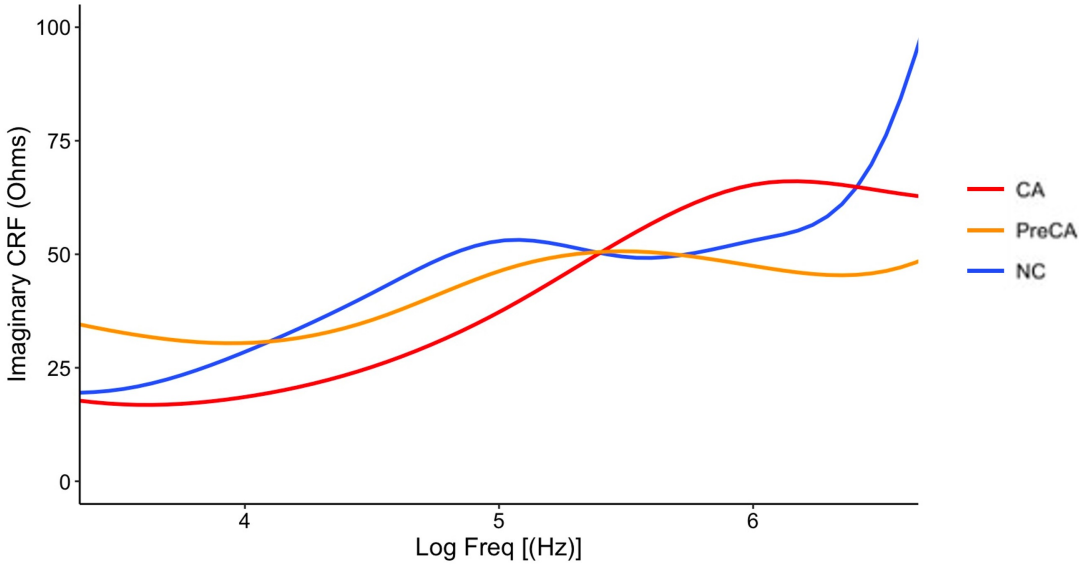

Figure 2. Example CRF curves from noncancerous (NC), precancerous (PreCA), and cancerous (CA) mice pancreases.

The CRF biomarker identified 4 out of 5 PanIN samples as cancerous. Considering the entire cohort for Study 1 (n=26), specificity and sensitivity were 100% and 94%, respectively. The sample determinations based on the CRF biomarker are reported in Table 1. If PanIN samples were excluded, specificity and sensitivity were both 100% (n=21). The Spearman correlation coefficient between percent fibrosis and CRF was r(15)= 0.82 (p <0.001), which indicates a strong positive correlation (Figure 3).

		Histology Assessment		
		CA	PreCA	NC
Biomarker Assessment	CA	12	1	0
	PreCA	1	4	0
	NC	0	0	9

Table 1. Confusion matrix for Study 1. NC: noncancerous; CA: cancerous; PreCA: precancerous.

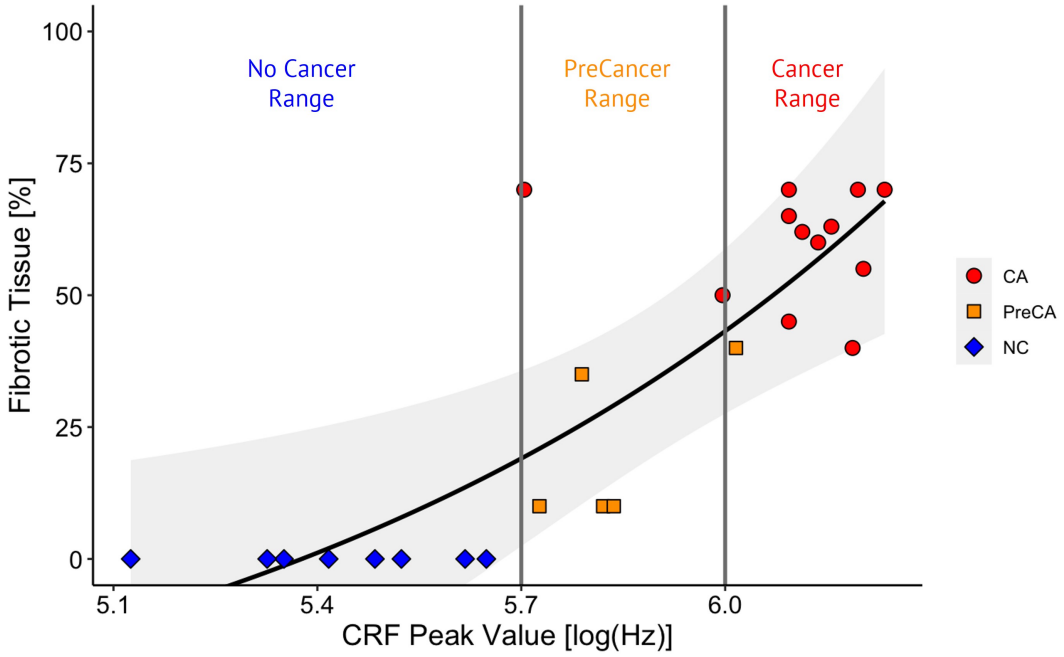

Figure 3. Spearman correlation between percent fibrosis and CRF for noncancerous, cancerous, and precancerous pancreases. Grey band shows the 99.99% confidence interval.

Discussion

This study found specificity and sensitivity of 100% and 94%, respectively, of the bioimpedance based biomarker in discerning between cancerous and noncancerous pancreas tissues from mice. Moreover, all pancreatitis samples were detected as noncancerous. The findings also determined a strong positive correlation between CRF biomarker and percent fibrosis in cancerous and precancerous samples. This feasibility study demonstrates the potential for the use of the CRF to predict PC and the level of fibrosis in PC. The identification of malignant precursors for PanIN samples indicates the biomarker capability to detect early-stage PCs. The biomarker was found to be strong against the confounding factor of pancreatitis, demonstrating that the CRF can decipher PC from normal and acute pancreatitis tissues making it an ideal clinical detection tool.

The positive correlation between CRF biomarker and pathologically analyzed cancer induced fibrosis in PC may be similar to that shown in breast cancer ***Gregory et al.*** (***2020***). Gregory et. al ***Gregory et al.*** (***2020***) previously reported using the CRF biomarker as a prognostic indicator for the aggressiveness of breast cancer. In that retrospective study, a strong correlation was found between the CRF values of tumor excisions measured at time of surgery and long term patient outcomes in terms of recurrence or time-cancer-free ***Gregory et al.*** (***2020***). According to their finding, when the CRF is below 5.3 log(Hz) it is likely that the cancer is nonrecurrent; when the CRF is in the range between 5.3 log(Hz) and 5.8 log(Hz) there is a high likelihood that cancer is recurrent not metastasizing; and when the CRF is above 5.8 log(Hz) then there is an increasingly greater likelihood that the cancer is recurrent with metastasis (see Figure 4) ***Gregory et al.*** (***2020***). A similar behavior was observed for the pacreatic tissues data presented in this currect study (see Figure 3). These findings suggest that the CRF may well be a universal property of cells as they transform regardless of organ origin and that the CRF biomarker may be studied as a prognostic indicator.

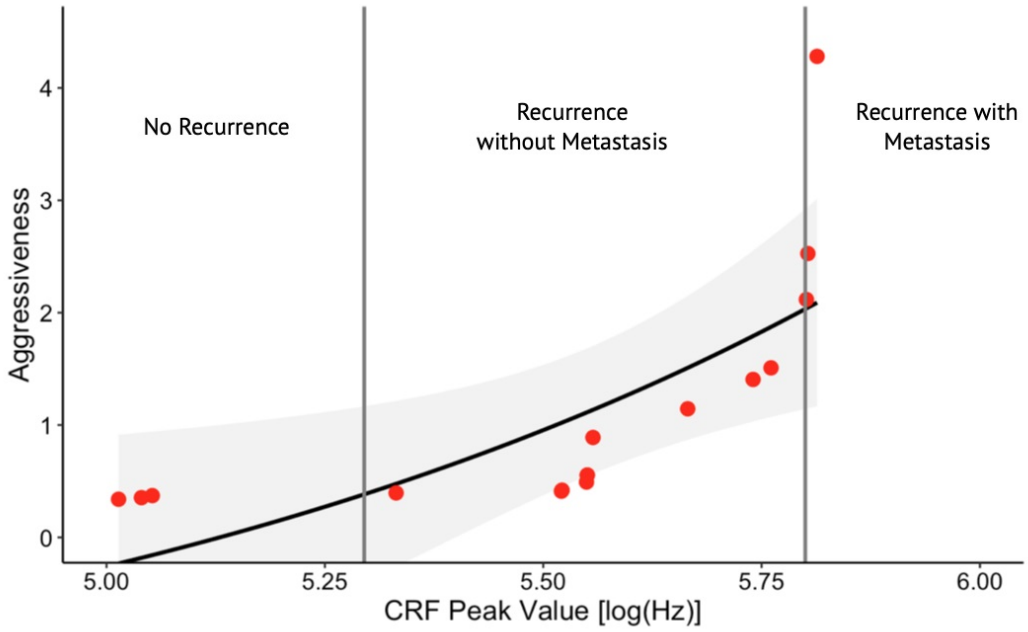

Figure 4. Gregory et al. ***Gregory et al.*** (***2020***) have showed that the CRF biomarker can retrospectively classify breast cancer data in 3 well-differentiated categories: nonrecurrent (NR); recurrent with no metastasis (RNM); and recurrent with metastasis (RM).

Once proven to be effective in a larger preclinical and clinical trial, the CRF based technology could be implemented into a medical device for clinical use. Indeed, the electrodes used to measure the bioimpedance could be developed into a rapid onsite evaluation device that would be used as an ex vivo decision support tool for real-time quantitative assessment of biopsy samples. Another future development can be seen in the implementation of the measuring electrodes on the tip of an endoscopic device for in vivo clinical use to assist endoscopists in the decision-making process and to guide them in margin assessment and biopsies acquisition.

This study is not without limitations. We expect some level of variability when transferring these results to a clinical trial. A larger sample size could allow for a deeper understanding of the potential use of the biomarker for early detection of PC. This study did not include chronic pancreatitis samples, however a standard model for this disease is already available and will be included in a future study by the group.

Methods and Materials

Background

Several studies ***Qiao et al.*** (***2010***); ***Han et al.*** (***2007***); ***Gregory et al.*** (***2012***); ***Svoboda et al.*** (***2018***); ***Shell and Gregory*** (***2017***); ***Gabriel et al.*** (***1996***) have demonstrated that different tissue types and cells behaviors, including cancer, can be identified by measuring frequency dependent bioelectrical properties. The cell membrane behaves like an electrical capacitor in that a charge (ion) brought up to the outside of the membrane causes charges of the opposite sign to deploy on the interior face of the membrane. This process then stores equal amounts of electrical charge of opposite sign on each side of the membrane. However, this charge can be neutralized by charges flowing in the opposite direction through resistive paths between the inside and outside of the cell membrane. Some possible paths are via proteins embedded in the membrane; further paths are possible by a split of the current passing through the cell or around the cell. The behavior of the

cell membrane has been described with the circuit diagram (Cole-Cole model). Current passing through the extracellular matrix encounters only resistive impedance to the current flow, as does the current passing through the proteins in the membrane wall with current passing around the cell. A portion of the current also passes through the capacitive membrane, and this has a complex behavior that can be mathematically modeled. The characteristic rate at which a cell redistributes electrical charge on and off the cell membrane, so that the charge gets equilibrated, is called Cole Relaxation Frequency (CRF). By examining the transmembrane cellular response in the frequency range of 1 KHz to 10 MHz, also known as the β region, cancerous tissues can be detected. To characterize spectral bioimpedance measurements, Novascan has developed an algorithm that utilizes the equivalent circuit proposed by Cole et al. ***Cole and Cole*** (***1941***). The circuit is described by the following equation: $Z = Z' + jZ'' = R_\infty + \frac{R_0 - R_\infty}{1 + (j\frac{f}{CRF})^\alpha}$, where Z is the complex sample impedance, Z' is the real, and Z'' is the imaginary component of Z, R_0 and R_∞ respectively represent the low and high frequency limits of Z, f is the measurement frequency, *CRF* is the Cole Relaxation Frequency, j is the imaginary unit and α is a dimensionless number that is inversely related to the broadening in the frequency domain of Z', and the spread of the peak seen in $-Z''$. The algorithm extracts the CRF that is used as an impedance spectroscopy biomarker to detect cancer. NovaScan has established proof-of-concept technologies to detect cancer in breast ***Gregory et al.*** (***2012***, 2020), skin ***Svoboda et al.*** (***2018***), and lung ***Bogdanowicz et al.*** (***2022***); ***Guidetti et al.*** (***2022***) tissues. Moreover, for each tissue kind, NovaScan has developed customized prototype devices that have been tested and validated *ex vivo* ***Gregory et al.*** (***2012***); ***Svoboda et al.*** (***2018***); ***Bogdanowicz et al.*** (***2022***); ***Guidetti et al.*** (***2022***). We based the feasibility of the current work on these previous studies and on the work by Subramanian et al., which illustrated cell architecture derangement across tumor formation, further explaining the physical foundation of CRF deviations observed for cancer ***Subramanian et al.*** (***2009***).

Mouse Model

The KPC (Pdx1-Cre/LSL-Kras^{G12D}/LSL-p53^{R172H}) murine model is the most employed *in vivo* preclinical tool for studying PC. Mutations in both endogenous KrasG12D (K) and p53R172H (P) alleles accompanied by the Lox-STOP-Lox (LSL) insert are simultaneously expressed following Cre (C) induction regulated by the Pdx1 promoter. The phenotypic result triggers the initiation of a high frequency of Pancreatic Intraepithelial Neoplasia (PanIN) lesions that can progress to pancreatic ductal adenocarcinoma (PDAC) ***Hu et al.*** (***2019***). In order to avoid variance in observations from chimeric strains, KPC mice in the B6 strain background develop PanINs at 4-5 weeks, local invasive cancer at 10-12 weeks and more advanced disease at 16-22 weeks, with metastasis in 40% of specimens. The KPC mouse model is among the most commonly used models for studying PDAC due to its faithful recapitulation of human pancreatic cancer biology ***Hu et al.*** (***2019***); ***Renz et al.*** (***2018***); ***Niknafs et al.*** (***2019***); ***Gabriel et al.*** (***2020***); ***Vernucci et al.*** (***2019***). A timely study of the prognostic value of CRF would be challenging with human tissue *ex vivo* (5-10 yr study) and almost impossible *in vivo*. KPC PDACs provide a unique opportunity to study the evolution of cancer in a controlled setting, not otherwise possible in human patients.

Study Design and Methods

We evaluated the biomarker in a double blind study on ex vivo pancreases of mice. An initial study included 15 K-ras;Trp53;Pdx-1-Cre, 2 K-ras;Pdx-1-Cre, and 9 wild type controls; to determine if the biomarker could distinguish between PC and acute pancreatitis (AP), in a secondary study we challenged it with 18 cerulein-induced AP (3 groups at 24, 48, 72 hours, n=6 for each group) and 6 saline-injected controls. All tests were performed in multiple locations of the pancreases using a custom-built bioelectrical impedance measurement device and tetrapolar electrodes. The tetrapolar configuration is comprised of 4 electrodes including a source electrode (for the generation of the stimulating high-frequency signal), a drain electrode (for the measurement of the current

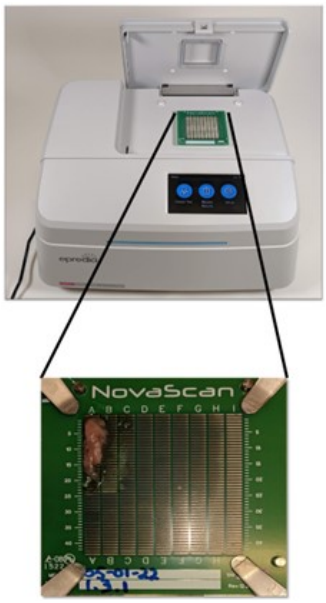

Figure 5. Bioimpedance spectroscopy scanning device with measurement electrode array used for a series of spectral bioimpedance measurements. A zoom in of the electrode with a pancreas sample is also shown.

through a precision 50 Ohm shunt resistor), and two pick up electrodes placed between the source and the drain (for the measurement of the voltage drop across the tissue). The device performed a bioelectrical impedance measurement of the samples over a frequency range of 1 KHz to 20 MHz. The measurements of the biological sample were done using an analog heterodyne-type circuit in which the measured high frequency signals from each electrode were demodulated to a low-frequency signal that was then sampled by analog-to-digital converters (ADC). This information was processed further by a micro-controller to extract the magnitude and phase of the measured voltages as complex numbers. The impedance was then computed as the complex ratio between the voltage drop across the pick-up electrodes and current passed through the drain electrode. The impedance values were then sent to a PC where they were displayed in their Real and Imaginary components for further analysis and determination of the Cole Relaxation Frequencies (CRF). An array of electrodes was used to map the tissue samples. The electrode array was manufactured on a standard PCB featuring 400 1x4mm cupper pads spaced by 0.5 mm, finished by immersion silver and chlorination to function as Ag/AgCl electrodes. The electrodes are electrically connected to contact pads on the back side of the PCB through vias. An XYZ motorized stage was used to move four pogo pins to make contacts to back-side contact pads. Each pogo pin is connected to the custom-made electronics for impedance measurement as described above. A custom GUI allowed for the synchronously motion the XYZ and recording the impedance at each location of the sample in order to build an impedance map.

Cancer determination was made when the CRF parameter was measured above 1 MHz (Figure 1). All samples were processed by standard histopathology after bioimpedance testing. Sensitivity and specificity of CRF based outcomes were determined against histopathology outcomes as ground truth. During histopathology pancreases were also assessed for percent fibrosis averaging over multiple fields of view. Spearman's correlation was used to determine if there was any correlation between percent fibrosis and CRF. An a priori a-value was set at 0.01 to indicate statistical significance. All statistical analyses were performed in R.

Acknowledgments

Additional information can be given in the template, such as to not include funder information in the acknowledgments section.

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