 doi:10.20944/preprints202108.0235.v1

Title: Metabolic and lipoprotein profiling of Pancreatic Ductal Adenocarcinoma patients of African ancestry.

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is a lethal cancer with a characteristic dysregulated metabolism. Abnormal clinicopathological features linked to defective metabolic and inflammatory response pathways can induce PDAC development and progression. In this study, we investigated the metabolites and lipoproteins profiles of PDAC patients of African ancestry. Nuclear Magnetic Resonance (NMR) spectroscopy was conducted on serum obtained from consenting individuals (34 PDAC, 6 Chronic Pancreatitis, and 6 healthy participants). Seventy-five signals were quantified from each NMR spectrum. The Liposcale test was used for lipoprotein characterization. Spearman's correlation and Kapan Meier tests were conducted for correlation and survival analyses respectively. In our patient cohort, the results demonstrated that levels of metabolites involved in the glycolytic pathway increased with the tumour stage. Raised ethanol and 3-hydroxybutyrate were independently correlated with a shorter patient survival time, irrespective of tumour stage. Furthermore, increased levels of bilirubin resulted in an abnormal lipoprotein profile in PDAC patients. Additionally, we observed that the levels of a panel of metabolites (such as glucose, lactate) and lipoproteins correlated with those of inflammatory markers. Taken together, the metabolic phenotype can help distinguish PDAC severity and be used in predicting patient survival and in informing treatment intervention.

Keywords: Pancreatic Ductal Adenocarcinoma, metabolites, cholestatic (obstructive) jaundice, lipoprotein, inflammation, tumour stages.

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Introduction

Pancreatic Ductal Adenocarcinoma (PDAC) is one of the most fatal cancers primarily due to late-stage presentation and resistance to therapy [1]. Over the past two decades, the number of deaths caused by pancreatic cancer has doubled to over 441,000 cases globally [2]. Surgery remains the only curative treatment strategy. However, over 80% of PDAC patients are diagnosed with locally advanced or metastatic disease and therefore cannot undergo surgery [3]. The 5-year survival rate stands at about 10% despite advances in management [4,5]. Classic symptoms of PDAC include weight loss, anorexia, abdominal pain, and obstructive jaundice [6]. Some of the risk factors of PDAC include age, obesity, smoking, excessive alcohol intake, chronic pancreatitis (CP), and Type 2 Diabetes Mellitus (T2DM) [7]. Although there is very little biological information on PDAC in the African population, they have been shown to have increased incidence and mortality attributed to a combination of social (such as excessive smoking and alcohol intake) and genetic factors [8-10].

As a hallmark of cancer, tumour cells reprogram their metabolism such as promoting glycolysis to maintain cell survival and increase proliferation rate [11-13]. Metabolites are products of metabolism that navigate important biological functions such as energy conversion [14,15] and signalling [16,17]. Blood metabolite concentrations can reflect the metabolic adaptation of tumour or highlight the host response to the tumour. In this sense, Nuclear Magnetic Resonance (NMR) spectroscopy was shown to be a powerful technique for the high-throughput analysis of blood samples [18]. NMR spectroscopy has been used to investigate the serum metabolome of patients with PDAC to distinguish malignant and benign diseased states and some metabolites such as leucine, valine, isoleucine, tyrosine, lysine, creatinine, triglycerides, and 3-hydroxybutyrate were dysregulated [19-22]. Although these authors reported on blood-based metabolomics biomarkers of PDAC, however, their findings did not make associations to outcomes and were conducted in other population groups [23,24].
PDAC is a complex and heterogeneous disease. Maladies associated with biological and metabolic processes such as obstructive jaundice, diabetes, and inflammation, can result in complications that could alter the course of the disease [25]. These maladies could also lead to changes in both metabolic and lipoprotein profiles. For instance, over 70% of PDAC patients at the time of their diagnosis present symptoms of cholestatic jaundice [26], a reduction or stoppage of bile flow. An abnormal lipoprotein profile has been linked to patients that present with cholestatic jaundice that is due to the increased bile acid and cholesterol levels [27,28]. T2DM is another common comorbidity that is well known to reflect changes in the serum metabolome. In PDAC, T2DM can promote tumour progression via changes in the transcriptome and metabolome [29]. Its close association with chronic inflammation adds an extra layer to the complexity of this disease [30].

To our knowledge, for the first time, this study shows the links between metabolomic and lipoprotein profiles in PDAC patients of African ancestry with disease staging and patient survival. Additionally, the impact of the metabolic and lipoprotein profile on T2DM, cholestatic jaundice, and inflammation, is reported.

Materials and Methods

Sample Collection and processing

The study was approved by the University of Witwatersrand Human Research Ethics Committee (Medical) (Study number- M190681). All participants gave written informed consent. Patient clinical data were collected using the REDCap v9.0 [31]. Sample and data collection were done between March 2019 and March 2020. The study site was the

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Hepatopancreatobiliary Unit at Chris Hani Baragwanath Academic Hospital, Soweto Johannesburg, South Africa.

Only patients with clinically and histologically proven PDAC were recruited for this study. Inclusion criteria included patients from 18 years old and above, of African ancestry, diagnosed with one of the three stages of PDAC. Patients undergoing chemotherapy at the time of the study were excluded. Stratification into resectable, borderline resectable, locally advanced, and metastatic disease was conducted with contrast-enhanced triple phased CT-scan of the abdomen following the National Comprehensive Cancer Network (NCCN) guidelines [32]. For this study, both resectable and borderline resectable were categorized as Resectable Pancreatic Adenocarcinoma (RPC). In this group, the tumour had either not invaded any vessel or had invaded the portal vein to 90° in which case neoadjuvant chemotherapy may be necessary before surgery. The Locally Advanced Pancreatic Adenocarcinoma (LAPC) group included cases where the tumour had invaded the superior mesenteric artery and/or portal vein to more than 180°. Lastly, the Metastatic Pancreatic Adenocarcinoma group (MPC) where the tumour had spread to other organs such as the liver [33]. Chronic pancreatitis (CP) patients and healthy volunteers (HC), also of African ancestry, were recruited as the control arm of the study. To be eligible, all the healthy participants confirmed that they were in good health and were not taking any regular medication. Blood samples were collected by venipuncture in clear vacutainer tubes (BD Biosciences, New Jersey USA) without anti-coagulant. The blood was centrifuged at 3000 rpm, 4°C for 10 mins after allowing it to clot for 30-60 mins at room temperature. All samples were processed within 2 hours of collection and immediately stored at -80°C until the time of analysis.

Serum sample preparation

Three hundred microliters of thawed serum samples were aliquoted into a microcentrifuge tube and followed by 300µl of a solution containing 0.75 M potassium phosphate buffer (pH 7.4), 5.81 mM of trimethylsilyl-2,2,3,3-tetradeuteropropionic acid (TSP; Sigma-Aldrich, St. Louis, MO, USA) and a trace amount of sodium azide dissolved in deuterium oxide. Samples were vortexed to ensure complete homogeneity and a final volume of 540 µl of each sample was transferred to a 5 mm NMR tube (Wilmad Lab Glass) and analysed.

Lipid extracts preparation

Three hundred microliters of BUME (butanol: methanol - 2:1) was added to a 100 µl serum aliquots in glass GC vials followed by 300 µl DIPE (diisopropyl ether:ethyl acetate -3:1) and 300 µl H_2O. Sample were vortexed for one minute after addition of BUME and H_2O and incubated on a shaker for 10min after DIPE addition to allow for lipid extraction. The samples were then centrifuged at 4000 rpm for 5 min after which the top layer was transferred to clean vials, dried under N_2 at 37°C and then resuspended in 600 µl solution of $CDCl_3$:CD_3OD:D_2O (chloroform-d:methanol-d:water-d; 16:7:1, v/v/v) containing 1.18 mM. TSP. Five hundred and forty microlitres of this final solution was then transferred to 5mm NMR tube (Wilmad Lab Glass) for analyses.

Nuclear Magnetic Resonance Spectroscopic Analysis

One-dimensional (1D) proton (^{1}H)-NMR spectra was acquired using different pulse sequences on a 500 MHz Bruker Avance III HD NMR spectrometer equipped with a triple-resonance inverse 1H probe head and x, y, z gradient coils. A standard nuclear overhauser effect spectroscopy (NOESY) pulse sequence presat (noesygppr1d.comp) was used on both serum and lipid extract samples. On serum samples, NOESY was used to detect both signals of small metabolites and high molecular weight macromolecules such as lipoproteins. Additionally, a standard diffusion-edited (DIFF) pulse sequence (ledbpgppr2s1d) was used on serum samples

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to detect only high molecular weight macromolecules, such as lipoproteins. Pooled samples were used as a quality control sample and was included in each batch for qualitative assessment of repeatability by overlaying the raw spectra.

Nuclear Magnetic Resonance profiling

NMR spectroscopy was used to quantify a panel of 75 signals, including metabolites, lipid groups, proteins, and the inflammatory markers, GlycA and GlycB. The peaks of the identified metabolites were fitted by combining a local baseline and Voigt functions based on the multiplicity of the NMR signal [34]. The assignment of quantified signals is reported in **Table S1**. To validate the efficacy of the different deconvolution models, the root-mean-square deviation was determined. The absolute concentration of each metabolite was calculated according to the equation previously reported [35]. The number of protons contributing to the unknown signals was imputed to 1. The concentration of carbohydrates was estimated also considering the equilibrium between their cyclic forms. A selected number of ratios between metabolite concentrations was selected and associated to one or more enzymatic reaction (**Table S2**).

GlycA and GlycB signals were quantified by integrating the areas between 2.00 and 2.05 ppm and between 2.09 and 2.05 ppm respectively, above a local baseline aimed to remove the signal of lipoproteins. The Liposcale test (Biosfer TesLab) was then used to determine lipoproteins HDL, LDL, and VLDL particle number, size, and concentration of each subtype [36]. Each DIFF spectrum in the range between 0.1 and 9.5 ppm, excluding the regions corresponding to the water signals between 4.40 and 5.00 ppm, was segmented into 0.001-ppm chemical shift bins, and the corresponding spectral areas under the curve giving a total of 8800 variables.

Statistic and data analysis

Statistical analysis and graphical illustrations of the data were generated in R (version 3.6.1) and R studio (version 1.1.456) software using scripts developed in-house. Wilcoxon and Kruskal-Wallis rank-sum test were used to compare differences in numerical covariates (e.g., age and metabolite concentration). Fisher's exact test was used to assess differences between categorical variables (e.g., gender). Spearman's rank test was used to calculate the correlation coefficient (rho) between variables. The Wald test was used to calculate the statistical significance (p-value) of the differences between Kaplan–Meier survival curves. Prognostic factors for overall survival were analysed using the Cox proportional hazard regression. P-values < 0.05 were considered to be significant. To account for multiple testing, a false discovery rate (FDR) of <10% was applied.

The KODAMA algorithm [37] was used to identify of patterns that demonstrate metabolic phenotypes across all samples [38]. Using the Partition around medoids (PAM) clustering [39] was applied to the KODAMA scores using the silhouette algorithm 10 [40] to verify results obtained. The silhouette median value was utilized to assess the ideal amount of clusters ranging from 2 to 10.

Using partial least-squares (PLS) analysis, regression was performed on DIFF spectra metabolic profiles. Furthermore, a cross-validation of 10-fold was performed to evaluate the predictive efficacy of the model [18]. Both the goodness of fit parameter (R^2) and the predictive ability parameter (Q^2) were also calculated using standard formulas [41]. The Q^2 value was calculated from p-value to assess the performance of the PLS regression model [42]. A P-value < 0.05 was regarded as significant.

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Results

Patients demographic and clinicopathological characteristics

Forty patients (6 CP, 22 RPC, 8 LAPC and 4 MPC) and 6 age-matched HC were recruited. The demographic and comorbidities features of the patients with PDAC, and CP are reported in **Table 1**. The demographic features were matched across the 4 patient groups (i.e., CP, RPC, LAPC and MPC). About 50% (n=21) of the patients are smokers (≥1 packet a day) and 18 patients are alcohol consumers (> 100 grams of alcohol, which corresponds to six bottles of beer, per day). The frequency of cholestatic jaundice was statistically significant with a high prevalence in PDAC patients while being absent in all of the CP patients. Of note, five of the PDAC patients developed cholangitis, an inflammation of the bile duct system often caused by bacterial infection, and this was higher in patients with more advanced stages of PDAC. As an expected consequence of cholestatic jaundice, abnormal values of bilirubin were observed in PDAC groups compared to the CP group as shown in **Table 2** (**Figure S1**). Although not statistically significant, the PDAC groups also displayed the typical profile associated with cholestatic jaundice, including increased alkaline phosphatase and gamma-glutamyl transferase activity and a lesser increase in the transaminase enzymes, when compared to the CP patients. Interestingly T2DM tended to be more frequent amongst CP patients when compared to PDAC patients although statistical significance was not achieved.

From the clinical data routinely collected, no statistical significance in either routine haematological (**Table S3**) or chemistry-parameters (**Table S4**) were observed between the PDAC and CP groups.

Table 1: Demographic features and clinicopathological of Chronic Pancreatitis and Pancreatic Ductal Adenocarcinoma patients

Feature	CP (n=6)	RPC (n=22)	LAPC (n=8)	MPC (n=4)	p-value
HIV status					0.831
Negative, n (%)	5 (83.3)	19 (86.4)	6 (75.0)	4 (100.0)	
Positive, n (%)	1 (16.7)	3 (13.6)	2 (25.0)	0 (0.0)	
Gender					0.286
female, n (%)	0 (0.0)	8 (36.4)	2 (25.0)	2 (50.0)	
male, n (%)	6 (100.0)	14 (63.6)	6 (75.0)	2 (50.0)	
Smoking					0.450
no, n (%)	1 (16.7)	12 (54.5)	4 (50.0)	2 (50.0)	
yes, n (%)	5 (83.3)	10 (45.5)	4 (50.0)	2 (50.0)	
Alcohol					0.962
no, n (%)	3 (50.0)	13 (59.1)	4 (50.0)	2 (50.0)	
yes, n (%)	3 (50.0)	9 (40.9)	4 (50.0)	2 (50.0)	
Age, median [IQR]	51 [46.25 57.25]	63.5 [50.25 66.75]	56.5 [48 62.5]	56 [46 69.75]	0.439
Cholestatic (obstructive) jaundice					0.013
no, n (%)	6 (100.0)	8 (36.4)	2 (25.0)	1 (25.0)	
yes, n (%)	0 (0.0)	14 (63.6)	6 (75.0)	3 (75.0)	
Cholangitis					0.145
no, n (%)	6 (100.0)	20 (90.9)	7 (87.5)	2 (50.0)	
yes, n (%)	0 (0.0)	2 (9.1)	1 (12.5)	2 (50.0)	
T2DM					0.322
no, n (%)	3 (50.0)	16 (72.7)	7 (87.5)	4 (100.0)	
yes, n (%)	3 (50.0)	6 (27.3)	1 (12.5)	0 (0.0)	
Hypertension					0.560
no, n (%)	6 (100.0)	17 (77.3)	6 (75.0)	4 (100.0)	
yes, n (%)	0 (0.0)	5 (22.7)	2 (25.0)	0 (0.0)	

IQR: interquartile range; T2DM: Type 2 Diabetes Mellitus; CP: chronic pancreatitis; RPC: Resectable Pancreatic Ductal Adenocarcinoma; LAPC: Locally Advanced Pancreatic Ductal Adenocarcinoma; MPC: Metastatic Pancreatic Ductal Adenocarcinoma

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Table 2: Liver Function Tests of the Chronic Pancreatitis and Pancreatic Ductal Adenocarcinoma groups.

Feature	CP median	RPC median	LAPC median	MPC median	p-value	FDR
Total.Protein (g/L)	66.00	59.00	66.00	69.00	0.289	0.330
Albumin (g/L)	36.50	30.00	27.00	32.50	0.361	0.361
Total.Bilirubin (µmol/L)	5.00	154.00	120.00	58.00	0.006	0.030
Conjugated Bilirubin (µmol/L)	2.00	141.00	112.50	45.00	0.008	0.030
Alanine transaminase (U/L)	18.00	88.00	29.50	38.00	0.051	0.082
Aspartate.transaminase (U/L)	28.50	104.00	55.00	75.00	0.019	0.052
Alkaline phosphatase (U/L)	74.00	615.00	337.00	314.50	0.025	0.052
Gamma.glutamyl.transferase.(U/L)	61.50	751.00	301.00	483.00	0.151	0.201

FDR: false discovery rate; CP: chronic pancreatitis; RPC: Resectable Pancreatic Ductal Adenocarcinoma; LAPC: Locally Advanced Pancreatic Ductal Adenocarcinoma; MPC: Metastatic Pancreatic Ductal Adenocarcinoma

Metabolic and lipoprotein signatures in the different tumour stages

To delineate the metabolic signatures for the different PDAC stages, serum samples analysis of the cohort was conducted using NMR spectroscopy. Three different sets of NMR experiments were conducted to collect a broad range of information (**Figure 1**).

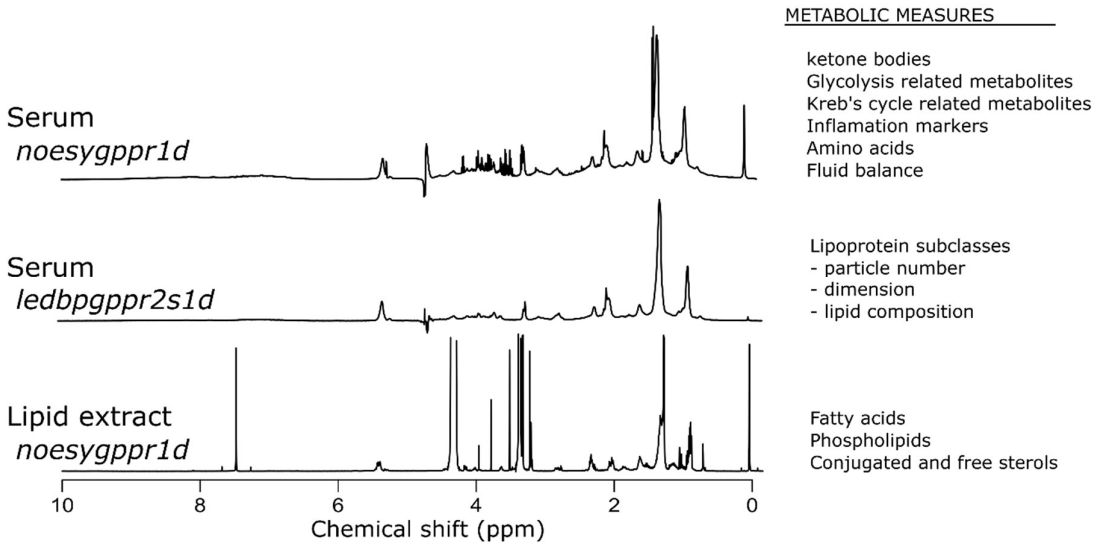

Figure 1: Nuclear Magnetic Resonance experiments showing their relative metabolic measures extracts.

The analysis of metabolic concentrations in serum samples (**Table S5**) and lipid extracts (**Table S6**) revealed that lactate, the end-product of glycolysis under anaerobic conditions, was strongly correlated with the disease stage (rho=0.50; p-value < 0.001; FDR=0.012). Although not significant, pyruvate, the precursor of lactate, showed a positive correlation with the tumor stage (rho=0.28, p-value=0.060, FDR=0.294). Lactate and glucose concentrations were not correlated (rho=0.06; p-value = 0.688). A strong positive correlation with tumor stage was noted with the glycine concentration (rho=0.52; p-value < 0.001, FDR=0.012). On the other hand, ascorbate (rho=-0.47; p-value=0.001, FDR=0.021) seems to be depleted or present in a reduced concentration in patients with PDAC. A comparison of the concentrations of lactate, glycine, ascorbate, and pyruvate across the groups HC, CP, RPC, LAPC, and MPC is shown in **Figure 2**.

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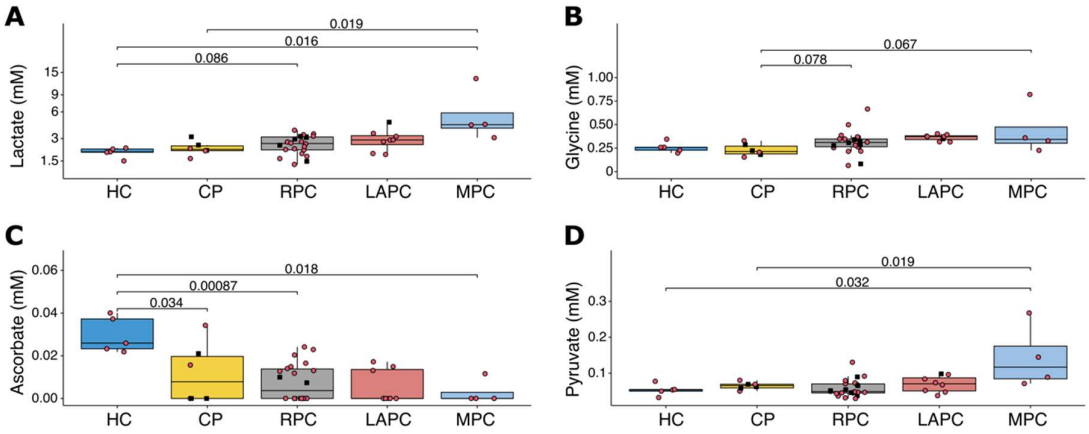

Figure 2: Boxplots showing the comparison of the concentration of (A) Lactate, (B) Glycine, (C) Ascorbate and (D) Pyruvate for HC, CP, RPC, LAPC, and MPC. Lactate is shown to be significantly elevated across the groups, glycine was significantly elevated in MPC when compared with CP. Ascorbate was significantly downregulated in CP, RPC, LAPC, and MPC when compared to HC while pyruvate was significantly upregulated in MPC when compared to HC, CP, HC, RPC, and LAPC. Black squares represent patients with Type 2 Diabetes Mellitus. HC: Healthy controls; CP: Chronic Pancreatitis; RPC: Resectable Pancreatic Adenocarcinoma; LAPC: Locally Advanced Pancreatic Adenocarcinoma; MPC: Metastatic Pancreatic Adenocarcinoma

The analysis of the metabolite ratios (**Table S7**) showed no association with disease stage while the lipoprotein parameters (**Table S8**) reported negative correlations of some parameters associated with the number of HDL-particles (P_{HDL}). Gamma-glutamyl transferase and the ratio between aspartate transaminase and alanine transaminase were not associated with the disease stage (result not included).

Dysregulated metabolites in patient survival

Wald test after adjusting for age, was used to identify the metabolites in serum samples as shown in **Table S9**, the lipid extracts (**Table S10**), the metabolite ratios (**Table S11**), and the lipoprotein parameters (**Table S12**) that correlated with the time of survival. Both 3-hydroxybutyrate (p-value= 0.015; FDR=0.370) and ethanol (p-value= 0.002; FDR=0.126) were independently correlated with the survival time in patients with PDAC. Cox hazard analysis showed that a statistically significant higher hazard ratio (HR) exists between the patients with 20% highest concentration of ethanol compared to the rest (HR=4.22 [95%CI: 1.44-12.32]; p-value=0.009) and between the patients with 20% highest concentration of 3-hydroxybutyrate compared to the rest (HR=2.88 [95%CI: 1.02-8.11]; p-value=0.045). Gamma-glutamyl transferase and the ratio between aspartate transaminase and alanine transaminase was not associated with the survival time (result not included).

Patients with 20% highest concentrations of ethanol and 3-hydroxybutyrate were grouped. This combined group showed significantly poorer survival than the remaining patients (HR=5.87

[95%CI: 1.92-17.92]; p-value=0.002). No correlation was observed between PDAC stages, lipid extracts, metabolite and lipoprotein levels, and survival time. **Figure 3** shows Kaplan–Meier plots of the survival time segregated according to the value of ethanol, 3-hydroxybutyrate, and a combination of them as described earlier.

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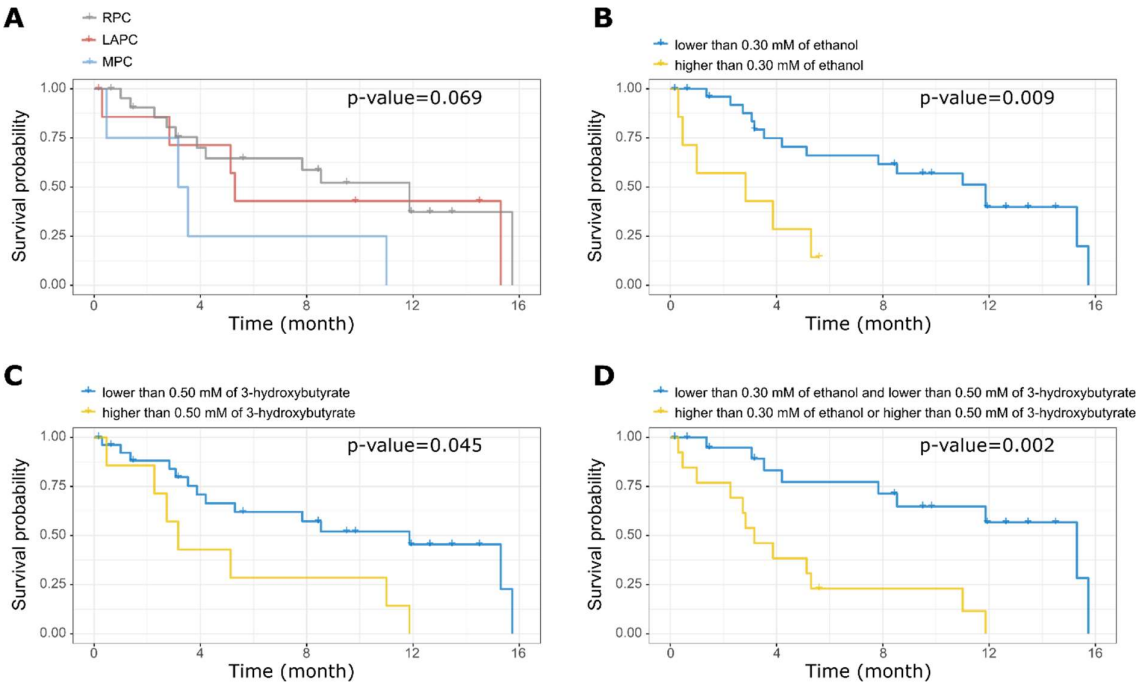

Figure 3: Impact of tumour stages and metabolites concentration on patient survival. Kaplan–Meier survival curves showing effect of **(A)** Tumour stage, **(B)** Ethanol, **(C)** 3-hydroxybutyrate, and **(D)** combination of ethanol & 3-hydroxybutyrate with survival time. There was no significant link between tumour stages and patient survival. PDAC patients with low levels of both ethanol and 3-hydroxybutyrate survived longer. RPC: Resectable Pancreatic Adenocarcinoma; LAPC: Locally Advanced Pancreatic Adenocarcinoma; MPC: Metastatic Pancreatic Adenocarcinoma

Impact of raised bilirubin levels on metabolites and lipoproteins in PDAC.

PAM clustering was performed on the KODAMA scores to identify any distinct lipoprotein phenotype (**Figure 4A**). The patients classified in cluster A or cluster B showed large differences in the lipoprotein profile when they were compared with cluster N, where the controls were classified. To verify if patients belonging to clusters A or B showed signs of cholestatic jaundice, as suggested by Lamiquiz-Moneo et al. [43], the concentration of cholesterol ester and free cholesterol was evaluated in the lipid extracts (**Figure 4B**). The ratio between free cholesterol and cholesterol ester was used as a marker to identify the presence of an abnormal lipoprotein produced in patients with cholestatic jaundice [27]. **Figure 4C** shows that patients belonging to the cluster A and B have a higher ratio indicative of the presence of abnormal lipoprotein. Using the ratio between free cholesterol and cholesterol ester, a threshold of 0.45 was identified to discriminate the clusters A and B from cluster N. All patients belonging to clusters A and B had values above 0.45. All patients belonging to cluster N had values below 0.45, except 3 patients.

Supervised PLS analysis was then performed to identify variance in the metabolic profiles of DIFF spectra associated with the ratio between free cholesterol and cholesterol ester; the resulting model demonstrated a clear and robust discrimination between patients ratio values below and above 0.45 (R2 = 0.81, 95% CI 0.81-0.86; Q2 = 0.70, 95% CI 0.69-0.72; p-value <0.001). The cross-validated model was able to discriminate the two groups with an accuracy of 90%, a sensitivity of 93.75%, and a specificity of 87.50%.

Both A and B clusters showed atypical lipoprotein expression and were then grouped as “AB”, to understand the effects of the altered ratio (free cholesterol/cholesterol ester) in lipoproteins,

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a comparison of the N versus AB clusters was performed for full blood count features (**Table S13)**, blood chemistry features (**Table S14**) and liver function parameters (**Table S15**). As expected, most of the liver function parameters were significantly altered; total bilirubin (p-value=0.003, FDR= 0.012), conjugated bilirubin (p-value=0.006, FDR= 0.016) and aspartate transaminase (p-value= 0.009, FDR= 0.018) increased in clusters AB. Furthermore, some metabolites (**Table S16**) such as total protein (p-value < 0.001, FDR< 0.001), glutamine (p-value < 0.001, FDR=0.007) reduced in concentration whereas lipid levels (p-value < 0.001, FDR= 0.001) were elevated in clusters AB. Lipid extracts (**Table S17)**, metabolites ratios (**Table S18**) and lipoproteins (**Table S19**) were significantly altered.

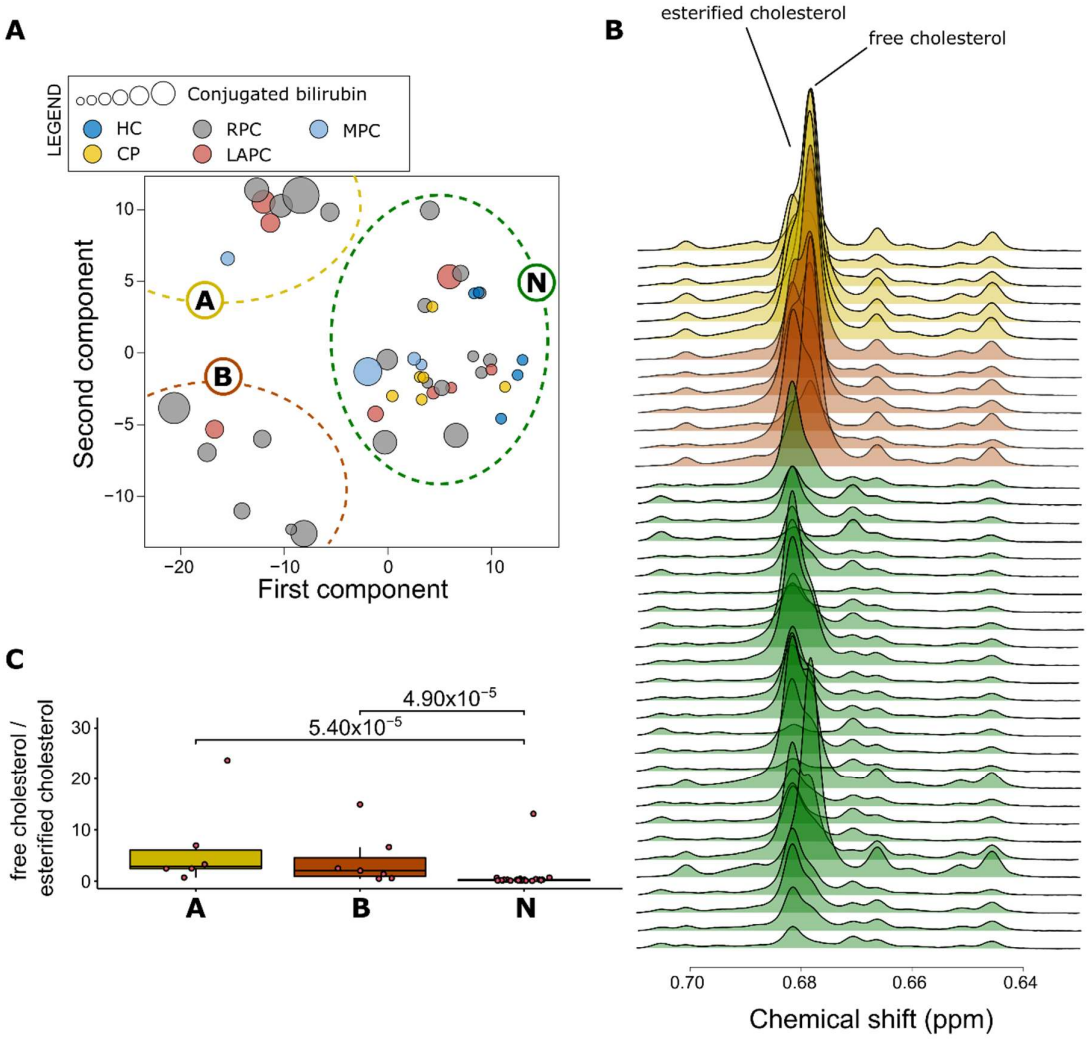

Figure 4: Measurement of Lipoprotein concentration in patient groups. (A) Lipoprotein particle concentrations were measured from the NMR spectra using LipoScale test and were separated into 3 clusters. Cluster N which is made up of all the controls and some PDAC patients have a normal lipoprotein profile while clusters A and B have atypical lipoprotein profiles with high bilirubin levels. **(B)** Spectra showing the abnormal lipoprotein profile associated with clusters A (yellow) and B (red) with a high concentration of free cholesterol which could be indicative of an abnormal lipoprotein profile while cluster N (green) all have normal lipoprotein profile; higher levels of esterified cholesterol except for one outlier. **(C)** Boxplot of the ratio of free cholesterol to

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esterified cholesterol was calculated for the three clusters to comprehend the level of lipoprotein abnormality in the serum. Cluster N has the least ratio. PDAC: Pancreatic Ductal Adenocarcinoma

Impact of diabetes and inflammation on metabolites and lipoproteins levels

In order to determine the impact of diabetes and inflammation on the metabolic signatures of PDAC patients and their link with the tumour stages, the serum metabolite concentrations between patients with and without T2DM were compared using Wilcoxon rank-sum test. With regards to patients with T2DM, no statistically significant difference between the CP and PDAC groups in the metabolite and lipoprotein concentrations was detected (results not included).

Then, the inflammatory status of the patients using both the Glasgow Prognostic Score (GPS) and the NMR inflammatory biomarkers, GlycA and GlycB were compared (**Figure 5**). GPS is a cumulative inflammation-based cancer prognostic marker based on elevated serum CRP and decreased albumin concentration [44]. The percentage of patients with GPS=2 is higher in PDAC than CP. The NMR inflammatory marker GlycA and GlycB were lower in HC compared to the pathology groups (**Figure 5B and 5C)**. Slightly higher values of GlycA and GlycB were observed in CP compared to PDAC. Over 14% of the PDAC patients had cholangitis and showed only slightly higher values of CRP (p-value=0.064) which was not significant.

However, we identified metabolites (**Table S20**) such as glucose, lactate, histidine, phosphorous, lipid extracts (**Table S21),** glucose/lactate, threonine/glycine ratios (**Table S22**) and lipoproteins (**Table S23)** that correlated with inflammatory markers: GlycA, GlycB, CRP, and Albumin. Glucose was shown to correlate directly with GlycB (**Figure S2)**.

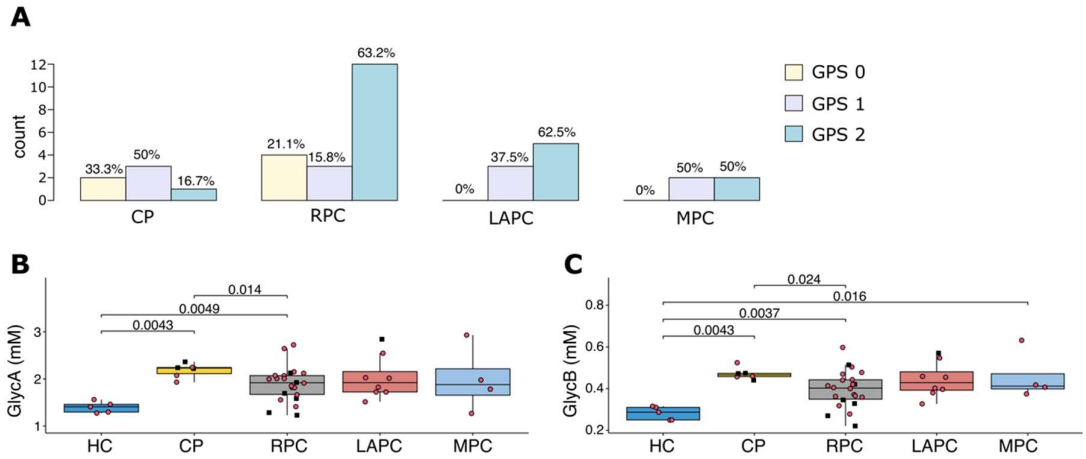

Figure 5: Inflammation status in patient groups A) shows the inflammation levels of PDAC and CP groups using Glasgow Prognostic Score (GPS), CP (6) has the least inflammation and PDAC (RPC:22; LAPC: 8; MPC: 4.) groups are all highly inflamed. GlycA **(B)** and GlycB **(C)** show the comparison of the inflammatory status of PDAC and control groups (HC and CP) using GlycA and GlycB biomarkers, respectively. There was no difference observed across the groups for both GlycA and GlycB levels except when compared with the HC. The black square boxes represent T2DM patients. T2DM: Type 2 Diabetes Mellitus; PDAC: Pancreatic Ductal Adenocarcinoma; HC: Healthy controls; CP: Chronic Pancreatitis; RPC: Resectable Pancreatic Adenocarcinoma; LAPC: Locally Advanced Pancreatic Adenocarcinoma; MPC: Metastatic Pancreatic Adenocarcinoma

 doi:10.20944/preprints202108.0235.v1

Discussion

PDAC has an almost equal number of new cases and deaths annually. Hence the need for more investigation of underlying molecular underpinnings especially in under-studied patient groups. Although patients of African descent have an elevated risk and poor survival rates of PDAC, there is little molecular and clinical information for this group. The combination of the analysis of metabolites and lipoproteins profiles with clinical parameters may improve management decisions and outcomes [45]. In this study, metabolomic and lipoprotein perturbations were observed at different stages of PDAC.

Elevated levels of lactate and glycine were shown to be associated with PDAC stages. In PDAC cells, there is an increased uptake of glucose to produce lactate and ATP under aerobic conditions, a phenomenon known as the Warburg effect [46]. Elevated pyruvate levels which is a product of glycolytic pathway, suggests the occurrence of Warburg effect which could promote PDAC progression [47]. Lactate is readily available to hypoxic tumour cells which are located far from blood vessels [48]. Furthermore, the correlation between glucose/lactate ratio with CRP, which was observed in this study, could suggest that glycolysis is elevated with inflammation. Glycine is formed from 3-phosphoglycerate an intermediate of glycolysis pathway [49], thus upregulated glycolysis could result in elevated glycine levels. Glycine is also the main substrate in glutathione and collagen production [50,51], which are essential in PDAC progression. Activation of serine/glycine biosynthesis promotes tumourigenesis by delivering a single carbon for 1-carbon metabolism for proteins, lipids, nucleic acids, and other biological macromolecules to support tumour growth [52]. Furthermore, this study observed that Threonine/Glycine ratio a direct association with albumin inferring an increase in glycine levels with inflammation.

This study also showed a link between reduced levels of ascorbate (vitamin C) with PDAC stages. Oxidized ascorbate (dehydroascorbate) is transported into cells via glucose transporters after which it is reduced to ascorbate using glutathione. [53]. It acts as a pro-oxidant triggering reactive oxygen species activities which inhibit a key glycolytic enzyme, Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH), in cancer cells [54]. Reduced levels of ascorbate may imply deregulation of the glycolysis rate, resulting in the Warburg effect, which in turn may favour PDAC progression. This hypothesis is supported by various studies that have identified the therapeutic roles of ascorbate in PDAC. Combination of ascorbate and gemcitabine achieved a more significant tumour growth inhibition in the mouse model than gemcitabine alone, also pharmaceutic doses of vitamin C act as pro-oxidant and reduced tumour growth in mice xenografts [55,56]. The anti-tumour effect was further observed when ascorbate inhibited epithelial to mesenchymal transition and consequently metastasis in both *in vitro* and *in vivo* models [55]. Administration of ascorbate was also demonstrated to improve survival in a stage IV PDAC patient with little toxicity observed [57].

Interestingly, this study showed that ethanol and 3-hydroxybutyrate (3-HB) have a negative correlation with survival time and are independent of the disease stage. Several studies have contradictory results on the role of 3-hydroxybutyrate in pancreatic cancer [22,24]. Excessive amounts of ketone bodies are usually found in individuals with diabetic ketoacidosis (DKA) or alcoholic ketoacidosis (AKA) [58]. This study suggests that high 3-HB levels could be linked to alcohol consumption or T2DM and not necessarily to the pathology. In DKA, lack of insulin contributes to ketogenesis in the liver. DKA is also linked to an altered ratio of 3-HB to acetoacetate [58], although there was no association with staging and survival in our cohort. One study showed that heavy alcohol consumption was a contributing risk factor of PDAC especially in black women [8]. Indeed, it is well-known that high concentrations of ethanol inhibit lipolysis while substantial production of ketone bodies such as 3-hydroxybutyrate occurs once with its decrease [59]. In liver cells, AKA causes a change in redox potential induced by alcohol and reduces oxaloacetate levels [59]. Although the mechanism leading to

 doi:10.20944/preprints202108.0235.v1

early deaths in PDAC patients who are alcohol consumers is unclear, one theory is that the use of 3-hydroxybutyrate by oxidative mitochondrial metabolism can induce the proliferation and migration of cancer cells [60,61]. In addition, ascorbate depletion in PDAC patients due to heavy alcohol consumption could both increase glycolysis rate thereby promoting the severity of the disease and inhibit glycogen synthesis in the tumour microenvironment [62].

Most of the PDAC patients in this study have elevated bilirubin levels reflecting an obstruction in the bile duct by the tumour. Clinically, cholestatic jaundice can be diagnosed when the ratio of total bilirubin to conjugated bilirubin is greater than 50% and there are elevated levels of other clinical liver parameters such as alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) [63]. Since these are parameters used to assess liver function, they can also be linked to liver diseases or injury [64]. Furthermore, chronic inflammation induces a variety of alterations in lipid metabolism which are accompanied by altered ratio of free cholesterol to cholesterol ester and associated with abnormal lipoprotein profile [27]. This study confirms the previously reported association between an atypical lipoprotein profile with cholestatic jaundice [43] suggesting that detection of abnormal lipoprotein profile might be more specific in identifying cholestatic jaundice in our PDAC patient cohort.

Although inflammation has emerged as an important player in pancreatic cancer development and progression [65], NMR inflammatory markers, GlycA and GlycB, were not able to discriminate between CP and PDAC. Although few PDAC patients developed cholangitis which is an inflammation of the biliary tract, they presented a generally high level of inflammation as expected. GlycA and GlycB were not able to stratify the patients based on the tumour staging. However, a fingerprint of the inflammatory processes has been observed in the metabolic profile. Interestingly, the positive correlation between GlycB and glucose concentration as well could enrich the long-standing debate on the link between inflammation and diabetes. The higher level of glucose detected in the blood could be due to the effect of chronic inflammation on decreasing insulin secretion and sensitivity [66].

Despite some of the statistically significant data, the small number of recruited patients in each stage might be a limitation. This pilot study is part of an ongoing project that aims to validate these findings in a larger patient cohort.

Conclusion

In our cohort, we demonstrated that obstructive jaundice, T2DM, and inflammation can contribute to defining the metabolic phenotype in PDAC, thus evaluating their patterns could help in predicting prognosis whereby high-risk patients for the late-stage disease may benefit from better management decisions. The depletion of vitamin C in PDAC patients with high alcohol consumptions rate reiterates its therapeutic role. Furthermore, evaluating the lipoprotein profiles in patients could help to identify more accurately those with obstructive jaundice that may require urgent treatment; however, this has to be verified.

Disclosure of Potential Conflict of Interest

The authors have no conflict of interest to disclose.

Acknowledgment

The authors would like to thank Sister Jorlina Kobue and the clinical team of the Hepatopancreatobiliary unit of Chris Hani Baragwanath Academic Hospital, Johannesburg,

South Africa, for their help with sample and clinical data collection. We are grateful to Dr Lungile Sithole and the technical staff at the NMR unit of the University of Johannesburg for their help with the initial on-site NMR training.

Funding

Research reported in this publication was supported by the South African Medical Research Council under a Self-Initiated Research Grant. The views and opinions expressed are those of the author(s) and do not necessarily represent the official views of the SAMRC. The research was further supported by the International Centre for Genetic Engineering and Biotechnology, Cape Town, South Africa.

Author contributions

Conceptualization, E.E.N; methodology, E.E.N, N.E and S.C; Bioinformatic analyses, N.E and S.C.; formal analysis, N.E, S.C, T.VZ and C.V; investigation, N.E, S.C, L.Z, J.OJ, P.F, and E.E.N; resources, L.Z, P.F, E.E.N, G.C, M.S; data curation, N.E, S.C, J.OJ, J.D, and E.E.N; writing—original draft preparation, N.E, L.Z, J.OJ, NE, SC, and E.E.N.; writing—review and editing, N.E, S.C, J.OJ, T.VZ, L.Z, C.V, J.D, M.S, P.F, G.C, and E.E.N; visualization, N.E, S.C.; supervision, E.E.N, P.F, J.OJ, and G.C.; project administration, E.E.N.; funding acquisition, E.E.N. P.F, and L.Z. All authors have read and agreed to the final version of the manuscript.

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