Urine metallomics signature as an indicator of pancreatic cancer

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Abstract

Pancreatic ductal adenocarcinoma (PDAC) is one of the deadliest types of cancer. Its high mortality rate is attributed largely to the difficulty of early diagnosis. Analysis of urine is an excellent non-invasive approach to trace changes in biochemical reactions due to cancer development. Here we show remarkable differences in concentration of several essential metals: significantly lower levels of urinary calcium and magnesium and increased levels of copper and zinc in PDAC when compared to healthy controls, and demonstrate that a combined analysis of these essential metals are accurate indicators (sensitivity = 99.5%) for metal dyshomeostasis in PDAC. In addition, natural stable zinc isotope composition ($\delta^{66/64}$Zn) in urine reveals the preferential excretion of isotopically light zinc in PDAC, likely supporting the deregulation of metalloproteins. These findings demonstrate for the first time that metallomics is a promising approach for discovery of biomarkers for detection of patients with PDAC, completely non-invasively, using urine samples.

A Introduction

Essential metals, such as calcium (Ca), magnesium (Mg), copper (Cu) and zinc (Zn) are essential for life and play a fundamental role in a number of vital physiological functions. Calcium and Mg are the most abundant divalent metals in the human body and are essential for a wide variety of metabolically important reactions such as protein synthesis and cell proliferation.[1] Copper binds to metalloproteins and acts as a cofactor for oxidative proteins where disruption of Cu metabolism is mainly caused by oxidative stress.[2] Likewise, Zn determines the catalytic and structural role of proteins[3], is essential for cellular growth and metabolism, and regulates the expression and distribution of Zn transporters.[4] Due to their important roles, the essential metals are tightly regulated in the human body, but their deregulation is found in several cancers.[5,6]

During our studies on pancreatic ductal adenocarcinoma (PDAC), one of the cancers with the poorest prognosis, we noticed the deregulation of several proteins involved in trace elements homeostasis. ATP7A, a Cu transporter was found to be exclusively over-expressed in PDAC (and not in chronic pancreatitis or healthy tissue) as well as ceruloplasmin, a plasma protein that binds and transports Cu and Zn.[7] We also highlighted the up-regulation of S100 proteins[8], which bind Ca, Mg, Cu and Zn.[9] Importantly, ATP7A and several S100 proteins are up-regulated early in PDAC precursor lesions,[7,10] indicating the potential of imbalance in trace elements to be a biomarker for early PDAC detection. This prompted us to study whether trace elements can provide a signature of early PDAC.

We analyzed urine, as it has been shown that it is an excellent non-invasive matrix for biomarker discovery.[11-13] As urine content is subjected to tight regulation, changes in biochemical reactions due to cancer development and progression may also be easier to identify.[14]

Here, we show that concentrations of several essential metals in urine can discriminate healthy controls from PDAC patients. Furthermore, we demonstrate that the intrinsic Zn isotope compositions ($\delta^{66/64}$Zn) in urine of these two groups are distinct. The analytical resolution of isotopic tracers is 100 times better than currently routinely achievable in hospitals and healthcare institutions[15], and the studies of stable metal

isotopes (*i.e.,* Zn and Cu) in different diseases were shown to provide a key additional information on the metabolism of essential metals which cannot be obtained by concentration analysis alone.[16-19] Thus, we wanted to establish if Zn isotope compositions can be used to trace changes in the molecular mechanisms caused by PDAC development, which has not been explored previously.

B Experimental

Study Participants and sample collection. Healthy (n = 46) and PDAC (n = 21) urine specimens were collected specifically for this study through Barts Pancreas Tissue Bank, after patient consent and with ethical approval (Reference number 13/SC/0593). All samples were collected in pre-cleaned 50 ml metal-free tubes to avoid any contamination with environmental trace elements. The basic demographic information for the recruited patients and the controls is summarized in Table S1. The sample size was calculated to be sufficient based on a previous study using a power calculation to give 80% statistical probability, in order to inform and justify a larger study.[18]

Sample preparation. Urine sample preparation was performed by microwave acid digestion. Quartz sub-boiled distilled HNO_3 (15.4 N) and 30% H_2O_2 (Romil Ltd) were used throughout the procedure. One milliliter of urine sample was transferred in acid cleaned XP-1500 Plus (PTFE) vessels and 3 ml of distilled HNO_3 and 2 ml H_2O_2 were added. A procedural blank consisting of 3 ml of distilled HNO_3 and 2 ml H_2O_2 was included for each microwave digestion run. After pre-digestion at room temperature overnight the samples were processed using the MARS 5 Digestion Microwave System (CEM Corp., UK) by ramping up the temperature stepwise to 210°C and 250 psi over 60 minutes, and held there for 30 minutes to ensure complete digestion. After cooling, the digested samples were transferred to Savillex Teflon vials and evaporated to complete dryness at 100°C. Each urine sample was digested twice where one set was used for major and trace element analysis and the other for Zn isotope analysis. For major and trace element analysis, the dried down samples were re-dissolved in 5 ml 2% HNO_3 refluxed at 80°C for 2 hours, cooled

down and transferred to acid-cleaned 'metal-free' centrifuge tubes (VWR). For Zn isotope analysis, the samples were re-dissolved in 1 ml 1N HCl for the subsequent step of matrix separation.

Major and trace element analysis. Major (K, Na, Mg, Ca, Rb) and trace elements (Li, Al, Fe, Co, Ni, Cu, Zn, Cr, As, Sr, Mo, Ba, Pb) in the urine samples were analyzed by a PerkinElmer NexION 350D Inductively Coupled Plasma-Mass Spectrometer (Q-ICP-MS) equipped with an Elemental Scientific (Omaha, USA) prepFAST. Trace elements were measured by flow-injection system to decrease the total dissolved solids (TDS) input to the plasma. Helium was used as a cell gas to perform kinetic energy discrimination (KED) for Cr, Fe, Co, Cu, As, and Pb, in order to reduce polyatomic interferences on the analyte mass. After every 10 samples, standard quality control and calibration blanks were analyzed to evaluate potential memory effects and cross contamination.

Zn isotope analysis. All steps of sample purification for Zn isotope analysis was carried out in a Class 10 laminar flow hood. Initially, 10% of each sample were used for concentration checks to ensure a correct sample/spike ratio prior Zn separation. Zinc separation from matrix solutes was performed with AG-MP1 resin (BioRad, 100 – 200 mesh) using Teflon columns with 10 ml reservoir. The columns were loaded with 250 µl resin volume and cleaned with one reservoir volume of 0.1N HNO_3 and deionized H_2O. The resin was conditioned with one reservoir 6N HCl and equilibrated with 4 x 0.5 ml 1N HCl. The spiked sample was re-dissolved in 1ml 1N HCl, loaded on the column and subsequently rinsed with 8 ml of 1N HCl. In the last step, Zn was eluted by 6 ml 0.01N HCl and collected in Savillex Teflon vials. After drying, matrix separation was repeated because of the relatively low Zn to matrix ratio. In particular, the high Na and K urine content can cause polyatomic interference ($^{23}Na^{39}K^{+}$) on mass 62. The signal on mass 62 is usually from ^{62}Ni, an interference we use to correct for the Ni interference on ^{64}Zn.

The Zn isotope ratios ($\delta^{66/64}Zn$) were measured with a Nu Plasma HR MC-ICP-MS and a DSN at low mass resolution. Zn isotope measurement for each sample was normalized using double-spike techniques and

sample-standard bracketing. All Zn isotope values were expressed in the delta notation as $\delta^{66/64}$Zn (‰) relative to the IRMM 3702:

$$\delta^{66/64}Zn = \left(\frac{(^{66}Zn/^{64}Zn)sample}{(^{66}Zn/^{64}Zn)IRMM3702}\right) \times 1000 \quad \text{(Eq. 1)}$$

Control blanks and the certified reference materials ERM BB184 (bovine muscle), ERM BB186 (pig kidney) and IRMM 3702 were processed with each batch of samples. The uncertainty on $\delta^{66/64}$Zn was estimated by calculating the twice root mean square (RMS) for samples prepared and analyzed in duplicate (n = 52).

Quality test and data normalization. To account for element variability based on hydration status and urine volume, element concentrations were normalized (C_{norm}) relative to the urine specific gravity (SG) using the Levin-Fahy equation[20]:

$$C_{norm} = C_{measured} \times (SG_{ref}-1)/(SG_{measured}-1)$$

where $C_{measured}$ is the sample element concentration and $SG_{measured}$ is the sample specific gravity. SG_{ref} is the median value for healthy humans with reference urinary SG of 1.02.[21,22] Normalization of trace element concentrations using SG appears to be more reliable than the creatinine parameter because of the erroneous assumption of constant creatinine excretion rates and larger inter-individual variability than SG.[23]

The detection limits (LOD) for each element was calculated by three times the standard deviation of the procedural blank from microwave digestion (n = 11). Limit of quantification (LOQ) was determined by ten times the standard deviation of the procedural blank. Element values below LOD were excluded from further statistical evaluations.

Statistics. The Mann-Whitney test was used to evaluate the significance of element concentrations and isotopic signature between PDAC and healthy controls. Hotelling's T^2 test was used to test for the difference in multivariate means for the Ca, Mg, Cu and Zn in cases and controls. For the elements Ca, Mg, Cu and Zn and their combination a receiver operating characteristic curve (ROC) was performed. The performance

characteristics of the elements were evaluated and compared in terms of the specificity (SP, proportion of correctly identified healthy controls), sensitivity (SN, proportion of correctly identified PDAC patients) and the area under the ROC curve (AUC). Confidence intervals (CI 95%) for AUCs were derived based on the DeLong's method[24], SP and SN 95% CI were derived using bootstrap replicates. Analysis of the SN, SP and the AUC of the elements was performed in R version 3.5.1.

C Results and Discussion

Quality control. In this discovery study, we have analyzed urine samples from 46 healthy controls and 21 patients diagnosed with PDAC (Table S1). The LOD values listed in Table S2 show that few samples of the PDAC pool for the statistical analysis had to be excluded for Cr (8), Al (5), Fe (4), Co (2), Ni (1), Ba (1) and Pb (1). For the healthy controls few samples had to be omitted for Al (9) and Cr (19).

Average external reproducibility for $\delta^{66/64}$Zn of the samples, determined as twice root mean square, were 0.13‰ (n = 52). The average $\delta^{66/64}$Zn data for reference materials ERM BB186 (pig kidney; -0.68 ± 0.14‰, n = 21) and ERM BB184 (bovine muscle; -0.29 ± 0.12‰, n = 14) are in good agreement with previously published values.[16,25] Total procedural Zn blanks were in average 1 ± 0.9% relative to the bracketing standard and sample concentration used for Zn isotope analysis.

Trace element concentrations of all samples are reported in Tables S3 and S4. While no significant variations in the element concentrations are observed for K, Li, Al, Rb, Ni, Cr, As, Mo and Pb, the element concentrations of Na, Mg, Ca, Fe, Cd, Cu, and Zn in urine differed between PDAC and healthy controls. Gender and age did not correlate with any of the urinary essential metals, indicating that they were not confounders in our findings.

Our data demonstrate significantly lower urinary Ca ($P < 0.0001$) and Mg ($P = 0.0002$) concentrations in PDAC patients compared to the healthy controls (Fig 1A). This seems to be reflecting disruption of cell proliferation and protein synthesis affects the Ca efflux and intake in the cell.[26] Significantly lower levels

of both macroelements have previously been reported in PDAC plasma,[27,28] which is now corroborated by our findings in urine.

Patients with PDAC exhibit a significantly higher levels of Cu ($P = 0.02$) and Zn ($P = 0.015$) (Fig 1B) than observed in healthy urine samples. Increased Cu levels were also described in both tissue and serum (together with ceruloplasmin) in PDAC patients,[29] the former being negatively associated with patient survival.[30] Moreover, reduction of Cu and ceruloplasmin using chelates is an approved therapeutic approach.[31] Increased urine Cu level seen in our study likely mimics increased levels in plasma.

The mean urinary Zn concentration for the healthy controls (313 ng ml^{-1}) is in the range of previously reported values (180 - 305 ng ml^{-1}),[21,32,33] however, the higher mean Zn concentrations (1302 ng ml^{-1}) in the PDAC urines could indicate the presence of cancer. Development and growth of PDAC cells require intracellular milieu with low Zn levels, which can be achieved through downregulation of metal-binding transporters, like ZnT (SLC30A) and ZIP3 (SLC39A).[34,35] Of note, downregulation of ZIP3 as well as the transcription factor RREB1, which regulates its expression, were found in PDAC precursor lesions, PanINs.[25] Furthermore, recurrent mutations of RREB1 were recently demonstrated in PDAC[35]; all of this indicates the important role of Zn homeostasis in PDAC pathogenesis. The lack of effective Zn uptake likely leads to increasing Zn excretion (also supported by previous studies in PDAC tissues, which contained approximately 40% less Cu and 65% less Zn than healthy pancreas,[34,35] which could explain our finding of increased levels of Zn in urine. Zinc is tightly regulated in the body which is reflected by a strong correlation between urinary Zn concentration and Zn/Cu ratio in the healthy controls ($r^2 = 0.66$, Fig. S1); this balance is disrupted in PDAC, which is evidenced by the lack of correlation between urinary Zn concentration and Zn/Cu ratio for PDAC samples ($r^2 = 0.0002$).

Hotelling's T^2 test indicated significant difference in the multivariate profiles of Ca, Mg, Cu and Zn between cases and controls ($p<0.001$); the results of univariate receiver operating characteristic (ROC) curve are shown in Fig 2. The combination of the four elements Ca, Mg, Cu and Zn showed a good discriminating power for PDAC patients from healthy controls (AUC 0.995,) which now warrants further confirmation in

much larger number of samples. This strongly indicates that the metal dyshomeostasis is caused by PDAC and supports the use of urine metallomics as a novel approach for pancreatic cancer detection.

The natural variation of metal isotopic ratios is a powerful and more sensitive indicator of the minute changes in metabolic processes, independent of the element concentration. We therefore aimed to establish Zn isotope ratios in urine of PDAC patients, and show that urinary Zn isotopic composition in PDAC samples is significantly different than in healthy controls (P = 0.002, Fig. 3A). The $\delta^{66/64}$Zn values for PDAC samples range between -0.33‰ to +0.15‰, with a median value of -0.15‰. The healthy control group tended to have higher $\delta^{66/64}$Zn values, ranging between -0.26 ‰ to 0.67‰ (median value of 0.02‰). The majority of PDAC samples (75%) showed the preferential excretion of isotopically light Zn while the healthy controls are predominantly isotopically heavy (60%, Fig 2B, Table S5). Neither age nor gender showed a significant relationship to Zn isotope composition.

A limited number of proteins can cause this observed difference in the Zn isotope composition. Mechanistically, Zn binds on readily exchangeable ligands including either nitrogen, oxygen or sulfur binding sites.[3] To minimize the overall energy of the biological system, heavy isotopes are preferentially substituted into the compound whose bonds are stronger, thus favoring complexation to nitrogen ligands, while isotopically lighter Zn preferentially binds to sulfur ligands present in cysteine-rich proteins like metallothionein.[36] Deregulation of metalloproteins can thus lead to shifts in Zn isotope composition.[17-19] Therefore, one putative scenario leading to the lighter Zn composition in PDAC can be the oxidation of metallothioneins. Oxidative stress caused by cancer development and progression oxidizes the sulfhydryl groups in cysteine,[37] which decreases Zn binding capacity. As sulfur-based cysteine binds preferentially isotopically light Zn due to weak electronegativity, oxidation of sulfhydryl-cysteine groups could ultimately result in an increase in free Zn with light Zn isotope signature, which we are detecting in PDAC urine specimens. In addition to further experimental confirmation of this hypothesis, as expression of metallothionein in PDAC has been shown to correlate with the disease stage,[38] it would also be interesting

to explore if Zn isotopic composition in urine changes over time with PDAC progression, and could serve as a prognostic and monitoring biomarker.

The results of this proof-of-concept study demonstrate that urinary concentration of several trace elements, as well as Zn isotope composition in urine of PDAC patients significantly differ from healthy controls. This suggests that metallomics studies have the potential to be a source of new biomarkers for detection and potentially monitoring of PDAC, completely non-invasively, using urine specimens. Larger, independent studies are now warranted to determine if such analysis can help unravel early changes in PDAC development that could lead to curative surgical resection and ultimately improve the currently poor survival of pancreatic cancer patients.

Conflicts of interest. There are no conflicts to declare.

Acknowledgments. K.S. conducted the analytical work; K.S., F.L. and T.C.J. designed the study. F.L. and T.C.J managed the study; AS, RR and HMK provided the samples from Barts Pancreas Tissue Bank; O.B. conducted the statistical analysis and all authors analyzed the data and contributed to writing the manuscript. The project was supported by a Pancreatic Cancer Action Early Diagnosis Challenge Award.

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Figure Legends

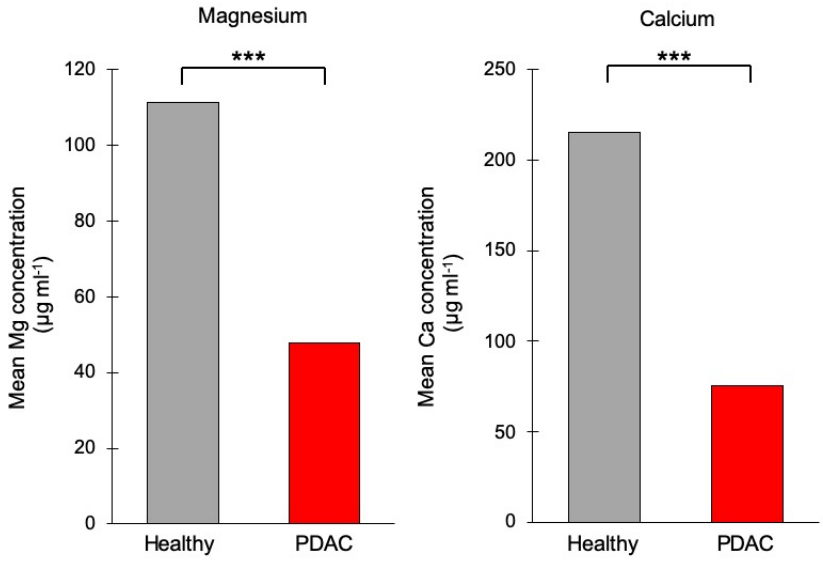
Figure 1: Element concentrations in urine in healthy control (grey, n = 46) and PDAC (red, n = 21) samples. For all panels, * P = 0.01-0.05, ** P = 0.001-0.01, and ***P < 0.0001

Figure 2: Performance characteristics for Ca, Mg, Cu and Zn and their combinations shown as receiver operating characteristics (ROC) curve. The area under the ROC curve (AUC), sensitivity and specificity and the 95% confidence interval are listed in the table. The area under the ROC curve (AUC) can vary between 0.5 (pure chance) and 1.0 (fully trustworthy test). The AUC values for the present data support the use of Ca, Mg, Cu and Zn and their combinations for detection of PDAC.

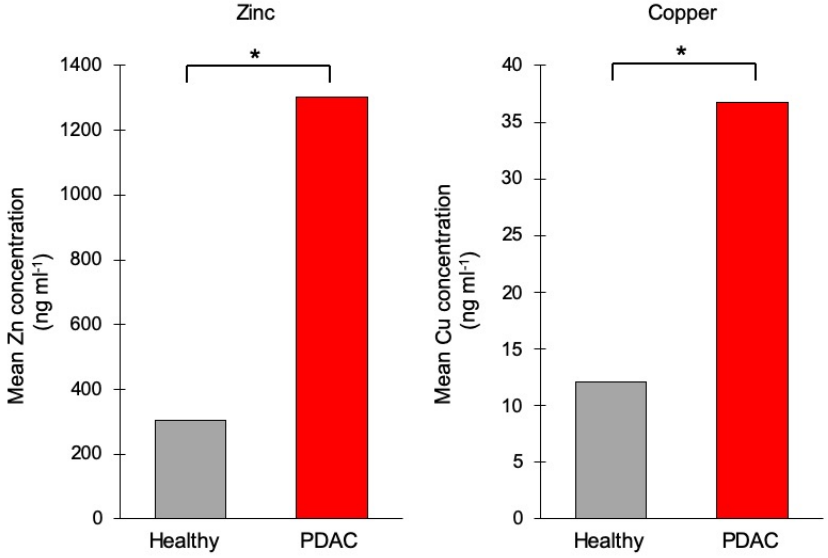
Figure 3: Distribution of urinary Zn isotope composition as **A:** Whiskers plot and **B:** Histogram ($\delta^{66/64}Zn_{IRMM3702}$) for healthy control (n = 33) and PDAC (n = 17). For the whiskers plot, the central line marks the median value. The lower quartile (25_{th} percentile) and upper quartile (75_{th} percentile) of the dataset and the whiskers present the most extreme data points. Red denotes PDAC samples and grey denotes urine from healthy controls.

Figure 1

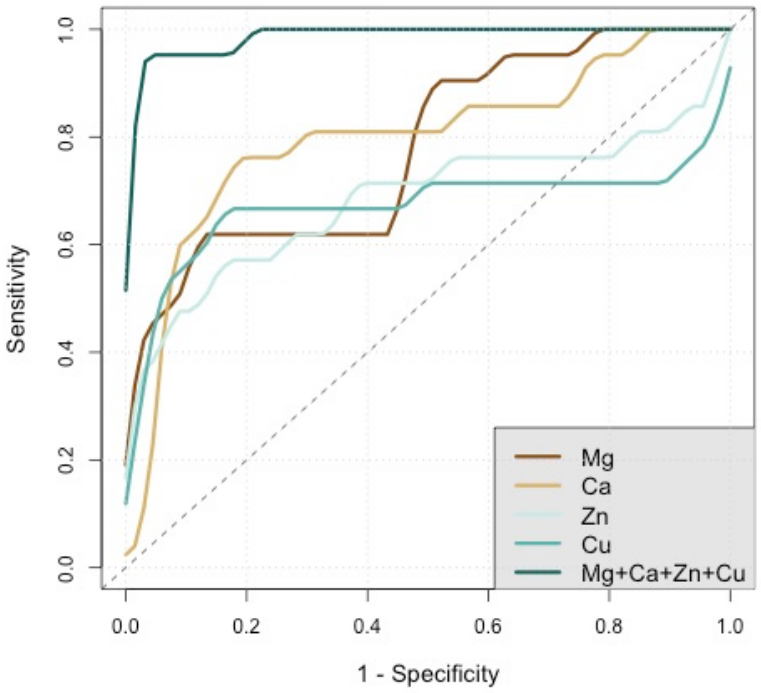
A)


Magnesium
***
Mean Mg concentration (µg ml-1)
120
100
80
60
40
20
0
Healthy
PDAC
Calcium
***
Mean Ca concentration (µg ml-1)
250
200
150
100
50
0
Healthy
PDAC

B)


Zinc
*
Mean Zn concentration (ng ml-1)
1400
1200
1000
800
600
400
200
0
Healthy
PDAC
Copper
*
Mean Cu concentration (ng ml-1)
40
35
30
25
20
15
10
5
0
Healthy
PDAC

318 Figure 2



319

320

Approach	Mg	Ca	Zn	Cu	Mg+Ca+Zn+Cu
AUC (95% CI)	0.783 (0.659-0.907)	0.796 (0.667-0.926)	0.685 (0.516-0.855)	0.671 (0.484-0.858)	0.99 (0.97-1)
Sensitivity (95% CI)	0.619 (0.381-0.81)	0.762 (0.571-0.905)	0.571 (0.381-0.762)	0.667 (0.476-0.857)	0.952 (0.857-1)
Specificity (95% CI)	0.891 (0.804-0.978)	0.826 (0.717-0.935)	0.848 (0.739-0.935)	0.848 (0.739-0.935)	0.978 (0.935-1)

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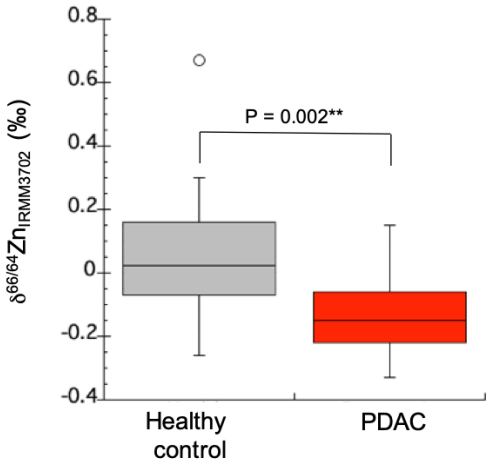
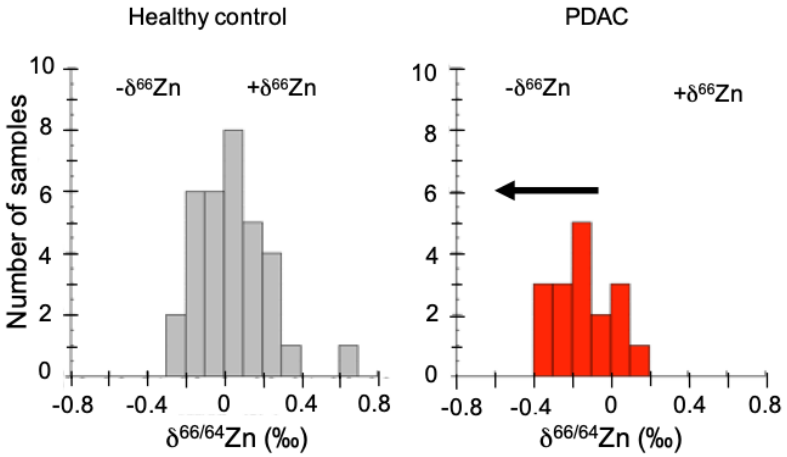
Figure 3

A)


0.8
0.6
0.4
0.2
0
-0.2
-0.4
δ66/64ZnIRMM3702 (‰)
P = 0.002**
Healthy control
PDAC

B)


Healthy control
PDAC
-δ66Zn
+δ66Zn
Number of samples
δ66/64Zn (‰)