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Phases of Metabolic and Soft Tissue Changes in Months Preceding a Diagnosis of Pancreatic Ductal Adenocarcinoma

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Abstract

Background & Aims: Identifying metabolic abnormalities that occur before pancreatic ductal adenocarcinomas (PDACs) are detected could increase chances for early detection. We collected data on changes in metabolic parameters (glucose, serum lipids, triglycerides; total, low-density, and high-density cholesterol; and total body weight) and soft tissues (abdominal subcutaneous fat [SAT], adipose tissue, visceral adipose tissue [VAT], and muscle) from patients 5 years before the received a diagnosis of PDAC.

Methods: We collected data from 219 patients with a diagnosis of PDAC (patients) and 657 healthy individuals (controls) from the Rochester Epidemiology Project, from 2000 through 2015.

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We compared metabolic profiles of patients with those of age- and sex-matched controls, constructing temporal profiles of fasting blood glucose, serum lipids including triglycerides, cholesterol profiles, and body weight and temperature for 60 months before the diagnosis of PDAC (index date). To construct the temporal profile of soft tissue changes, we collected computed tomography scans from 68 patients, comparing baseline (>18 months before diagnosis) areas of SAT, VAT, and muscle at L2/L3 vertebra with those of later scans until time of diagnosis. SAT and VAT, isolated from healthy individuals, were exposed to exosomes isolated from PDAC cell lines and analyzed by RNA-Seq. SAT was collected from KRAS^{+/LSL-G12D} P53^{flx/flx} mice with PDACs, C57/BL6 (control) mice, 5 patients and analyzed by histology and immunohistochemistry.

Results: There were no significant differences in metabolic or soft tissue features of patients vs controls until 30 months before PDAC diagnosis. In the 30 to 18 months before PDAC diagnosis (phase 1, hyperglycemia), a significant proportion of patients developed hyperglycemia, compared to controls, without soft tissue changes. In the 18 to 6 months before PDAC diagnosis (phase 2, pre-cachexia), patients had significant increases in hyperglycemia and decreases in serum lipids, body weight and SAT, with preserved VAT and muscle. In the 6 to 0 months before PDAC diagnosis (phase 3, cachexia), a significant proportion of patients had hyperglycemia compared with controls, and patients had significant reductions in all serum lipids, SAT, VAT, and muscle. We believe the patients had browning of SAT, based on increases in body temperature starting at 18 months before PDAC diagnosis. We observed expression of uncoupling protein 1 (UCP1) in SAT exposed to PDAC exosomes, SAT from mice with PDACs, and SAT from all 5 patients but only 1/4 controls.

Conclusions: We identified 3 phases of metabolic and soft-tissue changes that precede a diagnosis of PDAC. Loss of SAT starts 18 months before PDAC identification, is likely due to browning. Overexpression of UCP1 in SAT might be a biomarker of early-stage PDAC, but further studies are needed.

Graphical Abstract

Keywords

carcinogenesis; diabetes; disease progression; asymptomatic

Introduction

Profound metabolic and soft tissue changes occur in the pre-diagnostic phase of PDAC diagnosis that could aid in its early detection. For example, PDAC causes new-onset diabetes.^{1, 2} a median of 6-9 months before PDAC diagnosis^{1, 3, 4}. The objective of our study was to identify additional metabolic abnormalities that may provide clues to even earlier diagnosis of PDAC. For this we focused on defining the temporal changes in metabolic parameters (glucose, serum lipids (triglycerides [TG], total cholesterol [TC], low-density cholesterol [LDL] and high-density cholesterol [HDL] and total body weight), and soft tissues (abdominal subcutaneous, adipose tissue (SAT), visceral adipose tissue (VAT) and muscle) in the 5 years preceding PDAC diagnosis.

Dyslipidemia, characterized by elevation in serum TG, TC and LDL and decrease in HDL, is strongly associated with type 2 diabetes mellitus (DM) and obesity⁵. PDAC is associated with obesity⁶, insulin resistance^{7, 8} and DM^{8, 9}; therefore, one would expect dyslipidemia similar to that seen in type 2 DM in PDAC as well. However, weight loss, often profound, occurs in PDAC, which would normally lower serum TG, LDL and TC and increase HDL.¹⁰ To determine if, how and when the temporal lipid profile in PDAC is affected, we compared these metabolic parameters in PDAC vs. controls. To study the temporal profile of metabolic parameters we constructed a population-based cohort of all subjects with PDAC in Olmsted County between 2000 and 2015 and matched healthy controls; we recently described the glycemic profile of this cohort.¹¹

Weight loss and soft tissue changes (AT and muscle loss) are characteristic features of PDAC^{12, 13}. Temporally, while symptoms of cachexia and muscle loss (anorexia, fatigue, and reduced exercise tolerance) appear shortly before (<6 months) PDAC diagnosis, onset of objective weight loss precedes PDAC diagnosis by a year.^{3, 14} For a temporal profile of soft tissue changes we assembled a cohort of PDAC subjects with serial computerized tomography (CT) scans. Using this cohort we determined the time course of AT and muscle loss.

Recently AT loss in PDAC has been attributed to pancreatic exocrine dysfunction¹⁸. We, have previously shown that exosomes shed by PDAC have profound metabolic effects on a variety of tissues including beta cells,¹⁹ adipocytes²⁰ and peripheral blood mononuclear cells.²¹ We, therefore, tested the effect of PDAC exosomes on human SAT. We also studied SAT of PDAC-bearing mice as well as SAT from human PDAC subjects and controls. Our human, experimental and animal studies provide new insights into time course of metabolic and soft tissue changes in pre-diagnostic PDAC and provide preliminary evidence for a potential novel biomarker that has the potential for early diagnosis of PDAC.

Patients and methods

Selection of patients and controls, collection and handling of data and human specimens, consent for participation and all *in-vitro* experiments were in accordance to protocols approved by Mayo Clinic Institutional Review Board. All animal studies were compliant

with protocols approved by Mayo Clinic Institutional Animal Care and Use Committee (IACUC).

Cohorts assembled for clinical studies:

We compared metabolic profiles of PDAC vs. age- and gender-matched controls by constructing temporal profiles of fasting blood glucose (FBG), serum lipids including triglycerides (mg/dl), total cholesterol (mg/dl), low-density lipoprotein (LDL) cholesterol (mg/dl), high-density lipoprotein (HDL) cholesterol (mg/dl), body weight (Kg) and body temperature (°C) for 60 months before PDAC diagnosis (index date). To construct the temporal profile of soft tissue changes in PDAC we identified subjects with serial CT scans for 60 months before PDAC diagnosis date.

Case ascertainment for metabolic profile of PDAC: The Rochester Epidemiology Project (REP), a unique medical records linkage system funded by NIH since 1966, collects, collates, and indexes patient-level data from all health care providers in Olmsted County, Minnesota and allows for accurate population-based epidemiologic research.^{22, 23} We used REP index codes to identify all PDAC cases in Olmsted County between 2000 and 2015 (n=400), manually reviewed their medical charts to include only those (n=219) with a confirmed diagnosis of PDAC.¹¹ For each PDAC we selected 3 age- (same birth year) and gender-matched Olmsted County residents as controls blinded to all metabolic parameters who were seen at the Mayo Clinic in the same calendar month as the matched case's date of PDAC diagnosis (index date) (n=657). To construct the temporal metabolic profiles, we electronically retrieved all outpatient FBG, serum lipids, body weight and body temperature values at and up to 60 months before index date, grouped into 12-month time periods. To define our time interval groups, we plotted symptoms duration in our population-based PDAC cohort and observed that almost all symptoms occur in the last 6 months prior to cancer diagnosis (supplementary figure 1). Therefore, to understand the metabolic and soft tissue changes occurring in last 6 months, we studied this phase independently and all other time intervals were grouped into 12-month period from this period.

Study of changes in SAT, VAT and muscle in PDAC: SAT, VAT, and muscle were quantified from a single axial slice of abdominal CT scans (on contrast or non-contrast enhanced series) taken at L2-L3 vertebral level. In brief, imaging series DICOM data was converted to NIfTI using dcm2nii. Imaging was visualized and tissue areas were quantified (mm²) using custom software (Rilcontour, <https://gitlab.com/Philbrick/rilcontour>, Mayo Clinic, Rochester, MN). A cohort of 86 PDAC subjects with CT scans available at least in 2 out of 4 time points (−66 to −42, −42 to −18, −18 to −6 and at diagnosis of cancer) were identified. This PDAC CT cohort was then stratified based on weight change from baseline (−60 to −18) at subsequent time points as: weight loss (−2kg) or weight stable (+2kg) (n=68) and weight gain (−2kg) (n=18). Minimum cutoff value of body weight in Enriching New-onset Diabetes for Pancreatic Cancer (ENDPAC) score was used to define weight loss³. The muscle area of para-spinal and abdominal wall, total area of SAT, VAT, bone and organ were measured on the same CT section. We compared area of SAT, VAT and muscle at −42 to −18-month time interval to −66 to −42-month time interval and found all parameters to be similar (supplementary table 6). To construct the soft tissue temporal profile, we compared

the mean area of SAT, VAT and muscle at -18 to -6 and -6 to 0-month interval to -42 to -18-month interval.

Cell culture and isolation of exosomes: PDAC cell lines (Panc-1, MiaPaCa, ASPC and BXPC3) were cultured in DMEM with 10% FBS culture media to 70% confluence. After washing with PBS, cells were cultured in exosome free FBS containing DMEM for 72 hours followed by collection of the supernatant which was centrifuged twice at 3,000 rpm for 10 minutes at 4°C to remove debris. The supernatant was then centrifuged at 100,000g for 60 minutes to pellet exosomes. Exosomes were washed in PBS and centrifuged at 100,000g for 60 minutes. The pellet was re-suspended in PBS. Exosomes from 3 ml of heparinized blood from human subjects were isolated by the same method. Exosomes were passed through 250 nm filter before addition to culture media. Protein concentrations of exosomes were measured using the Bicinchoninic acid assay (Pierce).

Adipose tissue retrieval and isolation of preadipocytes: SAT and VAT were harvested from otherwise healthy donors (renal transplant) at the time of surgery. Pre-adipocytes were isolated by collagenase digestion and gradient separation method described elsewhere.²⁰

Culture of pre-adipocytes: Pre-adipocytes from SAT and VAT from one individual were cultured in parallel in adipocyte differentiation media (with constituents optimized for human pre-adipocytes as described elsewhere) for 4 weeks. Cultures were carried out in 12 well plates (Corning) with well volume of 6.9 ml. Exosomes from cancer cell lines were added on day 1 of culture in 1 ml culture media. No exosomes were added in control groups. Additional culture media (1ml volume) was added at 72 hour, 7 day, 10 day time points. Subsequently, culture media was exchanged (1 ml volume at a time) every 3 days. Various concentrations of exosomes were used in preliminary investigations on subcutaneous pre-adipocytes. Significant death of pre-adipocytes was observed at 50 µg/ml concentration of exosomes when cultured for > 72 hours. Concentration of 12.5 µg/ml exosomes seemed to be optimal and close to the physiologic exosome concentration in the plasma (16 ± 3 µg/ml in plasma in pancreatic cancer patients compared to 8 ± 3 µg/ml in healthy individuals as shown in our prior publication²⁰ which was used in all experiments of adipocyte differentiation.

Adipocyte cell lysates and western blot analysis: Mature adipocytes were washed with PBS twice and lysed with NP40 buffer (Atlanta biologicals) with protease and phosphatase inhibitors. Protein concentrations were measured by Bicinchoninic acid assay (Pierce) and 5µg protein was separated by polyacrylamide gel electrophoresis and transferred to PVDF membranes (Millipore). Blocking was done in 5% skimmed milk followed by incubation with primary antibody. HRP-conjugated secondary antibodies (Santa Cruz Biotech) were used and bands were detected by fluorescence. ImageJ (NIH, Bethesda, MD) software was used for quantification per developer's protocol.

Triglyceride assay: Triglyceride content was measured in adipocyte lysates using Infinity Triglyceride Kit (Thermo) per manufacture's protocol and triglyceride standard (Cayman chemical) was used for assay calibration.

RNA isolation: RNA extraction from the samples was performed following protocol of the RNeasy Mini kit (Qiagen, Valencia, CA). High-quality RNA for sequencing was confirmed on a Bioanalyzer 2100 (Bioanalyzer; Agilent Technologies, Santa Clara, California). RNA sequencing libraries were prepared at the Mayo Clinic Medical Genome Facility according to the manufacturer's instructions for the TruSeq RNA Sample Prep Kit v2 (Illumina, San Diego, CA). The concentration and size distribution of the libraries were determined. Libraries were pooled and diluted to 8-10 pM for flow cell clustering and sequenced using an Illumina HiSeq 2000 at 2×101bp settings.

RNA sequencing: The FASTQ files from the RNASeq samples sequenced were analyzed using Mayo Clinic's MAPR-Seq v. 2.0.0 application (<http://bioinformaticstools.mayo.edu/research/maprsea/>). MAPR-RSeq integrates a suite of open source bioinformatics tools along with in-house developed methods to analyze paired-end RNA-Seq data. Read alignment was performed with Tophat²⁴ which uses Bowtie,²⁵ a fast, memory efficient, short sequence aligner. The reads were aligned to the transcriptome (Ensembl GTF) and to the genome (hg19) to report both existing and novel expressed regions. The BAM file produced by Tophat was processed using featureCounts²⁶ to summarize expression at the gene and exon levels. DESeq,²⁷ an R package for performing differential expression analyses, was used to calculate; p-values, false discovery rates, and the amount of fold change between the group comparisons. The most significant differentially expressed genes were visualized by heat maps produced by the TM4 MeV application (<http://www.tm4.org/mev.html>). To identify possible quality control issues, RSeQC software²⁸ was used to detect abnormalities, such as; unsymmetrical gene body coverage, high levels of read duplication, and low saturation levels of known exon junctions, within each sample.

Pathway analysis: Pathway analysis of the differentially expressed genes in SAT and VAT exposed to exosomes profiled by RNA-Seq was performed using ingenuity pathway analysis (IPA; Qiagen, Redwood City, California, www.qiagen.com/ingenuity).

Microscopy: Axiovert 200M microscope (Zeiss) (with Axiovision software) was used for capture of bright-field images shown.

PDAC mouse model: KRAS^{+/LSL G12D} P53^{flox/flox} mice (C57/BL6 background) were injected with Ca5-Cre adenovirus in the pancreatic head. Mice were sacrificed at 45 days after injection. Using blunt dissection technique, subcutaneous adipose tissue was harvested from the lateral aspects of the ventral abdominal wall. C57/BL6 wild type mice were used as controls.

Histology and Immunohistochemistry (IHC): Hematoxylin and Eosin (H&E) staining were used for evaluation of histological features. For IHC, 4µm thick sections were cut from paraffin-embedded blocks. The sections were deparaffinized, dewaxed, and rehydrated. Antigen retrieval was performed in 10 mM sodium citrate buffer (pH 6.0) and blocking was performed with Protein Block Serum-Free Solution (Dako, Carpinteria, CA). Slides were then incubated overnight in primary antibody diluted in antibody diluent (Dako, Carpinteria, CA). For negative controls for each antibody, slides were incubated with diluent only. Background peroxidase activity was quenched with 3% hydrogen peroxide. Polymer-based

secondary antibodies (Biocare) were used for detection with DAB reagent (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin (Vector Laboratories). Images were captured using ImageScope software (Aperio).

Obtaining human SAT biopsy

Patient identification and sample acquisition: We reviewed the medical records of patients undergoing endoscopic ultrasound (EUS) and/or endoscopic retrograde choangiopancreatography (ERCP) under propofol sedation a day prior to procedure to identify subjects >50 years with either PDAC (n=6) or non-malignant pancreatic disorder (n=5) for procedure. The patients were consented for a SAT biopsy prior to the procedure. Using a 15 g core biopsy needle (BioPince™) we obtained SAT biopsies for qRT-PCR. Biopsies were obtained 2 inches lateral and inferior to the umbilicus in the left lower quadrant of the abdomen. The site was prepped with betadine and air dried. After a skin puncture using the tip of 15G scalpel, the needle was introduced in a tangential plane with the handle cocked. A median of 2 biopsies were taken from a single access site, with each biopsy aimed in an alternate direction. Approximate size of the biopsy specimens were 2-3 mm each. Sample were directly transferred to liquid nitrogen within 1 minute of obtaining biopsy and stored at -80°C until processing and analysis.

Quantitative reverse transcriptase PCR analysis from human SAT biopsy: We used qPCR which is widely considered as one of the most effective ways to quantify gene expression for low abundance mRNA transcripts and have been successfully used for clinical biomarker discovery in needle biopsy-obtained human specimens²⁹. RNA was isolated using methodology specifically validated for lipid rich tissues (RNeasy Lipid Tissue Mini Kit (Qiagen). Complement DNA (cDNA) was transcribed from 300 ng of RNA with the iScript cDNA Synthesis Kit (BioRad). qPCR analysis was performed using the ABI StepOnePlus Real-Time PCR System. Primers were designed utilizing NCBI primer-BLAST resource and used upon confirmation of the melting curve profile and the efficiency of amplification.

Statistical analysis

All Statistical analyses were carried out using commercial software (JMP, version 10-0, SAS Institute Inc, Cary, NC.). Additional details of statistical methods provided at the methods or results section pertaining to the analysis. All the results are expressed as mean (standard deviation [SD]) or median (interquartile range [IQR]) as appropriate. The Pearson's χ^2 test or Fischer's exact test was used to compare categorical variables as applicable. The two-tailed t test was used to compare continuous variables. Polynomial regression analyses were used to model the observed mean metabolic parameters ($\pm$ standard error of mean) in each time interval between cases and controls. A p value of 0.05 indicated statistical significance.

Results

Temporal profile of metabolic parameters of population-based PDAC:

The baseline demographic and clinical profile of cases and controls were comparable with no difference in the mean age, gender, body mass index and race (supplementary table 1).

Fasting blood glucose: The mean FBG was similar in cases and controls up to 30 months before index date (Supplementary table 2); it was higher in PDAC vs. controls (p 0.05) starting 30 to 18-months before index date and progressively increased until diagnosis (Figure 1 and supplementary table 2).

Serum lipids: The mean total cholesterol, triglycerides and LDL cholesterol levels were similar in cases and controls up to 18-months before index date (Figure 2A-C and supplementary table 3A-C); they were lower in cases vs. controls (p 0.05) starting 18 to 6-months before index date and progressively decreased until diagnosis (Figure 2A-C). The mean HDL levels were similar in cases and controls up to 6-months before index date, with a significant (p 0.05) decrease seen only at diagnosis (Figure 2D and supplementary table 3D).

Total body weight: The mean body weight was similar in cases and controls up to 18-months before index date (Figure 3A and supplementary table 4) and dropped lower than the mean body weight of controls (p 0.05) starting 18 to 6-month before index date and progressively decreased until diagnosis (Figure 3A).

Body temperature: The mean body temperature was similar in cases and controls up to 18-months before index date (Figure 3B and supplementary table 5); it was higher than the mean body temperature of controls (p 0.05) starting 18 to 6-month before index date and increased until diagnosis (Figure 3B). On stratifying PDAC subjects by weight loss (>2kg), mean body temperature of weight-losing subjects was significantly higher vs. weight stable/gaining subjects (37.1 vs. 37.4 °C; p=0.02) (supplementary figure 2).

Temporal adipose tissue and muscle changes in PDAC:

The mean area of SAT, VAT and muscle were comparable up to 18-month before index date in weight stable/weight losing PDAC subjects (supplementary table 6). Area of SAT and VAT in the -18 to -6 month period in those who gained weight (≥ 2kg) in the year before CT (n=18) showed increase in SAT with no significant change in VAT (supplementary figure 3A-C). In stable weight or weight losing subjects, the mean area of SAT was lower at -18 to -6-month interval compared to -42 to -18-month interval without any differences in the mean area of VAT and muscle (Figure 4A-C and supplementary table 6). In the 6 to 0-month period weight stable or losing PDAC subjects showed decrease in area of SAT, VAT and muscle compared to the 42 to 18-month interval (Figure 4A-C).

Since the -18 to -6-month pre-diagnostic period showed an unusual pattern of weight loss, SAT loss and decreasing serum triglycerides, total cholesterol, and LDL without change in VAT or muscle, we studied the SAT in *in vitro* experiments and in genetically engineered mouse models of PDAC.

Effect PDAC exosomes on SAT:

Decreased adipocyte size and decreased TG content were noted in SAT adipocytes exposed to PDAC exosomes. Similar TG content as controls with no exosomes was seen in SAT adipocytes exposed to exosomes from plasma of healthy donors (supplementary figure 4)

suggesting against non-specific or generic exosome effects. Further, SAT adipocytes exposed to exosomes from plasma of pancreatic cancer patients demonstrated reduced TG content as compared to adipocytes exposed to exosomes from healthy donors (supplementary figure 5). Pathway analysis based on m-RNA sequencing of SAT exposed to PDAC exosomes (Figure 5A-C) showed promotion of lipolysis, fibrosis, browning and acute inflammation.

Change SAT in mice with pancreatic cancer:

KRAS^{+/LSL} G12D P53^{flox/flox} mice developed tumors at 45 days after Cre-Adv injection; their median survival was 64 days (N=10). SAT in PDAC versus control mice (Figure 6A-D) showed increase in fibrosis and inflammatory infiltration and reduction in adipocyte size (Figure 6E). SAT had increased expression of UCP1 by IHC (Figure 6C-D).

qPCR analysis from human SAT biopsy:

Of the 6 PDAC cases and 5 controls, 1 PDAC case and 1 control did not yield adequate RNA for analysis and were excluded. A cutoff of 20-fold upregulation in *UCP1* mRNA levels distinguished PDAC from controls (5/5 vs. 1/4; p=.047) (Supplementary figure 6).

Discussion

Based on a comprehensive study of well annotated, population-based cohorts of PDAC and controls and another cohort of subjects with serial CT scans, we define the temporal profile of metabolic parameters and soft tissue changes in PDAC. Compared to controls, PDAC has three distinct metabolic phases, each marked by onset and significant (p<0.05) progressive worsening of one or more metabolic abnormalities. At baseline cases and controls were similar in all characteristics. The earliest metabolic change (Phase I) is new-onset hyperglycemia starting nearly 3 years before diagnosis. The next metabolic change (Phase II) is a decrease in lipids associated with weight loss starting 1-5 years before diagnosis. The first evidence of soft tissue change, namely SAT loss, is seen in this period. In the final phase (Phase III), starting <6 months before diagnosis, all parameters decrease (all lipids, SAT, VAT and muscle) except fasting glucose, which continues to rise.

Browning of white SAT is a well described mechanism of SAT loss in cancer³⁰. The combination of decrease in lipids, SAT, and weight seen in Phase II of our study is reminiscent of the effects of browning of SAT.³⁰ Since the purpose of browning is to generate heat,³¹ we mapped the temporal profile of body temperature. Indeed the body temperature rose at 18 months coinciding with drop in lipids and weight and body temperature progressively increased until diagnosis. The fact that this phenomenon was only seen in weight-losing PDAC (supplementary figure 2) suggested that browning caused the changes in temperature, weight, lipids and SAT.

We further tested this hypothesis in *in vitro* and *in vivo* experiments. In an earlier study we have characterized PDAC exosomes²⁰ and shown that they cause metabolic changes in adipocytes.²⁰ In current studies SAT pre-adipocytes differentiated with exosomes from PDAC showed a reduction in fat content (Figure 5A-B). Pathway analysis based on m-RNA sequencing (Figure 5C) showed upregulation of genes for lipolysis, fibrosis, browning and acute inflammation in SAT adipocytes by PDAC exosomes. SAT from mice with PDAC

(Figure 6A-D) confirmed increase in fibrosis and inflammatory infiltration. UCPI staining (Figure 6B-C) confirmed increased browning in SAT. In summary, browning of SAT in KRAS^{+/LSL} G12D P53^{flox/flox} mice bearing PDAC is suggested by reduced TG content (Figure 5A), reduced adipocyte size (Figure 5B), increased fibrosis (Figure 6A,B) and increased UCPI expression (Figure 6C); they corroborate results from RNA sequencing pathway analysis of adipocytes exposed to PDAC exosomes. Finally, *UCPI* was markedly upregulated (>20-fold) in SAT of human PDAC subjects (5/5) vs controls (1/4) (Supplementary figure 6).

In the 6-18 months before diagnosis PDAC patients have a clinical profile of advanced pre-diabetes (defined as FBG of 120-126 mg/dl or A1c of 6-6.5). While in type 2 DM this would be typically associated with weight gain and hyperlipidemia due to insulin resistance, in PDAC there is paradoxical decrease in weight and decrease in lipids despite rising glucose levels. Our data showing upregulation of *UCPI* in human SAT provides a potential biomarker for enriching the cohort of new-onset hyperglycemia for PDAC. A previous study in human cancer³⁰ supports our findings in PDAC subjects. In the previous study while 8/9 cancer subjects with weight loss had UCPI overexpression, none of 30 colon cancer subjects without weight loss had UCPI upregulation in SAT.³⁰ A large human study of UCPI in SAT before and after weight loss due to caloric restriction showed decrease in expression³², suggesting diet-induced weight loss is not due to browning of fat²⁸

The retrospective nature of our human studies is a limitation. However, currently these studies are not possible prospectively. Subjects in our study cohorts had excellent clinical annotation and at each studied time point 40-60% of patients had data, overcoming some of the limitations of the retrospective design. Other limitation of the study is the predominantly Caucasian population of Olmsted County and the metabolic profile needs validation in a more diverse population. Our studies raise many unanswered questions. What is the mechanism of hyperglycemia in Phase I? Why does the rise in glucose continue during Phases II through III despite two powerful anti-diabetic phenomena occurring at the same time (weight loss and browning of white SAT)? What is the mechanism of changes in Phase III? With our current dataset we are unable shed light on these matters, but we hope to continue to mine our clinical database for clues to answer these questions.

. A recent study, predominantly based on animal data, proposed that adipose tissue wasting in PDAC is due to exocrine dysfunction. We did not measure pancreatic exocrine function and therefore, have no way to confirm their findings. Their description of adipose tissue pathology and function does not rule out the possibility that their mice also had browning of SAT. It must be pointed out that there are significant differences in the KPC mice and humans regarding endocrine pancreatic function (no change vs. progressive and severe hyperglycemia, respectively). How this might impact soft tissue changes is unclear.

In summary, we constructed retrospective temporal profiles up to 60 months before index date of metabolic parameters of well annotated cohorts of population-based PDAC and matched controls and changes in soft tissue in a cohort of PDAC subjects with serial CT scans. These cohorts clearly demonstrate three distinct and progressive metabolic phases in pre-diagnostic PDAC starting 2-5 years before diagnosis. Weight loss in Phase II, starting 1-5

years before diagnosis and preceding the development of muscle loss, is likely due to browning of SAT. Experimental, animal and human data supporting UCP1 overexpression in SAT tissue needs prospective validation and may be a potential a biomarker of PDAC in weight-losing subjects with new-onset hyperglycemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Need to Know

Background:

We collected data on changes in metabolic parameters (glucose, serum lipids, triglycerides; total, low-density, and high-density cholesterol; and total body weight) and soft tissues (abdominal subcutaneous fat [SAT], adipose tissue, visceral adipose tissue [VAT], and muscle) from patients 5 years before they received a diagnosis of PDAC.

Findings:

We identified 3 phases of metabolic and soft-tissue changes that precede a diagnosis of PDAC. Loss of SAT starts 18 months before PDAC identification is likely due to browning. Overexpression of UCP1 in SAT might be a biomarker of early-stage PDAC.

Implications for Patient Care:

These phases might be used to identify patients at risk for PDAC who should undergo further evaluation.

Figure 1.

Temporal profile of fasting blood glucose of population-based controls and pancreatic cancer up to 60 months before index date (see also supplementary table 2)

Figure 2.

Temporal profile of population-based controls and pancreatic cancer up to 60 months before index date of: **A.** total cholesterol (see also supplementary table 3A). **B.** serum triglycerides (see also supplementary table 3B). **C.** LDL cholesterol (see also supplementary table 3C). **D.** HDL cholesterol (see also supplementary table 3D)

Figure 3.

A. Temporal profile of body weight of population-based controls and pancreatic cancer up to 60 months before index date (see also supplementary table 4). **B.** Temporal profile of body temperature of population-based controls and pancreatic cancer up to 60 months before index date (see also supplementary table 5)

Figure 4.

Temporal profile of soft tissue changes in weight losing/stable pancreatic cancer subjects up to 60 months before index date (see also supplementary table 6): **A.** subcutaneous adipose tissue (SAT) **B.** visceral adipose tissue (VAT) **C.** muscle mass

Subcutaneous adipocytes differentiated in the presence of pancreatic cancer exosomes and controls

Figure 5.

A. Brightfield images of adipocytes differentiated from subcutaneous pre-adipocytes with PDAC derived exosomes and controls (no exosomes) are shown. Fat droplets are seen here as vacuolar structures (confirmed with oil red staining 6). **B.** Reduced fat droplets were seen in subcutaneous adipocytes in response to PDAC exosomes (vs controls). The fat content was objectively quantified by measurement of triglyceride (TG) content for subcutaneous adipocytes. **C.** Key differentially regulated pathways based on m-RNA sequencing data of RNA isolated from subcutaneous adipocytes differentiated with and without PDAC exosomes.

Figure 6.

Changes in SAT in mice with pancreatic cancer: $KRAS^{+/LSL\ G12D}\ p53^{flox/flox}$ mice developed tumors weighing $0.82 \pm 0.1\text{g}$ ($746 \pm 145\text{ mm}^3$) at 45 days after Cre-Adv injection. Median survival of these mice was 64 days (N=10). Sample H&E sections of SAT in PDAC and control mice are shown in (A-B). Adipocytes in SAT were smaller while there was increase in fibrosis, browning and inflammatory infiltration as compared to control. Quantification of adipocyte size confirming these changes is shown in (E). Increase in brown adipose tissue (BAT) in SAT was confirmed by UCP1 staining as shown in (C-D).