Metabolome of pancreatic juice delineates distinct clinical profiles of pancreatic cancer and reveals a link between glucose metabolism and PD-1+ cells

Nina Cortese¹, Giovanni Capretti², Mario Luisa Barbagallo¹, Alessandra Rigamonti¹, Panteleimon G. Takis⁵, Giovanni F. Castino¹, Debora Vignali⁷, Giulia Maggi¹, Francesca Gavazzi², Cristina Ridolfi², Gennaro Nappo², Greta Donisi², Marco Erreni⁸, Roberta Avigni¹, Daoud Rahal⁹, Paola Spaggiari⁹, Massimo Roncalli³, Paola Cappello¹⁰, Francesco Novelli¹⁰, Paolo Monti⁷, Alessandro Zerbi², Paola Allavena¹, Alberto Mantovani¹,³,¹¹, Federica Marchesi¹,⁴*¹

¹ Department of Immunology and Inflammation, Humanitas Clinical and Research Center-IRCCS, Via Manzoni 56, 20089 Rozzano (MI), Italy
² Section of Pancreatic Surgery, Humanitas Clinical and Research Center-IRCCS, Via Manzoni 56, 20089 Rozzano (MI), Italy
³ Humanitas University, Department of Biomedical Sciences, Via Rita Levi Montalcini 4, 20090 Pieve Emanuele-Milan, Italy
⁴ Department of Medical Biotechnology and Translational Medicine, University of Milan, Milan, Italy
⁵ Giotto Biotech S.R.L., Sesto Fiorentino, Florence, Italy
⁶ Department of Metabolism, Digestion and Reproduction, National Phenome Centre, Faculty of Medicine, Imperial College London, London, UK
⁷ San Raffaele Diabetes Research Institute, IRCCS Ospedale San Raffaele, Milan, Italy
⁸ Unit of Advanced Optical Microscopy, Humanitas Clinical and Research Center-IRCCS, Via Manzoni 56, 20089 Rozzano (MI), Italy
⁹ Department of Pathology, Humanitas Clinical and Research Center-IRCCS, Via Manzoni 56, 20089 Rozzano (MI), Italy
¹⁰ Center for Experimental Research and Medical Studies, Città della Salute e della Scienza di Torino and Department of Molecular Biotechnology and Health Sciences, University of Torino, Torino, Italy
¹¹ The William Harvey Research Institute, Queen Mary University of London, London UK.
* Correspondence to:
Federica Marchesi,
Via Manzoni 56, 20089 Rozzano (MI), Italy
+39 02 8224 5113
federica.marchesi@humanitasresearch.it

Running Title: Immune-metabolic variables in pancreatic cancer patients

Keywords: Metabolomics, pancreatic juice, immunotherapy, biomarkers, pancreatic cancer, PD-1

Funding The research leading to these results has received funding from Associazione Italiana per la ricerca sul cancro (AIRC) under IG2016-ID.18443 project – P.I. Marchesi Federica, AIRC fellowship 18011 to NC, AIRC 5x1000 IG-12182 to PA and FN and IG 15257 to FN, AIRC 5x1000 21147 to AM. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflict of interests AM is a recipient of commercial research grants from Novartis, is a consultant/advisory board member for Novartis, Roche, Ventana, Pierre Fabre, Verily, AbbVie, Compugen, Macrophage Therapeutics, Astrazeneca, Biovelocita, BG Fund, Third Rock, and Versau, is an inventor of patents related to PTX3 and other innate immunity molecules and receives royalties for reagents related to innate immunity.
Abstract

Better understanding of pancreatic diseases, including pancreatic adenocarcinoma (PDAC), is an urgent medical need, with little advances in preoperative differential diagnosis, preventing rational selection of therapeutic strategies. The clinical management of pancreatic cancer patients would benefit from the identification of variables distinctively associated to the multiplicity of pancreatic disorders. We investigated, by $^1$H nuclear magnetic resonance, the metabolomic fingerprint of pancreatic juice (the biofluid that collects pancreatic products) in 40 patients with different pancreatic diseases. Metabolic variables discriminated PDAC from other less aggressive pancreatic diseases and identified metabolic clusters of patients with distinct clinical behaviors. PDAC specimens were overtly glycolytic, with significant accumulation of lactate, which was probed as a disease-specific variable in pancreatic juice from a larger cohort of 106 patients. In human PDAC sections, high expression of the glucose transporter GLUT-1 correlated with tumor grade and a higher density of PD-1$^+$ T cells, suggesting their accumulation in glycolytic tumors. In a preclinical model, PD-1$^+$ CD8 tumor-infiltrating lymphocytes (TILs) differentially infiltrated PDAC tumors obtained from cell lines with different metabolic consumption, and tumors metabolically rewired by knocking down the phosphofructokinase ($Pfk$) gene displayed a decrease in PD-1$^+$ cell infiltration. Collectively, we introduced pancreatic juice as a valuable source of metabolic variables that could contribute to differential diagnosis. The correlation of metabolic markers with immune infiltration suggests that upfront evaluation of the metabolic profile of PDAC patients could foster the introduction of immunotherapeutic approaches for pancreatic cancer.
INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of death, and projected to be the second within a decade (1–3). Although surgical resection is the only curative option, PDAC is affected by a high frequency of early relapse events and postsurgical complications. Preoperative diagnostic tools that improve patient profiling could limit unnecessary surgery in rapidly evolving diseases and could promote the use of neoadjuvant therapies. Current diagnostic strategies are not always adequately efficient in discriminating between PDAC and other benign pancreatobiliary diseases with overlapping symptoms for which surgical resection may not be necessary. The complex and challenging process of surgical decision-making would benefit from the identification of variables distinctively associated to the multiplicity of pancreatic disorders. So far, considerable advances towards a better definition of pancreatic cancer profiles have been primarily achieved through genomic analyses (4–6). However, pancreatic cancer cells are renowned for their extensively rewired metabolism, largely driven by intrinsic oncogenic events and by interactions with non-cancerous stromal cells (7). Metabolic reprogramming is considered a hallmark of cancer progression (8–10), and its analysis could represent a tool to identify novel disease-specific biomarkers. Previously, global analysis of metabolites by metabolomic platforms performed on body fluids has emerged as an information-rich tool that can ultimately reveal indicators of perturbed biological systems and disease-specific profiles. Blood and urine metabolic variables can discriminate between chronic pancreatitis and PDAC (11–13), suggesting an important connection between tumor metabolic alterations and diagnosis.

The pathways that regulate tumor metabolism and immune cell function are tightly linked (14–16). In homeostatic conditions, correct functionality of T cells requires a finely regulated metabolic network that provides energy and biosynthetic precursors (17). As a result, the metabolic dysfunction frequently observed in pathological states can affect the efficacy of the immune response. Nutrient deprivation is commonly present in tumor settings, suggesting that metabolic microenvironmental cues could impact the mounting and implementation of an effective antitumor immune response (9,18). In pancreatic cancer, PDAC cells escape immune recognition and
orchestrate an immunosuppressive microenvironment by various mechanisms, including limited immunogenicity, inadequate immune cell infiltration, and myeloid immune regulatory networks (19–21). Surprisingly, despite the well-known metabolic derangements that characterize pancreatic cancer (22), the influence of metabolic cues on immune cell function has not been addressed. Notwithstanding the compromised nature of immune cells in PDAC, this type of cancer has not emerged at the forefront of immunotherapeutic strategies. In particular, although checkpoint inhibitors represent an important breakthrough in the field of immunotherapy, they still have not been proven effective in PDAC patients (19,23), suggesting that a better comprehension of the immune-metabolic microenvironment could improve therapeutic strategies.

The immune microenvironment of human pancreatic cancer has been the object of intense investigations (24–28). Along this line, we have previously reported the clinical relevance of immune cells as potential novel biomarkers of prognosis (24) and response to therapy (25) in pancreatic cancer patients. In an attempt to characterize environmental cues affecting immune cell function, in this study we have investigated the metabolic profile and how it relates to immune infiltration in patients with pancreatic pathologies. In order to unearth early metabolic alterations, we analyzed pancreatic juice, a relatively unexplored body fluid collected during surgical procedures and enriched with pancreatic tumor-derived metabolites. Our results identified discriminative metabolic variables that allowed us to single out PDAC from other pancreatic pathologies, which were associated with distinct clinical behaviors. Also, we revealed a link between glucose metabolism and accumulation of PD-1+ cells in pancreatic tumors and provide preclinical evidence that metabolic rewiring of cancer cells results in modification of the immune contexture. Collectively, in this work we uncovered the diagnostic and prognostic relevance of measurable metabolic parameters in pancreatic juice.
METHODS

Human samples and study design

The study population included two cohorts of patients (referred to as Cohort 1 and Cohort 2), who underwent surgery at the Humanitas Clinical and Research Center from 2015 to 2018. All patients were aged more than 18 and non-metastatic, except for one patient for whom a hepatic metastasis was found intraoperatively. For all patients enrolled, peripheral blood, tumor tissue, and adjacent normal tissue were collected at the time of surgery according to protocols approved by the Ethical Committee of the Institution. Cohort 1 comprised 40 patients, from which pancreatic juice was withdrawn during pancreasectomy and used for metabolomics analysis (Supplementary Table S1). Patients were diagnosed postoperatively with pancreatic adenocarcinoma (n=31), pancreatitis (n=2), papillary-ampulla tumors (n=4), neuroendocrine tumors (n=2), IPMN (n=1) (Supplementary Table S1). Cohort 2 comprised 106 patients (inclusive of Cohort 1), including 79 PDAC patients and 27 non-PDAC patients, from which pancreatic juice was withdrawn during pancreasectomy and lactate measured by colorimetric assay (Supplementary Table S2). Clinicians prospectively assembled a clinical retrospective database by collecting patient demographics, clinical, and histopathological data, as detailed in Supplementary Tables. T (tumor) and G (grade) were categorized according to the 7th edition of the TNM staging system, as follows: T1-T2 as low-T tumors, T3 as high-T tumors; G1-G2 as low-grade tumors and G3-G4 as high-grade tumors.

CD8+ T cells were obtained from buffy coats of healthy donors (n=3) obtained from the blood bank of the Institution. All the patients were enrolled in the study after signed informed consent including collection of biological specimens and clinical data. The study was approved by the Ethical Committee of the Institution (protocol number ICH-595, approval issued on May 2009).

Plasma and pancreatic juice collection and preparation

Blood was collected at the time of surgery in EDTA-containing BD Vacutainer vials (BD Biosciences, 366473). Plasma was then obtained via centrifugation and stored at -80°C until further use. Pancreatic juice was collected intraoperatively by Wirsung puncture immediately before pancreas transection and stored in EDTA-containing BD Vacutainer vials (BD Biosciences,
366473) at 4°C until transportation to the laboratory of the Biobank Unit within the Hospital for processing. Once in the laboratory, the sample was centrifuged within 5 hours of collection, aliquoted, and stored at -80°C. Patients fasted overnight prior to collection.

**Nuclear magnetic resonance (NMR) spectroscopy**

NMR spectra of pancreatic juice and plasma samples were acquired using a Bruker IVDr 600 MHz spectrometer (Bruker BioSpin), operating at 600.13 MHz proton Larmor frequency, equipped with a 5 mm PATXI H/C/N with $^2$H-decoupling probe, including a z-axis gradient coil, an automatic tuning-matching (ATM), and an automatic refrigerated sample changer (SampleJet). 180 μL of pancreatic juice were mixed with 20 μL of D$_2$O buffer (1.5 M KH$_2$PO$_4$ dissolved in 99.9% D$_2$O, pH 7.4; 2 mM sodium azide; and 0.1% 3-(trimethyl-silyl) propionic acid-d$_4$ (TSP)). A total of 200 μL of this mixture was transferred into a 3-mm NMR tube (Bruker BioSpin) for the analysis. Plasma samples were prepared as previously described (29). Temperature was regulated to 300 ± 0.1 K for pancreatic juice and 310 ± 0.1 K for plasma, with a BTO 2000 thermocouple. Both one-dimensional (1d) Nuclear Overhauser Effect Spectroscopy (NOESY) and 1d Carr-Purcell-Meiboom-Gill (CPMG) spectra (30) were collected for plasma and pancreatic juice samples, whereas 1D-diffusion experiment was additionally acquired for plasma samples. Both pancreatic juice and plasma spectra were bucketed (0.02 ppm buckets width) by AMIX software (Bruker Biospin). After the OPLS-DA (orthogonal projection latent structure discriminant analysis), the produced latent variables plot (LV) components and the estimated variable importance in the projection (VIP) score of each group (variables with VIP > 1.0 were initially considered significant for each model) were used for the detection of the weighted variables (NMR signals–buckets) responsible for the group classification. These signals corresponded to the metabolites reported in the Results section. The statistical significance ($P$) of each metabolite was calculated by univariate one-way ANOVA analysis refined by a $n$-fold cross-validation implementation by splitting the data into $n$ sub-datasets while performing the ANOVA test (e.g. cross-validated ANOVA). Metabolite signals presenting a strong overlapping with EDTA were removed before multivariate statistical analysis.
Multivariate statistical analysis applied to metabolite quantification by NMR

All multivariate and univariate analyses of the NMR-based metabolomics study were performed by PLS_Toolbox 8.2.1 and homemade scripts in Matlab 2016a. Bucketed NMR data was normalized by total area normalization for both plasma and pancreatic juice spectra. Total area normalization is a “mild” normalization method and proved suitable for our data because no extreme outliers (identified as values with high distance to the model (DModX) or values outside the 95% CI of the multivariate analysis) were observed in any of our multivariate analyses (31). Deconvolution and integration of metabolite signals were accomplished by an in-house developed software in MATLAB programming suited by Dr. P.G. Takis. The algorithm is based upon the unconstrained non-linear minimization (fitting) of the metabolite NMR signals, employing a combination of lorentzian-gaussian functions. By this approach, each NMR region of interest is decomposed and deconvoluted into its component parts, and then integrated to obtain the metabolite concentrations in arbitrary units. For the $n \times$ Fold concentration changes for each metabolite the following equation was employed:

$$n \times \text{Fold} = \log_2 \left( \frac{\text{median of group 1}}{\text{median of group 2}} \right)$$

Lactate and glucose measurement

Concentration of lactate in pancreatic juice was assessed by a colorimetric assay (Sigma Aldrich, MAK064), following manufacturer's instruction, without prior pretreatment of samples. Samples were diluted 10-fold or 25-fold and concentration was calculated on standard curve. Concentration of glucose in interstitial fluid was measured by a colorimetric assay (Sigma Aldrich, MAK263), following manufacturer's instructions. Samples were diluted 10-fold or 30-fold and concentration was calculated on standard curve.

Immunohistochemistry

Human FFPE-PDAC tissues from 18 patients included in Cohort 2 were provided as tissue blocks, by the Pathology Department of the Humanitas Clinical and Research Center. 2 μm thick consecutive tissue sections were obtained from each specimen and stained as previously
described (24). In brief, sections (one section for each marker) were stained with primary antibodies raised against CD8 (DAKO, clone C8/144B, diluted 1:100), PD-1 (Abcam, clone NAT105, diluted 1:50), GLUT-1 (Abcam, clone EPR3015, diluted 1:2000), CD68 (DAKO, KP-1 clone, diluted 1:1000), CD20 (DAKO, L26, diluted 1:200). Investigators who performed the assessment of immune variables were blinded to the clinical data.

Quantification of the immune-reactive area

After staining procedure, tissue sections were digitalized using the slide scanner VS120 dotSlide (Olympus). To evaluate the percentage of immune-reactive area (IRA%) of immune cells, at least two independent operators blinded to any patient clinical data selected three non-overlapping and non-contiguous areas comprising of approximately 50% of tumor and 50% of stromal tissue. Evaluation of GLUT-1 was performed by selecting three pictures inclusive of pancreatic ducts and corresponding to the hotspot regions. Both sampled microscopic area and light density were maintained throughout the analysis. Selected areas were quantified by computer-assisted image analysis, with ad hoc software (Image Pro Premiere), to obtain the IRA% of the digitalized tissue surface. The mean value, obtained from the three different regions, was calculated for each marker and subsequently used for analyses.

Cell lines

Human BxPC3 were purchased from ATCC in 2007, and PT45 cell lines were kindly provided by Prof. Lorenzo Piemonti in 2001. Neither was authenticated. Cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 2 mM L-glutamine in a humidified incubator with 5% CO₂. Cells were routinely tested for Mycoplasma contamination. For supernatant collection, 5x10⁶ cells were seeded with fresh medium, and medium was collected after 24 hours, centrifuged to remove debris, and stored at −80°C until further use. The murine Panc02 cell line (32) was obtained from Prof. Lorenzo Piemonti as previously described (24) and DT6606 from Prof. Franco Novelli as described (33). Briefly, DT6606 is a cell line obtained from the LSL-KrasG12D-Pdx1-Cre mouse, a genetically modified mouse developing pancreatic cancer due to conditional expression.
of the Ras\textsuperscript{G12D} mutation under the Pdx pancreatic specific promoter. Cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 2 mM L-glutamine in a humidified incubator with 5% CO\textsubscript{2} for one passage before \textit{in vivo} injection. Cells were routinely tested for Mycoplasma contamination.

**Knockdown of Pfkm in Panc02 cells**

Stable genetic inhibition of glycolysis in Panc02 cells was achieved by silencing the phosphofructokinase gene (\textit{Pfkm}) with a lentiviral vector coding for a short hairpin RNA (shPFK) (Sigma Aldrich, SHCLNV-NM_021514). A scramble shRNA (shSCR) was used as control (Sigma Aldrich, SHC016V). For transduction, Panc02 cells were cultured to 60–80% confluence in Dulbecco Modified Eagle’s Medium (Lonza) with 10% FBS and 2 mM L-glutamine in a humidified incubator with 5% CO\textsubscript{2}. Transduced cells were selected by addition of puromycin (1 \(\mu\)g/mL) to the medium 72 hours after infection. Gene silencing was verified by real-time qPCR.

**Glycolytic rate assay**

Glycolytic rates of PT45 and BxPC3 cells were assessed using a commercially available kit (Agilent Seahorse XF Glycolytic Rate Assay Kit, Agilent Technologies, 103344-100) following manufacturer’s protocol. Briefly, \(10^4\) cells/well were seeded in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 2 mM L-glutamine in a humidified incubator with 5% CO\textsubscript{2}. The following day, oxygen consumption rates (OCRs) and extracellular acidification rates (ECARs) of PT45 and BxPC3 cells were measured in XF medium (non-buffered RPMI medium containing 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine) using an XF24 analyzer (Seahorse Bioscience). OCR and ECAR were measured under basal conditions and in response to 500 nM rotenone/antimycin A and 50 mM 2-deoxy-D-glucose (2-DG) (all Agilent Technologies), mitochondrial and glycolytic inhibitors, respectively.

**\textit{In vitro} T-cell cultures**
PBMCs were isolated from buffy coats of healthy donors by gradient centrifugation using Ficoll. CD8+ T cells were then separated using immunomagnetic positive selection (CD8 MicroBeads, Miltenyi Biotec) and seeded at 2x10^7 cells/well in glucose-free RPMI 1640 medium (Lonza) containing 10% FBS. D-glucose (Sigma) was added at concentrations varying from 90 mg/dL (referred to as “normal”) to 10 mg/dL (referred to as “low”). Cancer cell line supernatants from PT45 or BxPC3 cells (33% concentration), lactic acid (5 mM), or sodium lactate (5 mM) were added from day 0 for 3 days before measuring PD-1 expression by flow cytometry. Cells were stained with CFSE (10 μM for 10 minutes at 37°C in PBS), stimulated with anti-CD3/anti-CD28-coupled beads (Dynabeads, ThermoFisher Scientific) at a 1:100 bead/T-cell ratio, and cultured for 7 days before measuring PD-1, CD25, CD127 expression and CFSE dilution by flow cytometry. The following antibodies were used: PD-1 (clone EH12.1, BD Biosciences), CD25 (clone M-A251, BD Biosciences), CD127 (clone HIL-7R-M21, BD Biosciences). Sample acquisition was performed on a BD LSR FORTESSA (BD Biosciences), and data analyzed with FlowJo software.

Pancreatic ductal adenocarcinoma (PDAC) murine models

All mice used for the subcutaneous implantation of murine cell lines were 8-week-old C57BL/6J females purchased from Charles River (Calco, Italy) and housed in a specific pathogen–free animal facility of the Humanitas Clinical and Research Center in individually ventilated cages. Procedures involving mice and their care were conformed to EU and Institutional Guidelines (Protocol ID 121/2016-PR). Pancreatic cancer–prone LSL-KrasG12D-Pdx1-Cre mice (KC mice) were generated by crossing single-mutated KrasG12D with C57BL/6 mice expressing Cre recombinase. Mice were screened by PCR using tail DNA amplified by specific primers to the Lox-P cassette flanking Kras and wild-type Kras genes and Cre recombinase genes. Mice were bred, maintained, and treated at the saprophytic and pathogen-free animal facility of the Molecular Biotechnologies Center (Torino, Italy), as previously described (33).

For subcutaneous implantation of all murine cell lines (Panc02, DT6606, shPFK-Panc02, shSCR-Panc02), cells were cultured in RPMI 1640 medium (Lonza) supplemented with 10% FBS and 2
mM L-glutamine in a humidified incubator with 5% CO₂ for one passage before in vivo injection. 10⁶ cells were injected subcutaneously in the right flank, and tumor growth was monitored using a caliper twice a week. In all experiments, mice were sacrificed 21 days after injection of tumor cells. Once excised, tumor weight and size (using caliper) were recorded.

Isolation of tumor interstitial fluid (TIF)
Tumor interstitial fluid (TIF) was collected from subcutaneously implanted Panc02 and DT6606 tumors via a centrifugation method as previously described (34). Briefly, tumors were excised, rinsed in PBS, blotted on filter paper, placed on 40 μm nylon filters on top of 50 mL conical tubes and centrifuged for 10 minutes at 4°C at 400 x g. Flow through from this step was collected as tumor interstitial fluid and analyzed for the indicated metabolite concentrations.

Optical imaging
In vivo evaluation of glucose uptake was assessed on subcutaneously implanted Panc02 and DT6606 tumors using the fluorescent glucose analogue XenoLight RediJect 2-DG-750 probe (Perkin Elmer), following manufacturer’s instructions. The probe was injected i.v. 24 hours prior to sacrifice of the mice. Imaging was performed ex vivo on excised tumors using the IVIS Lumina III InVivo Imaging System (Perkin Elmer). Acquisition was performed with four filter pairs to allow spectral unmixing (excitation 680, 700, 720 and 740 nm; emission 790 nm). Binning: 8; f/stop: 2. Images were analyzed with Living Image 4.3.1 software (Perkin Elmer).

Flow cytometry of tumors
Single-cell suspensions were obtained by incubating fragmented tumors with collagenase type IV (0.5 mg/mL; Sigma Aldrich) for 30 minutes at 37°C. Tumor-infiltrating leukocytes were isolated by immunomagnetic positive selection (TIL CD45 MicroBeads, Miltenyi Biotec). The following fluorophore-conjugated primary antibodies were used: anti-CD45 (BD Pharmingen, clone 30-F11), anti-CD3 (eBiosciences, clone 145-2C11), anti-CD8 (BioLegend, clone 53-6.7), and anti–PD-1 (Biolegend, clone 29F.1A12). Sample acquisition was performed on a BD LSR FORTESSA (BD
Biosciences), and data analyzed with FlowJo software. Zombie Aqua (Biolegend) amine-reactive fluorescent dye was used to perform dead cell exclusion. 2-NDBG (2-Deoxy-2-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-D-glucose) uptake was measured by incubating digested tumors in a 200 μM 2-NBDG (Sigma Aldrich) solution for 30 minutes at 37°C.

**Gene expression**

RNA was extracted with RNeasy Plus Mini Kit (QIAGEN) following manufacturer’s instructions and reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) following standard protocol. cDNA was then used for real- time qRT-PCR, performed using SYBR® Green PCR Master Mix (Applied Biosystems) and SYBR® Green RTPCR Reagents Kit (Applied Biosystems) following kit protocol. Real-time qPCR reactions were performed on ViiA7 Real- Time PCR (Life Technologies) instrument with standard protocol. The results were normalized to Hprt expression. Differences in gene expression were analyzed by the comparative threshold cycle (Ct) method (\(2^{-\Delta\Delta Ct}\)). The following primers were used: Pfkm (forward: TCCATGAGGGTTACCAAGGC; reverse: GCACTTCCAATCACTGTGCC), Cd274 (forward: TCTCCTCGCCTGCAGATAGT; reverse: AGCCGTGATAGTAAACGCCC), Hprt (forward: CTCAGACCCTTTTTTGCCG; reverse: CGCTAATCACGACGCTGGG).

**Statistical analysis**

Correlation between lactate concentration measured by different assays and between IRA% values of GLUT-1 and PD-1 were estimated by a non-parametric Spearman Rank correlation coefficient test and linear regression analysis. Differences between experimental groups were estimated by a non-parametric Mann Whitney U test. Differences between distribution of patients according to clinical variables were estimated by a Chi square test. Survival analysis was performed by Kaplan-Meier curves. Differences in overall survival time was calculated by Mantel-Cox log rank test using the R software. For each test, only two-sided \(P\) values lower than 0.05 were considered statistically significant. All the analyses were done using GraphPad Prism software (Version 4.1) or R software.
RESULTS

Alteration in glucose metabolism in pancreatic juice from PDAC patients

Cancer cells have metabolic alterations that can result in biochemical changes in biological fluids and, therefore, represent a source of relevant metabolic variables. In order to capture a comprehensive overview of the metabolic profile of pancreatic specimens, we collected pancreatic juice from patients who underwent pancreatectomy at our institute. Pancreatic juice is the liquid that collects pancreatic products and is readily accessible during endoscopic procedures (35) or pancreatectomy. Due to its proximity to the pancreas, it could be more informative compared to plasma or urine. We performed a metabolomics analysis by $^1$H- nuclear magnetic resonance (NMR) on pancreatic juices from 40 patients (Cohort 1), including 31 PDAC and 9 other non-PDAC clinically relevant controls (2 chronic pancreatitis, 4 papillary-ampulla tumors, 2 neuroendocrine tumors, 1 intraductal papillary mucinous neoplasia (IPMN)) (Supplementary Table S1). Availability of limited information of this biofluid led us to use $^1$H-NMR spectroscopy, which despite low sensitivity, presents with high reproducibility and accuracy in detection (31). 1d Carr-Purcell-Meiboom-Gill (CPMG) spectra obtained from $^1$H-NMR analysis of pancreatic juices allowed us to appreciate in detail small molecular weight metabolites by filtering the broad NMR signals of macromolecules (e.g. lipoproteins, lipids etc.) (Fig.1A). Supervised OPLS-DA analysis indicated that the metabolic profile of pancreatic juice could discriminate between PDAC patients and non-PDAC controls, which differentially segregated with an accuracy of 82.4% obtained by cross-validation (Fig. 1B, Supplementary Fig. S1A). In the NMR profile of the pancreatic juice samples, 18 metabolites (L-phenylalanine, citrate, L-glutamine, L-tyrosine, acetate, L-tryptophane, L-valine, succinate, L-leucine, lactate, L-isoleucine, 3-hydroxy-butyrate, glycine, glucose, formate, L-alanine, acetone acetoacetate) were responsible for the group classification (Fig. 1C). By analyzing the contribution of each metabolite, lactate, the end product of glycolysis, was the only one significantly increased in PDAC patients (1.57-fold) compared to non-PDAC patients ($P=0.011$), whereas the concentration of most of the other metabolites detected was decreased (Fig. 1C). This result suggests a selective alteration of glucose metabolism in PDAC cells. The concentration of lactate
was not significantly different according to the occurrence of PDAC-associated diabetes (Supplementary Fig. S1B) or according to tumor size (Supplementary Fig. S1C), and metabolomics analysis on 29 plasma samples from the same patients did not achieve equal discriminative power (Supplementary Fig. S2).

**PDAC accumulates lactate and upregulates the glycolytic marker GLUT-1**

We then asked whether the concentration of a single metabolite could discriminate the two groups of patients. In a preliminary analysis on 27 juices, we confirmed that the concentration of lactate, measured by $^1$H-NMR, correlated with the concentration of lactate measured by a colorimetric assay ($r=0.954, P<0.0001$) (Fig. 2A). We then analyzed pancreatic juice samples from 106 patients (Cohort 2, Supplementary Table S2) by the colorimetric assay and found that the concentration of lactate was significantly enriched in pancreatic juice from PDAC patients ($n=79$) compared to juice from non-PDAC patients ($n=27; P=0.012; 95\%\ CI\ 5.93\ \text{to}\ 61.86$) (Fig. 2B). No difference was seen in lactate concentration in plasma from PDAC and non-PDAC patients (Supplementary Fig. S2).

We next tested pancreatic tissues to confirm our findings obtained in pancreatic juice. Because lactate is the end product of glycolysis and accelerated glucose metabolism in tumor cells (the so-called "Warburg effect") relies on modulation of key glycolytic enzymes (10), including glucose transporter 1 (GLUT-1), we assessed the rate of glycolysis in human PDAC tissues by analyzing the expression of GLUT-1 (22,36) in 18 PDAC specimens by immunohistochemistry. The expression of GLUT-1 on pancreatic ducts was significantly higher compared to the healthy region of the same patient and increased expression was seen in regions where tumor cells invaded normal pancreas (Fig. 2C-D). Despite a certain degree of heterogeneity of GLUT-1 expression within the same specimen, GLUT-1 expression was higher in specimens from high-grade (G3-G4) compared to low-grade (G1-G2) tumors (Fig. 2E-F), confirming an association between a more undifferentiated phenotype and a higher glycolytic activity of pancreatic tumor cells (22).

**Metabolic profile of pancreatic juice identifies patients with distinct clinical outcomes**
We, thus, investigated whether any association of the metabolic profile with the clinical behavior of PDAC patients exists (Cohort 1; n=31). Unsupervised K-means cluster analysis of the metabolomics data, without imposing any threshold, identified 4 metabolic clusters (Fig. 3A). By pairing the groups with clinical data, we found a distinct correlation of specific metabolic clusters (Cluster 1, Cluster 2, Cluster 3) with clinical parameters (i.e. tumor size (T) and grade (G)). In particular, although metabolic Cluster 1 comprised primarily patients with T1-T2 tumors, patients belonging to metabolic Clusters 2 and 3 had, for the most part, T3 tumors (Fig. 3B, top; P=0.02). Similarly, metabolic Cluster 1 comprised primarily patients with G1-G2 tumors, whereas patients belonging to metabolic Clusters 2 and 3 had, for the most part, G3-G4 tumors (Fig. 3B, bottom; P=0.009). Kaplan-Meier survival curves confirmed that the metabolic profile detected in pancreatic juice associated with different clinical outcomes, with Cluster 1 having the best and Cluster 2 having the worst survival (Fig. 3C, P=0.00079). In addition to lactate, L-valine, succinate, L-phenylalanine, L-leucine, and acetoacetate were significantly increased in clusters 2 and 3.

Nonetheless, the clustering was ascribable to the total metabolic fingerprint rather than to any one metabolite in particular (37), possibly due to the small number of samples, thus, resulting in a composite biomarker.

**Tissue metabolic alterations correlate with distinct immune contextures in human PDAC**

We next analyzed whether the increased glucose metabolism correlated with the immune contexture of PDAC. To this aim, we investigated potential associations of GLUT-1 expression with the presence of tumor-infiltrating leukocyte populations. By immunohistochemistry, we quantified the density (immunoreactive area (IRA%)) of GLUT-1 (Fig. 2C) and of immune cells, including CD8+ tumor-infiltrating lymphocytes (CD8-TILs), CD20+ B lymphocytes (CD20-TILs), CD68+ tumor-associated macrophages (CD68-TAMs) (Supplementary Fig. S3A), and PD-1+ cells (Fig. 4A). The density of PD-1+ TILs in 18 PDAC specimens correlated with the expression of GLUT-1 (r=0.52, P=0.023) (Fig. 4B). Other tumor-infiltrating immune cells did not show a correlation with the expression of GLUT-1 (Supplementary Fig. S3B). To identify the single metabolic determinants involved in PD-1 upregulation, we cultured sorted CD8+ T cells in control medium (containing
90mg/dL of D-glucose) or in low-glucose medium (containing 10 mg/dL of D-glucose), and added supernatants from two pancreatic cancer cell lines with different glycolytic profiles (PT45 and BXPC3; Supplementary Fig. S4), lactate, or lactic acid (Fig. 4C). Low-glucose concentration was the main driver for PD-1 up-regulation (P<0.0001) (Fig. 4C). Based on these preliminary results, we additionally performed dose-response experiments on the effect of glucose on CD8+ T-cell proliferation and expression of PD-1, CD25 and CD127 (Fig. 4D). Although proliferation progressively declined with decreasing glucose concentrations (Fig. 4D), a glucose concentration of 30 mg/dL induced a significant upregulation of PD-1 (P=0.002 versus control), and only marginally affected the expression of CD127 and CD25 (Figure 4D).

We confirmed the correlation between GLUT-1 expression by pancreatic ducts and PD-1 infiltration in the genetic preclinical model of PDAC Pdx1-Cre;Kras<sup>LSLG12D</sup>. Although very low or absent expression of GLUT-1 was detected in the pancreas of wild-type (WT) mice (Fig. 4E, left), weak to very intense immunoreactivity was present in the tumors of Pdx1-Cre;Kras<sup>LSLG12D</sup> mice (Fig. 4E, right). An analogous expression pattern was shown by PD-1<sup>+</sup> TILs (Fig. 4F) and, consistent with our observations in human PDAC, a positive correlation between GLUT-1 expression and PD-1<sup>+</sup> TIL density was present (r=0.79, P=0.0007) (Fig. 4G).

**Targeting glycolysis impacts the accumulation of PD-1<sup>+</sup> TILs in PDAC preclinical models**

To further investigate the association between the tumor metabolic state and PD-1<sup>+</sup> cell infiltration, we modeled the immune-metabolic axis observed in human specimens in preclinical models of PDAC. We analyzed implanted tumors obtained from two different pancreatic cell lines, DT6606 and Panc02, derived respectively from early and late stage murine PDAC. Despite the fact that tumors obtained from the two cell lines were not significantly different in size (Supplementary Fig. S5A), they displayed a different glycolytic activity. In particular, Panc02 tumors displayed a higher glycolytic activity measured by both *in vitro* uptake of a fluorescent glucose analogue (2-NBDG) (Fig. 5A) and *ex vivo* by optical imaging on the excised tumor mass, after injection of the fluorescent probe deoxy-D-glucose (2-DG-750) (Fig. 5B-C). The increased metabolism was
reflected in an increased glucose consumption, which led to decreased glucose concentration in the interstitial fluid obtained from Panc02 tumors compared to DT6606 tumors (Fig 5D). Tumor cells accounted for most of the glycolytic activity (Supplementary Fig. S5B), as assessed by glucose uptake ex vivo after tumor mass digestion. Consistent with the correlation between GLUT-1 expression and PD-1+ cell infiltration observed in both human and murine PDAC, Panc02 tumors contained a higher percentage of PD1+ cells within CD8+ lymphocytes compared to DT6606 tumors (Fig. 5E-G), despite a similar density of total CD3+ and CD8+ cells (Supplementary Fig. S5C). This suggests a selective increase of PD-1 expression in highly metabolic tumors.

To probe whether the increased metabolism of Panc02 cells was sufficient to determine PD-1+ TIL accumulation, we blunted the glycolytic metabolism of tumor cells. We selectively silenced glycolysis in Panc02 cells by genetically targeting the rate-limiting glycolytic enzyme phosphofructokinase-m (PFK-m), which, despite its relatively low expression (Supplementary Fig. S6A) is exclusively involved in the glycolytic reaction. We generated Panc02 cells stably knocked-down with an shRNA targeting Pfk (shPFK) and the corresponding control cell line stably expressing a scramble sequence (shSCR) (Fig. 5H). The two cell lines were implanted into mice, to originate tumors with opposite glycolytic rates (Supplementary Fig. S6B). Knockdown of Pfk did not affect tumor growth (Fig. S6C) nor the frequency of CD3+ and CD8+ lymphocytes (Fig. S6D). Genetic targeting of glucose metabolism significantly reduced the frequency of PD-1+ TILs within CD8+ cells infiltrating Panc02 tumors (Fig. 5I) and decreased Cd274 (PD-L1) expression in tumor cells (Fig. 5J), ultimately confirming the link between glucose metabolism and PD-1+ cell infiltration. This result also suggests that targeting tumor metabolism could be used as a strategy to alter immune infiltration.
DISCUSSION

Metabolomic analyses of cancer bio-specimens grant the possibility to appreciate the variety of molecules downstream of cancer metabolic adjustments. In pancreatic cancer, well-known for its metabolic alterations, metabolomic analyses of blood (12,38,39), urine (11), salivary fluids (40), and exhaled breath (13) have been performed, primarily documenting differences between PDAC and control subjects or patients with pancreatitis. In this study, we tested the value of pancreatic juice – a far less explored body fluid - as a source of metabolic markers of disease. $^1$H-NMR spectral data indicated significant group discrimination based on a disease-specific metabolic profile that singled out PDAC from clinically relevant controls, including benign pancreatitis and other pancreatobiliary diseases with overlapping symptoms (tumors of the ampullary region, neuroendocrine, and cystic tumors). This result suggests that upfront evaluation of the metabolic profile could implement diagnostic accuracy and help in detecting early-stage tumors. The metabolic fingerprint of pancreatic juice associated with distinct clinical outcomes, confirming that it could represent a composite biomarker (31,37) capable of prognostic classification, in contrast to plasma samples. This could be important in identifying rapidly evolving diseases, avoiding, when unnecessary, considerable morbidity still associated to surgical resection. Despite in this study pancreatic juice was collected intra-operatively, in the perspective of identifying early informative clinical markers, the collection of pancreatic juice could also be performed by minimally-invasive procedures, such as endoscopic ultrasound, retrograde cholangio-pancreatography, or by endoscopic collection of duodenal juice secretion (35), although such techniques could alter the composition of the fluid collected and results should be, therefore, carefully evaluated. Also, in order to detect important biochemical details and potential biomarkers, it may be beneficial to perform a more comprehensive metabolic assessment by other metabolomics assays, such as mass spectrometry.

Among the 18 metabolites identified in pancreatic juice, only lactate exhibited a higher concentration in samples from PDAC patients, whereas a number of other metabolites were decreased. The distinct metabolic profiles could suggest a rewiring of PDAC tumors towards
aerobic glycolysis (lactate accumulation) and increased lipid and nucleotide biosynthesis (decreased concentration of essential amino acids, glycine, acetoacetate). This comprehensive analysis does not allow to investigate in detail the biochemical pathways ongoing in tumor cells, as it is the result of all the different cell types that contribute to the tumor metabolism, including stromal and immune cells. Nevertheless, the increased expression of the glucose transporter GLUT-1 in PDAC paraffin-embedded specimens compared to healthy regions of the same patient reflects an increased glucose uptake (36). GLUT-1 expression in PDAC cells has been implicated in resistance to chemotherapy (41) and metastasis (22), confirming the clinical relevance of alterations in glucose metabolism that can be detected in the biological fluid immediately adjacent to the tumor. The concentration of lactate in the pancreatic juice collected from a larger cohort of patients also increased. This finding suggests that, with further development, the assessment of this metabolite could be feasible in clinical routines, where the performance of other biomarkers (such as carbohydrate antigen 19-9) is disappointing in terms of differential diagnostic power. It is important to underline that in our cohort of patients, we were not able to detect in plasma the specific alterations seen in pancreatic juice.

Our data suggest that the glucose metabolism of human PDAC affects its immune contexture. Metabolic reprogramming, a hallmark of cancer progression, is known to severely impact on the efficacy of the antitumor immune response (8–10). Our results entail clinical consequences because pre-surgical identification of markers correlating with immune variables could represent a considerable advancement in the introduction and choice of immunotherapeutic approaches for pancreatic cancer. When we attempted to define the mechanism behind the metabolic modulation of PD-1, we found that low glucose concentration was the main driver of PD-1 upregulation in T cells. The mechanisms of resistance and variable response to checkpoint inhibitors remain poorly understood and suggest that factors other than the immunologic ones could be important dictators of response to PD-1–targeted therapies, including metabolic pathways (42). Along this line, baseline blood concentration of lactate dehydrogenase (LDH) has been identified as a predictive biomarker of response to anti–CTLA-4 treatment (43–45) and intratumor expression of metabolic
genes was associated to resistance to anti–PD-1 therapy (46). Because anti–PD-1 works predominantly within tumors, looking for circulating biomarkers of response could be unrevealing, and pancreatic juice could represent a valuable option as a body fluid, collecting metabolites released in the microenvironment of the tumor mass. However, differently from the other tumor types mentioned, issues related to checkpoint inhibitors are still out of reach in the case of PDAC, for which these therapeutic approaches have been introduced to a minor extent, and patients still have not achieved tumor regression (19,23).

Genetic inhibition of glycolysis in a preclinical model resulted in a decreased accumulation of PD-1+ cells, without impairing CD8+ T-cell recruitment, and in decreased expression of Cd274 (PD-L1) in tumor cells. This provides rationale to metabolic targeting of tumors as an effective option to reboot the antitumor response. Other studies have shown that neutralization of tumor acidity improves antitumor responses to immunotherapy with checkpoint inhibitors (47), although further studies are needed to dissect the function of PD-1+ T cells in tumors with different metabolic activity.

Collectively, our analysis promotes metabolomics as a valuable diagnostic tool, while introducing pancreatic juice as a source of metabolic biomarkers to advance surgical management and clinical decision-making of pancreatic diseases. Although promising, our metabolic analysis cannot be considered definitive and further development is needed to design a clinically feasible method based on our results. The correlation of metabolic markers with immune infiltration suggests that implementation of preoperative patient metabolic profiling could foster the introduction of immunotherapeutic approaches for pancreatic cancer.
Authors’ contributions

Conception and Design: F. Marchesi, G. Capretti, A. Zerbi

Development of methodology: P. Takis, GF. Castino, M. Erreni

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Cortese, G. Capretti, M. Barbagallo, A. Rigamonti, P. Takis, GF. Castino, D. Vignali, M. Erreni, F. Novelli, P. Cappello

Analysis and interpretation of data (e.g. statistical analysis, biostatistics, computational analysis): G. Capretti, P. Takis, G. Maggi, F. Marchesi

Writing, review, and/or revision of the manuscript: N. Cortese, G. Capretti, F. Novelli, P. Cappello, P. Monti, A. Zerbi, P. Allavena, A. Mantovani, F. Marchesi

Administrative, technical, or material support (i.e. reporting or organizing data, constructing databases): G. Capretti, G. Maggi, R. Avigni, F. Gavazzi, C. Ridolfi, G. Nappo, G. Donisi, D. Rahal, P. Spaggiari, M. Roncalli

Study supervision: F. Marchesi, A. Zerbi, P. Allavena, A. Mantovani

Acknowledgments We thank Roberta Migliore for technical assistance; Daniela Pistillo and the Biobank within the Humanitas Clinical and Research Center; Dr. Antonio Inforzato for helpful discussion on biochemical pathways, Dr. Floriana Farina for assistance in glycolytic rate assessment.

Patient consent Obtained

Ethic approval The study was approved by the Ethical Committee of the Institution (protocol number ICH-595, approval issued on May 2009).
REFERENCES


Figure Legends

Figure 1. Metabolomic analysis of pancreatic juice indicates an alteration in glucose metabolism in pancreatic ductal adenocarcinoma patients. A, $^1$H-NMR CPMG spectra of PDAC samples ($n=31$, in blue) and non-PDAC samples ($n=9$, in green) providing details on the small molecular weight metabolites contained in the pancreatic juices. Ppm: parts per million. B, Score plots of supervised multivariate OPLS-DA analysis on CPMG spectra from PDAC samples (green squares) and non-PDAC samples (red diamonds). The analysis segregates the two groups (each one outlined by a spider plot), with an accuracy of 82.4% obtained by cross-validation. C, Calculated median $n$-fold concentration changes for different metabolites. Blue bars indicate a $P$ value<0.05, grey bars $P>0.05$ by T-test.

Figure 2. Pancreatic adenocarcinoma accumulates lactate and upregulates the glycolytic marker GLUT-1. A, Correlation between lactate as measured by colorimetric assay and $^1$H-NMR in pancreatic juice ($n=27$, $r=0.954$, $P<0.0001$ by Spearman’s simple linear regression analysis). Red solid line represents the line of best fit, dotted lines represent the 95% CI. B, Concentration of lactate in pancreatic juice from PDAC patients ($n=79$) compared to juice from non-PDAC patients ($n=27$). Values are mean± 95% CI ($P=0.012$ by Mann-Whitney test; 95% CI (5.93 to 61.86)). C, Immunohistochemical evaluation of GLUT-1 on PDAC paraffin sections. Representative images showing expression of GLUT-1 on pancreatic tumor ducts (lower left) compared to the healthy region of the same patient (upper left) and increased expression of GLUT-1 in tumor cells (dotted line) invading normal regions (right). D The expression of GLUT-1 (IRA%) in tumor ducts compared to an adjacent healthy region from the same patient ($n=6$ patients, 3 pictures each analyzed; $P=0.031$ by Wilcoxon matched paired test). E, Two representative specimens of low-grade (G2, left) and high-grade (G4, right) specimens, with low and high marker expression, respectively, shown. F, Expression of GLUT-1 (IRA%) according to tumor grade; low (G1-G2), high (G3-G4) ($n=8$ low group; $n=12$ high group; $P=0.038$ by Mann Whitney). Line represents median. Bars: 20 $\mu$m in C (left), 100 $\mu$m in E and in C (right).
Figure 3. Metabolic profile of pancreatic juice identifies clusters of patients with distinct clinical outcomes. A, Unsupervised K-means clustering on the $^1$H-NMR of pancreatic juices from PDAC patients ($n=31$). 4 metabolic clusters identified: Cluster 1 ($n=9$), Cluster 2 ($n=9$), Cluster 3 ($n=10$) (only Cluster 1, Cluster 2, and Cluster 3 were considered for further analysis due to the small number of patients in the grey cluster). B, Pie charts depicting for three of the clusters identified by K-means analysis distribution of patients according to clinical variables related to tumors, i.e. tumor size (T1-T2 versus T3) and grade (G1-G2 versus G3-G4). Distribution of patients in T1-T2 and T3, as well as in G1-G2 and G3-G4, groups was significantly different in the three clusters ($P=0.02$ by Chi square test). C, Kaplan-Meier survival analysis of the three metabolic clusters identified. Cluster 1: light blue; Cluster 2: blue; and Cluster 3: green ($P=0.00079$ by log rank Mantel-Cox).

Figure 4. Tissue metabolic alterations correlate with distinct immune contexts in pancreatic adenocarcinoma. (A-B), Correlation between GLUT-1 and PD-1$^+$ cells. A, Immunohistochemical evaluation of PD-1 on PDAC paraffin sections. Two representative specimens with low (left) and high (right) marker expression are shown. B, Quantitative evaluation of the density of GLUT-1 and PD-1 (immunoreactive area (IRA%)) in 18 PDAC patients ($r=0.52$, $P=0.023$ by Spearman’s simple linear regression analysis). Red solid line represents the line of best fit, dotted lines represent 95% CI. C-D) Changes in the expression of PD-1 on sorted healthy donor CD8$^+$ T cells in the presence of pancreatic cancer cell lines’ supernatants or in the presence of lactate (NaLac) or lactic acid (Lac) in media containing normal (90 mg/dl) or low (10 mg/dl) glucose concentrations for 3 days. C, One representative of three experiments performed; values are mean±SEM of triplicates. D, Changes in proliferation and expression of PD-1, CD25, and CD127 on sorted healthy donor CD8$^+$ T cells cultured for 7 days in media containing decreasing concentrations of glucose. One representative of three experiments performed; values are mean±SEM of triplicates. $P$ value by T-test ($**P<0.005; ***P<0.001$). (E-F) Histological sections of pancreata of wild-type mice (left) and PDx1-Cre;Kras$^{G12D}$ mice (right) stained with E, an anti–GLUT-1 or F, anti–PD-1. G, Quantitative evaluation of the immunoreactive area (IRA%) reveals a
linear correlation between the density of GLUT-1 and PD-1 in murine PDAC specimens (n=23, r=0.79, P=0.0007, by Spearman’s simple linear regression analysis). Red solid line represents the line of best fit, dotted lines represent 95% CI. Bars: 100 µm A, 200 µm in E and F.

Figure 5. Targeting of glycolysis impacts on the accumulation of PD-1+ TILs in PDAC preclinical models. (A-C), Evaluation of glucose metabolism in in vitro and in vivo. A, Glucose uptake by Panc02 cells derived from late-stage murine PDAC, measured as uptake of the fluorescent glucose analogue 2-NDBG, in vitro compared to DT6606 cells derived from early-stage PDAC. Graph represents mean±SEM of three independent experiments (**P<0.0001 by Mann Whitney) B, Fluorescent signal generated by representative excised tumor specimens; control was recorded after saline injection. C, Quantification of the fluorescence generated by XenoLight RediJect 2-DG-750, expressed as average radiant efficiency, of subcutaneously implanted DT6606 tumors (n=11) compared to Panc02 tumors (n=12). Graph represents mean±SEM of two independent experiments (**P<0.01 by Mann Whitney). D, Glucose concentration in interstitial fluid from Panc02 (n=10) and DT6606 tumors (n=5) (TIF). Box plots give median, lower and upper quartile by the box, and minimum and maximum by the whiskers. Graph represents two independent experiments (n=5 DT6606; n=10 Panc02; P=0.075 by Mann Whitney). (E-G) PD-1 expression on CD8+ T cells infiltrating Panc02 tumors compared to DT6606 tumors. E, Representative flow cytometry dot plots showing percentage of PD-1+ cells in CD8+ TILs isolated from DT6606 and Panc02 tumors. F, Frequency of CD8+ T cells expressing PD-1 in Panc02 tumors compared to DT6606 tumors. G, Mean fluorescence intensity (MFI) of PD-1 in CD8+ cells from Panc02 and DT6606 tumors. Graphs represent mean±SEM of two independent experiments (n=10 mice DT6606; n=11 mice Panc02; **P<0.01 by Mann-Whitney test). (H-J) Genetic inhibition of glycolysis by silencing the phosphofructokinase (Pfk) gene using a Pfk-targeting or scrambled shRNA in Panc02 tumors. H, Expression of the Pfk gene in Panc02 cells stably knocked-down with an shRNA targeting Pfk (shPFK) and the corresponding control cell line stably expressing a scramble sequence (shSCR). *P<0.05 by Mann-Whitney test. I, The frequency of PD-1+ TILs within CD8+ T cells infiltrating shPFK compared to shSCR tumors, as well as J, Cd274 (PD-L1)
expression. Graph represents mean±SEM of two independent experiments ($n=11$ mice shSCR; $n=11$ mice shPFK; *$P<0.05$ by Mann-Whitney test).
Figure 1
Figure 3
Figure 4

A. PD-1 expression in tumor tissues.

B. Correlation between PD1-TILs (IRA%) and GLUT-1 (IRA%).

C. Bar graph showing % PD-1 expression vs Control.

D. Graph showing % change vs Control with glucose concentration.

E. Immunohistochemical staining of GLUT-1 in WT and Pdx1-Cre;KrasG12D mice.

F. PD1-TILs in WT and Pdx1-Cre;KrasG12D mice.

G. Correlation between PD1-TILs (IRA%) and GLUT-1 (IRA%).

Downloaded from cancerimmunolres.aacrjournals.org on April 19, 2021. © 2020 American Association for Cancer Research.
Figure 5

A. Glucose uptake

B. Average radiant efficiency

C. Tumor metabolism

D. Glucose in TIF

E. DT6606 and Panc02

F. PD1-TILs

G. PD-1 (MFI)

H. Fold change (AU)

I. % of CD8+ cells

J. C2274 mRNA (AU)
Cancer Immunology Research

Metabolome of pancreatic juice delineates distinct clinical profiles of pancreatic cancer and reveals a link between glucose metabolism and PD-1+ cells

Nina Cortese, Giovanni Capretti, Marialuisa Barbagallo, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/2326-6066.CIR-19-0403

Supplementary Material
Access the most recent supplemental material at:
http://cancerimmunolres.aacrjournals.org/content/suppl/2020/02/04/2326-6066.CIR-19-0403.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerimmunolres.aacrjournals.org/content/early/2020/02/04/2326-6066.CIR-19-0403. Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.