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Title: Increased tissue and circulating levels of dipeptidyl peptidase-IV enzymatic activity in patients with pancreatic ductal adenocarcinoma

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Abstract

Background/Objectives: Pancreatic ductal adenocarcinoma (PDAC) is frequently heralded by an impairment of glucose homeostasis. Dipeptidyl peptidase-IV (DPP-IV) and fibroblast activation protein alpha (FAP) are aminopeptidases that regulate several bioactive peptides involved in gluco-regulation, and are frequently dysregulated in cancer. The present study analyzes blood plasma levels and the quantity and localization of DPP-IV and FAP in PDAC tissues.

Methods: DPP-IV and FAP concentration and enzymatic activity were evaluated in the plasma from 93 PDAC, 39 type 2 diabetes mellitus (T2DM) and 29 control subjects, and in matched paired non-tumorous and tumor tissues from 48 PDAC patients. The localization of DPP-IV and FAP was determined using immunohistochemistry and catalytic histochemistry.

Results: The enzymatic activity and concentration of DPP-IV was higher in PDAC tumor tissues compared to non-tumorous pancreas. DPP-IV was expressed in cancer cells and in the fibrotic stroma by activated (myo)fibroblasts including DPP-IV⁺FAP⁺ cells. FAP was expressed in stromal cells and in some cancer cells and its expression was increased in the tumors. Plasmatic DPP-IV enzymatic activity, and in particular the ratio between DPP-IV enzymatic activity and concentration in PDAC with recent onset DM was higher compared to T2DM. In contrast, the plasmatic FAP enzymatic activity was lower in PDAC compared to T2DM and controls and rose after tumor removal.

Conclusions: DPP-IV-like enzymatic activity is upregulated in PDAC tissues. PDAC patients with recent onset diabetes or prediabetes have increased plasmatic DPP-IV enzymatic activity. These changes may contribute to the frequently observed association of PDAC and recent onset impairment of gluco-regulation.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) represents a malignancy with a dismal prognosis and limited therapeutic options¹. Only 3-5% of patients survive longer than 5 years mostly because the diagnosis is often established at an advanced stage when surgical resection is not possible. Newly diagnosed impairment of glucose homeostasis, in particular when combined with weight loss or persistently low BMI, is a well-established early symptom of PDAC. However, the pathogenetic mechanisms leading to increased insulin resistance and to beta cell dysfunction in PDAC are incompletely understood. There are currently no markers to differentiate whether the newly diagnosed impairment of glucose homeostasis is a presenting symptom of PDAC, or the initial stage of the much more prevalent type 2 diabetes mellitus².

A strong desmoplastic reaction is characteristic for PDAC and the stroma in fact represents large part of the tumors^{3,4}. Several proteases including the serine proteases uPA (urokinase-type plasminogen activator), tPA (tissue plasminogen activator) and fibroblast activation protein alpha (FAP) as well as the matrix metalloproteinases MMP-2, MMP-9 and MMP-11 are dysregulated in PDAC and may contribute to its pathogenesis possibly by extracellular matrix remodeling (reviewed in³).

Dipeptidyl peptidase-IV (DPP-IV, CD26, EC 3.4.14.5) is an exopeptidase that cleaves the N-terminal X-Pro/Ala dipeptide from several bioactive peptides including the glucoregulatory peptides glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) and thereby regulates their bioavailability⁵.

DPP-IV is constitutively expressed as a dimeric transmembrane protein by various epithelial and some endothelial cells⁵ and its expression is upregulated in activated T cells⁶. A soluble form is present in blood plasma and other body fluids, but its origin is largely speculative⁷. The expression of DPP-IV is frequently dysregulated in cancer both on the tissue and systemic (plasma) level in a cancer type specific manner⁸.

Few other proteases, including the paralogues FAP and the intracellular dipeptidyl peptidase (DPP)8 and DPP9 were reported to exhibit the characteristic X-Pro/Ala aminopeptidase activity of the canonical DPP-IV (CD26).

The physiological substrates of these proteases largely remain to be identified, but the FAP mediated cleavage of the glucoregulatory peptides GIP and GLP-1 was confirmed *in vitro*⁹.

We previously proposed that the dysregulated enzymatic activity of DPP-IV and FAP may contribute to the pathophysiology of PDAC associated impairment of glucose homeostasis by affecting the availability of their biologically active substrates relevant for gluoregulation¹⁰. In this study we analyzed the expression of DPP-IV in the tumor tissue and blood plasma in PDAC patients and their association with the early symptoms of PDAC.

Methods

Patients, sample collection

Plasma samples were obtained from 93 subjects with pancreatic ductal adenocarcinoma (PDAC) as confirmed by aspiration biopsy or by surgical resection, 39 patients with type 2 diabetes mellitus lasting more than 2 years without any signs of malignant disease (T2DM group) and 29 control subjects without gastrointestinal, liver, renal or endocrine disorders. PDAC was diagnosed at an advanced stage (T3-4) in 84/93 (90%) patients. In 59/93 (63%) patients, PDAC was accompanied by impaired gluco-regulation (diabetes or prediabetes) lasting less than 2 years, 15/93 (16%) patients had long lasting diabetes according to the standard criteria¹¹ and 18/93 (19%) had normal fasting plasma glucose (<5.6 mmol/l). Main characteristics of the patient cohorts are shown in Table 1. Peripheral blood was collected into tubes containing sodium heparin as an anticoagulant; plasma was separated by centrifugation and stored at -78°C till analysis.

Pancreatic ductal adenocarcinoma tissue and non-tumorous pancreatic tissue distant from the tumor were obtained from 48 PDAC patients operated at the Military University Hospital Prague. The study was approved by the local Ethics Committee, the patients signed informed consent. Pancreatic tissue samples were divided into two parts, one of them being immediately frozen on dry ice and then stored at -70°C. The adjacent part of the sample was processed for routine hematoxylin–eosin staining. All specimens were histologically examined by a certified pathologist to identify samples containing normal pancreatic tissues as well as those with signs of malignancy, non-specific inflammatory changes or chronic pancreatitis.

Enzyme activity measurement

Tissue samples were homogenized in liquid nitrogen using the Retsch MM200 ball mill (Haan, Germany) at 30 strokes/s for 90s, and resuspended in a homogenization buffer (Na₂HPO₄·2H₂O 2 mmol/l, KH₂PO₄ 0.6 mmol/l, NaCl 22.4 mmol/l, pH 6.0) to produce a 15% homogenate. DPP-IV activity was measured by a fluorimetric assay using 7-(glycyl-prolylamido)-4-methylcoumarin as a substrate (Bachem, final concentration 50 μmol/l in 2 ml of PBS pH 7.4, 0.1 % Triton-X100, 37°C) on Fluoromax-4 (Horiba Jobin Yvon, Kyoto, Japan, excitation/emission 380/460nm). All samples were measured in quadruplicates, the enzyme activity values were normalized per 1 mg of total protein as quantified by the Lowry method. To determine the proportion of the DPP-IV-like enzymatic activity attributable to canonical DPP-IV (CD26), a highly selective¹² DPP-IV inhibitor sitagliptin (Biovision, Milpitas, USA, final concentration 300 nmol/l) was added to the reaction mixture.

The enzymatic activities of DPP-IV and FAP in the heparinized blood plasma were evaluated in 96 well plates using fluorimetric substrates at 37°C; the release of 7-amino-4-methylcoumarin was monitored at excitation/emission 380/460 nm (multilabel plate reader Victor Perkin Elmer, PE Systems, Prague, Czech Republic). For the DPP-IV enzymatic assay, 4µl plasma in a final volume of 200µl PBS pH 7.4 were incubated with 7-(glycyl-prolylamido)-4-methylcoumarin as a substrate (Bachem, Bubendorf, Switzerland, final concentration 50 µmol/l). For the FAP enzymatic assay, 10µl of plasma were incubated with N-carbobenzoxycyl-prolylamido -4-methylcoumarin (Z-Gly-Pro-AMC, Bachem, final concentration 100 µmol/l) in 25 mmol/l phosphate buffer with 1mmol/l EDTA and 5 % methanol, pH 7.5¹³. 84±7 % (mean±SD) of the enzymatic activity measured by this assay was inhibited by a highly selective FAP inhibitor¹⁴ and was considered to correspond to the FAP enzymatic activity.

DPP-IV and FAP ELISA

For the DPP-IV a FAP concentration measurement, the tissue homogenates were mixed 1:1 with the lysis buffer (Tris-HCl 10mmol/l pH 7.5, Triton X-100 1 %, SDS 0.1 %, NaCl 100 mmol/l, EDTA 1 mmol/l, EGTA 1 mmol/l, glycerol 10 %) with protease inhibitors (AEBSF final concentration 200 µmol/l, pepstatin A final concentration 25 µmol/l, E64 final concentration 50 µmol/l) and incubated for 30minutes at 4°C. The lysates were cleared by centrifugation (14 000 g, 30 min, 4 °C), aliquoted and stored at -78 °C till analysis.

Concentration of DPP-IV and FAP in both the lysates and plasma was assessed in triplicates by a specific ELISA (DuoSet, R&D Systems, Minneapolis, USA) according to the manufacturer's recommendation with a 7-point calibration curve (range 0-2000 pg/ml for DPP-IV and 0-4000 pg/ml for FAP). The lysates and plasma were diluted 1:500 and 1:2000 for DPP-IV and 1:300 and 1:10 for FAP, respectively. The absorbance at 450 nm was determined using the microplate reader Sunrise (Tecan, Mannedorf, Switzerland); the measured absorbance values were corrected by subtracting the absorbance values at 570 nm. The resulting differential absorbance values were used for the construction of the calibration curves and data evaluation.

Immunohistochemistry

Immunohistochemistry was done on 5 µm-thick formalin-fixed, paraffin-embedded tumor sections. The immunolabeling was performed using specific antibodies (DPP-IV polyclonal antibody, Abnova, Taipei City, Taiwan, 1:50, FAP alpha polyclonal antibody, LSBio, Seattle, USA, 1:50, α-SMA, monoclonal anti-human α-Smooth Muscle Actin, Dako, Glostrup, Denmark, 1:50). Tissues were blocked against endogenous peroxidase

activity with the dual endogenous enzyme blocking agent (Dako, Denmark). Antigen retrieval was performed in Tris buffer (pH 9.0) in a microwave oven. EnVision™ (Dako, Denmark) was used as a detection system. A 4-tiered semiquantitative system (0- negative, 1- 1- 30% positive cells, 2- 31- 60% positive cells, 3- 61- 100% positive cells) was used for the scoring of the DPP-IV and FAP immunopositivity in tumor and stromal cells. The immunopositivity of the stromal cells (irrespective of its intensity) was assessed in regions adjacent (up to 0.2 mm) and more distant (greater than 0.2 mm) from the tumor cells using specimen linear measurement (microscope field magnification 200x). Only the cytoplasmic staining was interpreted as immunopositive. Double immunofluorescence labeling was performed as described¹⁵ with minor modifications. Briefly, 10 µm-thick frozen sections were fixed with 4 % paraformaldehyde, permeabilized by 0.1 % Triton-X100, blocked with 10 % fetal calf serum plus 1 % bovine serum albumin in TBS and incubated overnight at 4 °C with the rat anti-human DPP-IV primary antibody (clone E19, Vitatex, Stony Brook, USA, 1:150). After washing away the non-bound antibodies, the slides were incubated for 1 h at room temperature with the corresponding secondary antibody (AF 488 goat anti-rat IgG, A11006, Life Technologies Czech Republic s.r.o., Prague, Czech Republic, 1:500). Subsequently, the primary antibody against α-SMA (1A4, Abcam, Cambridge, UK, 1:200) was applied for 1 hour followed by the AF 546 donkey anti-mouse IgG secondary antibody (A10036, Life Technologies, 1 hour at room temperature, 1:500). A parallel (simultaneous) double labeling protocol was used for DPP-IV and FAP using rat anti-human DPP-IV (clone E19, Vitatex, 1:150), and mouse anti-human FAP (clone F19 isolated from a hybridoma supernatant, at 56 µg/ml), both incubated overnight at 4 °C. 400 µmol/l ToPro (Life Technologies) added to the solution of the secondary antibodies was used for nuclear counterstaining. The primary antibodies were omitted in the staining controls. Slides were mounted in Aqua Polymount (Polysciences, Eppelheim, Germany) and viewed on the Olympus IX 81 confocal microscope equipped with the 488, 543 and 633 nm lasers (FluoView 300, Olympus Czech Group, Czech Republic).

DPP-IV catalytic histochemistry

In situ detection of the DPP-IV enzymatic activity was performed as described previously¹⁵. 10 µm-thick frozen sections were fixed in a 1:1 mixture of acetone and chloroform for 2 min at 4 °C and incubated with 7-(glycyl-L-prolylamido)-4-methoxy-L-naphthylamide hydrochloride (0.83 mmol/l, Sigma-Aldrich, Czech Republic) as a substrate and Fast Blue B (Sigma-Aldrich) in PBS (pH 7.4) at 4 °C. Nuclei were counterstained with hematoxylin. Under the conditions used, the contribution of other proteases exhibiting the DPP-IV-like enzymatic activity is minimal; to further prove that canonical DPP-IV (CD26) is responsible for the observed

DPP-IV-like enzymatic activity, the slices were preincubated with the DPP-IV inhibitor sitagliptin (Biovision, 1 $\mu\text{mol/l}$ in PBS) for 15 min at room temperature prior to the reaction and 1 $\mu\text{mol/l}$ sitagliptin was added to the reaction mixture.

Statistical analysis

For the plasma DPP-IV and FAP enzymatic activities and concentrations, the values were logarithmically transformed prior to statistical evaluation and differences between the groups were analyzed by one-way analysis of variance (ANOVA) and the Tukey post hoc test. Correlations between variables were assessed by the Spearman's coefficient. For statistical analyses, the software "Statistica 12" (StatSoft, Dell Software, Tulsa, USA) and R (R core team 2015) were used. When interpreting test results, $p < 0.05$ was considered as statistically significant. Semiparametric logistic regression based on a generalized additive model (GAM, ¹⁶) was used to evaluate the effects of the plasma DPP-IV and FAP values on the probability of PDAC. Data from patients with PDAC and recent onset diabetes/prediabetes ($n=59$), and type 2 diabetes mellitus without PDAC (T2DM group =39) were used (as positive and negative groups, respectively) and analyzed in the following models: model 1- logistic GAM with spline in the selected explanatory variable as a single predictor, model 2- logistic GAM with splines in the selected explanatory variable and in the cancer marker CA19-9 as two predictors (additive on the logistic scale). AIC (Akaike information criterion) was used to compare the two models.

Results

DPP-IV and FAP in pancreatic ductal adenocarcinoma (PDAC) tissue

DPP-IV-like enzymatic activity and its portion corresponding to the canonical DPP-IV (CD26) were analyzed in 48 tumor samples with histologically verified PDAC, and 45 samples of pancreatic tissue from PDAC patients distant from the tumor and without the evidence of infiltration by malignant cells. A variable proportion (56.2% [32.3-84.3] and 57.6% [36.7-87.5], median and range, $p = 0.83$) of the overall DPP-IV-like enzymatic activity was sensitive to a highly specific inhibitor sitagliptin in tumor tissue and non-malignant pancreatic tissue, respectively. The overall DPP-IV-like enzymatic activity was increased in the PDAC tumor tissue compared to the non-tumorous pancreatic tissue due to an increase of both the enzymatic activity attributable to canonical DPP-IV (i.e. sensitive to sitagliptin) as well as to other proteases exhibiting the DPP-IV-like activity. In 45 patients with PDAC, direct comparison of the matched paired tumor and non-tumor tissue samples revealed significantly higher overall DPP-IV-like and canonical DPP-IV enzymatic activity also on an intraindividual

basis (Wilcoxon paired test, $p < 0.05$). These results were confirmed by the observation of higher CD26 antigen concentration in the tumor tissue as determined by ELISA (Figure 1). The biochemically determined enzymatic activity of canonical DPP-IV correlated positively with the amount of CD26 measured by ELISA ($r = 0.65$, $p < 0.001$).

The localization of DPP-IV was analyzed using immunohistochemistry in 39 PDAC tumor samples. There was large variability among individual patients and regions of the samples regarding the expression of DPP-IV in cancer cells as well as in the stroma. DPP-IV was detected in cancer cells in 95% of the tumors, although the proportion of DPP-IV positive cells varied (Figure 2A, B, Table 2). DPP-IV was also expressed- albeit to a lesser extent- by the stromal cells both adjacent to cancer cells as well as in those more distant from them. A trend for a larger proportion of DPP-IV expressing cells was seen in the stromal cells adjacent to the cancer cells (Table 2). We further utilized an *in situ* enzymatic activity assay with a specific DPP-IV chromogenic substrate and a highly selective DPP-IV inhibitor sitagliptin to analyze the distribution of DPP-IV enzymatic activity in matched paired tumorous and non-tumorous tissue samples from PDAC patients. The DPP-IV-like enzymatic activity detected by this assay was virtually completely abrogated with the use of sitagliptin, confirming canonical DPP-IV (CD26) as its source. In the tumor tissues, DPP-IV positive cancer cells were detected in 80% (8/10) of the samples. In the stroma of the tumors, DPP-IV positive elongated cells as well as isolated round stromal cells corresponding to lymphocytes were identifiable in all analyzed samples, nevertheless with wide intra- and inter-individual variability. In several tumors, large stromal regions were completely DPP-IV negative, whereas others exhibited substantial DPP-IV positivity (Figure 2C-E), confirming the immunohistochemistry data. In the non-malignant paired tissues, similar DPP-IV stromal positivity was observed with pronounced positivity in fibroblastoid cells in several cases (Figure 2G, H). DPP-IV was also present in epithelial cells lining excretory ducts (Figure 2H). When present, the Langerhans islets in the pancreatic tissue from PDAC patients exhibited strong DPP-IV enzymatic activity as we previously reported in normal pancreas¹⁵.

In concordance with the previously published data^{17, 18}, we observed increased expression of fibroblast activation protein alpha (FAP) in the PDAC tumor tissue. Interestingly, FAP protein was also elevated in several non-tumorous pancreatic tissue samples in PDAC patients, in particular when the histological features of chronic pancreatitis were present (Figure 3B). Using immunohistochemistry, we confirmed the abundant expression of FAP in the stromal fibroblasts, although similarly to DPP-IV there was substantial inter- and intraindividual variability and in 36% (14/39) of the samples, no FAP staining in the stroma was observed. The expression of FAP in cancer cells was also variable (Table 2, Figure 3C, D). Strong stromal immunopositivity of α -SMA was

present in the tumor tissues (data not shown), confirming the presence of activated myofibroblasts typical for PDAC. Using double immunofluorescence labelling, co-expression of α -SMA and FAP was detectable in part of the DPP-IV expressing stromal cells (Figure 3F-H); these DPP-IV⁺ α -SMA⁺ and DPP-IV⁺FAP⁺ cells were also present in non-tumorous samples from PDAC patients. A large proportion of the α -SMA positive myofibroblasts in tumorous and non-tumorous samples were however DPP-IV negative.

Blood plasma DPP-IV and FAP in pancreatic ductal adenocarcinoma patients

We evaluated whether the levels of circulating DPP-IV and FAP are changed in PDAC patients with a special focus on the possible association of these changes with the early symptoms of PDAC, i.e. new onset diabetes and weight loss¹⁹. Plasmatic DPP-IV enzymatic activity correlated with DPP-IV (CD26) protein concentration determined by ELISA in PDAC ($r= 0.43$, $p < 0.05$, $n= 93$) and controls ($r= 0.58$, $p < 0.05$, $n= 29$), but not in type 2 diabetes mellitus patients without PDAC (T2DM, $r= -0.15$, not significant, $n= 39$). Compared to T2DM, the DPP-IV enzymatic activity was mildly, but statistically significantly increased in the PDAC subgroup with recently diagnosed diabetes/prediabetes; the highest levels were observed in the subgroup with a more pronounced weight loss (Table 3). In contrast, the DPP-IV (CD26) protein concentration determined by ELISA in PDAC as well as in the subgroup with recent onset diabetes/prediabetes was substantially lower compared to T2DM. As a result, the ratio between the DPP-IV enzymatic activity and the DPP-IV concentration (i.e. specific DPP-IV/CD26 enzymatic activity) was significantly higher in PDAC compared to the T2DM. Interestingly, of the parameters evaluated in our study, the specific DPP-IV (CD26) enzymatic activity was the best predictor for discriminating PDAC patients with recently diagnosed diabetes/prediabetes from T2DM ($p<0.001$, test performed in model 1- see Methods). The predictive ability of this parameter was preserved even when the concentration of the routinely utilized CA19-9 marker were included in the model ($p=0.054$, model 2, Figure 4) and improved the predictive ability of CA19-9 (AIC /Akaike information criterion/ 108.0 for DPP-IV specific enzymatic activity, 77.5 for CA19-9 alone and 70.9 for the combination of both parameters).

Plasma FAP enzymatic activity was slightly, but statistically significantly decreased in PDAC and its subgroups compared to the T2DM and controls, with similar levels in PDAC patients with both normal glucoregulation and recent onset diabetes/prediabetes. FAP protein concentrations quantified by ELISA were not significantly different among PDAC, the T2DM group and control subjects (Table 3).

In 13 patients, the plasma levels of DPP-IV and FAP were also determined following the surgical removal of the tumor (approximately after 3 months). There was a significant rise in the FAP enzymatic activity as well as protein concentration following surgical removal of the tumor ($p < 0.01$, Wilcoxon paired test, Figure 4).

Discussion

Changes in the expression of DPP-IV and FAP are a frequent phenomenon in cancer patients. Upregulation or downregulation of DPP-IV and FAP in the tumor tissue was observed in various malignancies in a cancer type specific manner and in several cases, a direct pathogenetic role of these proteases was demonstrated (reviewed in ^{8, 20}). In addition, changes in the plasma levels of DPP-IV and FAP were observed in cancer patients and both proteases were suggested to represent potential biomarkers ^{7, 21 22}.

In the current work we show that the circulating and tissue levels of DPP-IV enzymatic activity are higher in patients with PDAC. In the non-tumorous pancreas, DPP-IV is abundantly expressed in part of the pancreatic duct cells and in adult human pancreatic alpha cells ^{15, 23}. In the majority of PDAC tumor tissues, the DPP-IV protein concentration and enzymatic activity was increased. In addition, the DPP-IV-like enzymatic activity insensitive to a highly specific DPP-IV inhibitor was also elevated. We confirmed the raised FAP levels ^{17, 18} in the PDAC tumors, but the almost 100- fold lower enzymatic activity towards the fluorogenic substrate used ²⁴ strongly argues that other proteases such as the ubiquitously expressed DPP8 and/or DPP9 ^{25, 26} are increased in PDAC. Thus, the enzymatic activity and expression of several DPP-IV-like molecules is higher in PDAC tissue. DPP-IV (CD26) was detected in the PDAC cancer cells and also in the desmoplastic stroma. The immunohistochemical detection of DPP-IV and an enzyme activity based approach gave comparable results, although the catalytic histochemistry seemed more sensitive. Based on the morphological appearance, part of the DPP-IV positive stromal cells were most likely infiltrating immune cells such as activated lymphocytes that are known to express DPP-IV ²⁷. DPP-IV (CD26) was also present in elongated cells corresponding to cancer associated fibroblasts as suggested by its presence in α -SMA and FAP positive cells. Some of the DPP-IV and FAP doublepositive fibroblastoid cells were found in close contact with Langerhans islets and may therefore influence the humoral signals regulating insulin secretion. In the tumor tissue, a slightly more frequent expression of both DPP-IV and FAP was observed in stromal cells located in closer contact with the cancer cells. Cohen et al. ¹⁷ previously reported that FAP expression is higher in adjacent tumor associated myofibroblasts in PDAC. Our data indicate that the same is true for DPP-IV. Possibly, expression of both proteases may be affected by paracrine factors secreted from cancer cells ²⁸. Nevertheless, we observed DPP-positive stromal cells

and a substantial increase of FAP expression also in the non-tumorous tissue in PDAC patients suggesting a widespread reaction of the pancreatic stroma to the tumor. The proteases may participate on the fibroproduction characteristic for PDAC. Macromolecular complexes comprising DPP-IV and FAP were detected at invadopodia in fibroblasts contributing to their migration²⁹ and the association of FAP with fibroproduction is well established³⁰. Interestingly, a recent study showed that inhibition of the DPP-IV enzymatic activity can inhibit liver fibrosis in an animal model by suppressing the synthesis of collagen and proliferation of hepatic stellate cells³¹. The expression of DPP-IV may also be characteristic of a separate and possibly functionally different subpopulation(s) of fibroblasts as was shown in the mammary gland, where DPP-IV is typically present in interlobular fibroblasts, but is absent in intralobular fibroblasts³².

In the majority of malignancies, the DPP-IV plasma levels are decreased, the possible exceptions being hepatobiliary carcinomas and pancreatic cancer^{21,33}. Our results revealed statistically significantly higher DPP-IV plasma levels in comparison with diabetes mellitus without PDAC. The increase was modest, with highest levels in patients with a recently diagnosed disorder of glucoregulation and weight loss greater than 2 kg. In contrast, the levels of circulating enzymatically active FAP were lower in PDAC. Although there is currently no explanation for this observation, a similar and somewhat counterintuitive decrease of the circulating levels of FAP was previously reported in colorectal cancer and was suggested to represent a new diagnostic marker with the sensitivity comparable to CEA³⁴. The rise of the plasmatic FAP levels after the removal of the PDAC observed in this study strongly suggests that the change is directly linked to pancreatic carcinogenesis. It is currently unknown, whether the above mentioned changes contribute to the pathogenesis of PDAC. Mildly higher plasma DPP-IV enzymatic activities observed in morbidly obese³⁵ and in some studies in type 2 diabetes mellitus patients³⁶ were suggested as a possible factor contributing to the increased GLP-1 inactivation. In PDAC patients, the observed combination of higher levels of DPP-IV and FAP in the pancreatic tissue together with the higher circulating levels of DPP-IV enzymatic activity may thus lead to increased local as well as systemic inactivation of insulin secretion promoting mediators. This may contribute to the functional impairment of beta cells and type 3c diabetes mellitus (T3cDM) characteristically observed in PDAC². The use of DPP-IV inhibitors (gliptins) may seem advantageous in this setting, but there is persisting suspicion that these drugs may in fact be associated with an increased risk of pancreatic cancer³⁷.

An interesting finding of the current study is that the DPP-IV specific activity, i.e. the ratio between the plasmatic DPP-IV enzymatic activity and concentration of DPP-IV (CD26) as determined by specific ELISA, has a potential discriminatory ability to differentiate between type 2 diabetes mellitus without PDAC (T2DM)

and a recent onset diabetes or prediabetes associated with PDAC and improves the discriminatory ability of CA19-9. The DPP-IV specific activity was significantly lower in the T2DM group in comparison to the other patient groups due to substantially increased immunoreactive DPP-IV (CD26) protein in the T2DM group. The molecular basis of the difference remains to be identified. Our results nevertheless suggest that determination of the DPP-IV (CD26) antigen concentrations in T2DM may not accurately estimate the plasma levels of the enzymatically active DPP-IV (CD26).

There are certain limitations to our study. It remains to be established whether the lower DPP-IV specific activity is present in the early stages of type 2 diabetes mellitus without PDAC. It is also currently unclear, whether the changes of tissue and plasma levels of DPP-IV and FAP are present in the early stages of PDAC as the vast majority of the patients (91%) in our study had T3-T4 disease. Lastly, highest plasmatic levels of DPP-IV enzymatic activity were present in PDAC with recent onset diabetes/prediabetes (and weight loss >2kg), but the estimation of diabetes duration in these patients brings the same uncertainty as in the general population of patients with T2DM.

In conclusion, the current work shows increased levels of the proteases exhibiting the DPP-IV-like enzymatic activity in the microenvironment of pancreatic carcinoma and increased plasmatic DPP-IV enzymatic activity in PDAC patients with recent onset diabetes or prediabetes. These changes may reflect the activation of mesenchymal cells leading to fibroproduction associated with tumor growth and may contribute to the frequently observed association of PDAC and recent onset impairment of glucoregulation.

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Conflict of interest: The authors declare no conflict of interest.

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Table 1 Main characteristics of the patients with pancreatic ductal adenocarcinoma (PDAC), type 2 diabetes without PDAC (T2DM) and healthy controls.

The PDAC group is further subdivided based on the presence of the early symptoms of PDAC, i.e. diabetes or prediabetes (DM, recent onset= less than 2 years, long lasting= longer than 2 years) and weight loss. Results are expressed as median (range). BMI= body mass index, FPG= fasting plasma glucose. Statistical significance for the difference between PDAC and its subgroups as compared to type 2 diabetes mellitus without PDAC (^a p<0.05, ^b p<0.01, ^c p<0.001) and controls (^x p<0.05, ^y p<0.01, ^z p<0.001), and for type 2 diabetes mellitus without PDAC compared to controls (** p<0.01, *** p<0.001), Kruskal-Wallis test. ‡In 4 patients, the information on the weight loss and/or the duration of diabetes was not available and thus they were not included in the corresponding subgroups.

	PDAC (n=93) ‡	PDAC with recent onset DM (n=59)			PDAC with long lasting DM (n=15)	PDAC with normal glucoregulation (n=18)	Type 2 diabetes mellitus without PDAC (T2DM, n=39)	Healthy controls (n=29)
			PDAC with recent onset DM and weight loss >2kg (n=39)	PDAC with recent onset DM and weight loss <2kg (n=17)				
Male/Female	51/42	29/30	18/21	10/7	13/3	9/9	32/7	22/7
Age (years)	68 (46- 84) ^{b,x}	67 (53- 84)	65 (53- 82)	70 (54- 84)	70 (46- 78)	70 (50- 81)	63 (51- 73)	64 (49- 73)
BMI kg.m ⁻²	25.5 (13.6- 40.6) ^c	24.7 (13.6- 40.6) ^c	23.8 (13.6- 40.6) ^{c,x}	25.6 (23.8- 38.7)	26.5 (19.6- 36.2)	25.5 (17.4- 33) ^a	29.9 (23- 45.4)	27.7 (19.4- 76.5)
HbA1c (mmol/mol)	45 (28- 124) ^y	45 (29- 83) ^y	45 (30- 83) ^x	46 (29- 58)	74 (37- 124) ^z	36 (28- 53) ^c	50 (24- 90) ***	38 (27- 49)
FPG (mmol/l)	6.5 (2.2- 23.8) ^z	6.7 (4.1- 18.6) ^y	6.8 (4.5- 18.6) ^y	6.7 (4.1- 9.4)	11.6 (5.1- 23.8) ^z	5.3 (2.2- 5.9) ^c	7.8 (5.2- 11.2) ***	5.4 (3.8- 8.7)
CA 19-9 (kIU/l)	95.1 (0.6- 62320) ^{c,z}	122.8 (0.6- 62320) ^{c,z}	137 (0.7- 62320) ^{c,z}	48.2 (0.6- 884.7) ^x	74.1 (0.6- 3378) ^{b,z}	77.5 (0.6- 2540) ^{b,z}	8.3 (2- 50.1)	4.9 (0.6- 15)

Table 2 DPP-IV and FAP staining patterns in pancreatic cancer tissue (n= 39)

% positive cells	DPP-IV immunopositivity			FAP immunopositivity		
	cancer cells	stroma adjacent to cancer cells *	stroma more distant from cancer cells	cancer cells	stroma adjacent to cancer cells *	stroma more distant from cancer cells
0%	2	17	24	11	14	16
1-30%	17	19	15	6	9	8
31-60%	7	3	0	4	6	13
61-100%	13	0	0	18	10	2

* $p < 0.05$, Stuart-Maxwell Marginal Homogeneity Test for the comparison of staining in stroma adjacent to and more distant from cancer cells.

Table 3 Plasma levels of DPP-IV and FAP in the patients with pancreatic ductal adenocarcinoma (PDAC), type 2 diabetes without PDAC (T2DM) and healthy controls.

The PDAC group is further subdivided based on the presence of the early symptoms of PDAC, i.e. diabetes or prediabetes (DM, recent onset= less than 2 years, long lasting= longer than 2 years) and weight loss. Results are expressed as means with SD ranges after logarithmic transformation. Statistical significance for the difference between PDAC and its subgroups as compared to type 2 diabetes mellitus without PDAC (^a p<0.05, ^b p<0.01, ^c p<0.001) and controls (^x p<0.05, ^y p<0.01, ^z p<0.001), and type 2 diabetes mellitus without PDAC compared to controls (** p<0.01, *** p<0.001), ANOVA, Tukey post hoc test. [‡]In 4 patients, the information on the weight loss and/or the duration of diabetes was not available and thus they were not included in the corresponding subgroups.

	PDAC (n=93) [‡]	PDAC with recent onset DM (n=59)			PDAC with long lasting DM (n=15)	PDAC with normal glucoregulation (n=18)	Type 2 diabetes mellitus without PDAC (T2DM, n=39)	Healthy controls (n=29)
			PDAC with recent onset DM and weight loss >2kg (n=39)	PDAC with recent onset DM and weight loss <2kg (n=17)				
Canonical DPP-IV (CD26) enzymatic activity (pkat/ml)	201.9 (135- 302)	205.4 ^a (136.8- 308.6)	218.3 ^a (137.9- 345.5)	179.8 (143.7- 225)	179.5 (120- 268.6)	202.3 (139.9- 292.4)	175.8 (141- 219.2)	182.2 (153.2- 216.7)
DPP-IV (CD26) concentration (ng/ml)	923.3 ^b (523.7- 1627.8)	851.6 ^c (479.3- 1513)	883.6 ^a (516.3- 1512.4)	848.9 (442.4- 1628.9)	958.5 (529.9- 1733.8)	1127.3 (694.3- 1830.4)	1338.8 ^{**} (634.2- 2825.9)	806.6 (403.1- 1614)
specific DPP-IV (CD26) enzymatic activity (pkat/ng)	0.219 ^c (0.131- 0.365)	0.241 ^c (0.142- 0.411)	0.247 ^c (0.147- 0.414)	0.212 (0.12- 0.374)	0.187 (0.122- 0.288)	0.179 (0.115- 0.281)	0.131 ^{***} (0.059- 0.293)	0.226 (0.122- 0.419)
FAP enzymatic activity (pkat/ml)	5.72 ^{cz} (3.37- 9.71)	5.67 ^{cy} (3.3- 9.75)	5.59 ^{cy} (3.14- 9.93)	5.82 ^a (3.8- 8.91)	6.5 (4.76- 8.86)	5.29 ^{cy} (2.78- 10.05)	8.76 (7.05- 10.88)	8.11 (6.32- 10.42)
FAP concentration (ng/ml)	13.51 (8.25- 22.11)	13.86 (8.6- 22.34)	13.97 (9.02- 21.64)	14.61 (8.49- 25.15)	13.38 (7.65- 23.4)	12.44 (7.4- 20.92)	16.56 (9.94- 27.61)	15.42 (8.28- 28.71)

Figure legends:

Figure 1 Quantification of DPP-IV in pancreatic ductal adenocarcinoma (PDAC) and non-tumorous pancreatic tissue in PDAC patients.

A) Overall DPP-IV-like enzymatic activity, B) Enzymatic activity attributable to the canonical DPP-IV (CD26), C) DPP-IV-like enzymatic activity insensitive to a specific DPP-IV inhibitor sitagliptin, D) DPP-IV (CD26) concentration determined by ELISA. \pm Median and range of 25-75% of the values; lines connect the values measured in paired non-tumorous and tumorous tissue samples in individual patients. * p<0.05, ** p<0.01, Mann-Whitney U test for non-tumorous pancreatic tissue vs. PDAC.

Figure 2 Localization of DPP-IV in tumorous and non-tumorous tissue in pancreatic ductal adenocarcinoma (PDAC).

A, B) Detection of DPP-IV in PDAC by immunohistochemistry (original magnification 200x) and C-H) by catalytic histochemistry (in red). C- F) Tumorous tissue, G- H) Non-tumorous pancreatic tissue in PDAC patients. DPP-IV is present in malignant cells (+), stromal cells (++) and Langerhans islets (+++). Inset- section incubated in the presence of a highly specific DPP-IV inhibitor sitagliptin.

Figure 3 Expression and localization of fibroblast activation protein (FAP) in tumorous and non-tumorous pancreatic tissue and co-expression of DPP-IV in FAP positive fibroblasts.

A) FAP expression in pancreatic ductal adenocarcinoma (PDAC) and non-malignant pancreatic tissue in PDAC patients. \pm Median and range of 25-75% of the values; lines connect the values measured in paired non-tumorous and tumorous tissue samples in individual patients. *** p<0.001, Mann-Whitney U test. B) FAP expression in non-tumorous pancreatic tissues in PDAC patients depending on the presence and absence of histological features of chronic pancreatitis as compared to non-malignant pancreatic tissue from patients with other pancreatic tumors (2x microcystic serous cystadenoma of the pancreas, 3x pancreatic neuroendocrine tumors, 2x carcinoma of the ampulla of Vater, 2x solid pseudopapillary neoplasm of the pancreas, 1x cholangiocarcinoma). — median, \square 25%-75%, $\bar{\Gamma}$ range of non-remote values, Δ source data, \circ remote values, * extremes, *p<0.05, *** p<0.001, Kruskal-Wallis test. C, D) Localization of FAP in cancer and stromal cells adjacent to the cancer cells in pancreatic ductal adenocarcinoma (PDAC). Double immunofluorescence labeling of E) DPP-IV and α -smooth muscle actin (α -SMA), and F-H) DPP-IV and FAP in pancreatic ductal adenocarcinoma. A Langerhans islet

surrounded by stromal cells expressing DPP-IV and FAP is depicted in H. ⁺DPP-IV expressing cancer cells, ⁺⁺FAP positive stroma, ⁺⁺⁺stromal cells co-expressing DPP-IV and FAP.

Figure 4 Probability of pancreatic ductal adenocarcinoma (PDAC) based on the generalized additive models: A) effect of specific DPP-IV enzymatic activity in model 1, B) effect of specific DPP-IV enzymatic activity in model 2, C) effect of CA19-9 in model 2. Data from patients with PDAC and recent onset diabetes/prediabetes (n=59), and type 2 diabetes mellitus without PDAC (n=39). Semiparametric logistic regression based on an additive logistic model. D) Plasma FAP enzymatic activity and E) concentration in PDAC patients before and after the surgical removal of the tumor. ** p<0.01, Wilcoxon paired test, n=13.