Expression of ornithine decarboxylase in peripheral blood mononuclear cells from patients with pancreatic adenocarcinoma: A preliminary report

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Abstract. Ductal adenocarcinoma represents 90-95% of pancreatic cancer (PC) cases and it is an aggressive disease with asymptomatic evolution at early stages, non-specific symptoms and a typical late diagnosis with a 5-year survival rate estimated to be 8%. A window of opportunity lies in early diagnosis as there are currently no reliable biomarkers. CA 19-9 is one of the most frequently used biomarkers of PC, with 75 and 77.6% sensitivity (Se) and specificity (Sp), respectively, and the carcinoembryonic antigen (CEA) shows 39.5 and 81.3% of Se and Sp, respectively. A case-control study was conducted including adult patients with a histological diagnosis of PC (n=11) without previous treatment at the Oncology Service of the CMNO-IMSS between 2019 and 2020, and a control group of adult volunteers (n=11) who were clinically healthy or with controlled disease including hypertension, hypothyroidism and diabetes. Clinical, laboratory and sociodemographic data as well as blood, urine and saliva samples were collected following patient consent. Polyamines were quantified using high-performance liquid chromatography with fluorescence detection, CA 19-9 and CEA were evaluated using enzyme-linked immunosorbent assay, and the protein expression of ornithine decarboxylase (ODC) was evaluated using western blotting. Polyamine metabolism and modulation by means of ODC were increased in the serum and saliva of patients with PC, and the expression of ODC alone was increased in peripheral blood mononuclear cells (PBMCs). The present study focused on the evaluation of putrescine, spermine, spermidine and ODC in PBMCs associated with CA 19-9 and CEA as an auxiliary tool in PC diagnosis.

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Abbreviations: CEA, carcinoembryonic antigen; CS, clinical stage; DM2, diabetes mellitus type 2; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ERCP, endoscopy retrograde cholangiopancreatography; HFH, hereditary family history; HPLC, high-performance liquid chromatography; ODC, ornithine decarboxylase; PAC, pancreatic adenocarcinoma; PBMCs, peripheral blood mononuclear cells; PC, pancreatic cancer; RIPA, radioimmunoprecipitation assay; SDS, sodium dodecyl sulfate

Key words: ornithine decarboxylase, pancreatic adenocarcinoma, polyamines, peripheral blood mononuclear cells, biomarker

Introduction

Pancreatic cancer (PC) is the 4th leading cause of cancer-related death in male and female patients in developed countries, with an incidence of 12.5/100,000 individuals in the American continent (7% of all types of cancer) (1). PC is associated with poor prognosis, and the mortality rate is similar to that of the incidence (2). Long-term survival remains poor, and the 5-year survival rate for patients with resectable tumors is 15-20%, while patients with unresectable tumors at the time

of diagnosis have a 5-year survival of ~0% (3,4); it is expected that by 2030, this tumor type will become the 2nd leading cause of cancer-related death (4).

PC is a tumor that grows slowly and silently for ~10 years (5), and most cases occur in patients with no evidence of hereditary family history (6). However, there are still no reliable biomarkers used in the clinic. It is noteworthy that the serum tumor biomarker CA 19-9 is a pentose related to the carbohydrate antigen Sialyl Lewis located on erythrocyte surfaces, which is absent in 10-15% of the population due to the lack of the enzyme necessary for its synthesis (7-9). Elevated levels of CA 19-9 (<1,000 IU/ml) are suggestive of pancreatic adenocarcinoma (PAC); a decrease in values through treatment suggests a good response, and at present, this is the only serum tumor marker for PAC (10). The sensitivity (Se) of CA 19-9 is 69-98%, while the specificity (Sp) is 46-98% (11). This marker has also been found to be increased in patients with other upper gastrointestinal tumors, diseases that give rise to biliary obstruction, inflammatory pathology and other benign conditions such as primary sclerosing cholangitis (12), but it can also be detected at normal levels in some patients with PAC (13). At present, CA 19-9 is mainly used as a monitoring tool to assess patient response to treatment (10).

Another tumor biomarker is carcinoembryonic antigen (CEA), a member of surface glycoproteins located at the apical pole of erythrocytes; this molecule is the most commonly used tumor marker in colon carcinoma (14). However, CEA levels can also be increased in other pathological conditions, such as gastric, pulmonary, pancreatic and breast neoplasias, in the medullary carcinoma of the thyroids, as well as in other conditions such as cirrhosis, ulcerative colitis, pancreatitis and even in smokers. The totality of its functions remains unknown (15).

In the clinic, CEA is associated with colorectal cancer prognosis, staging, treatment response and recurrence; however, it plays a notable role in other neoplasias too such as PC. The positive predictive value (PPV) of CEA for PC is 78.6%, and the negative predictive value (NPV) is 75% (16).

The tumor microenvironment of PC should be investigated further, and biomarkers with high predictive potential and Sp are urgently required.

Polyamines are aliphatic molecules consisted of various amine groups (17). In humans, polyamines are derived from two sources, including endogenous biosynthesis by *de novo* biosynthesis and interconversions among themselves, and also through digestive secretions, especially intestinal, pancreatic and catabolism products from intestinal cells. In addition, polyamines are also produced by intestinal microorganisms using dietary intake, an exogenous source of polyamines (18).

These small molecules are associated with numerous cell processes, such as cell division, nucleic-acid packaging, DNA replication and others. In mammals, polyamines are produced from ornithine by ornithine decarboxylase (ODC), which produces putrescine, which in turn gives rise to spermine and spermidine through the spermidine and spermine synthases, respectively (17).

One of the first events in cell proliferation is the induction of polyamine biosynthesis; it is known that the overexpression of ODC beyond the minimal threshold can induce cell transformation and tumor promotion (19). However, ODC expression increases the biosynthesis of putrescine and the subsequent biosynthesis of spermine and spermidine. Thus, the activity of this enzyme is sufficient for tumor promotion, making ODC a proto-oncogene (20).

Consequently, high levels of polyamines were found in the biological fluids of patients with cancer, and these have been investigated as a biomarker in ovarian cancer (21), colorectal cancer (22,23), breast cancer (23), lung cancer (24), prostate cancer (25) and PC (26). These levels decrease after tumor eradication and increase again in case of relapse (27); although these molecules are present in some normal tissues, such as the bone marrow, pancreas, intestinal mucosa and prostate, their expression is increased in tumor tissues. Although it has been shown that blood or urine polyamine concentrations are elevated in patients with cancer, it is not known if ODC is expressed in peripheral blood mononuclear cells (PBMCs) (28).

Asai *et al* (6) quantified polyamine levels in the saliva of patients with PC, in patients with pancreatitis and in healthy controls, showing that polyamines, especially spermine, were notably increased in patients with PC compared with those in the control group. A combination of the four metabolites spermine, N1-aceylspermidine, N1-acetylspermine and 2-aminobutanoate also exhibited marked increase in patients with cancer compared with patients with benign pancreatic pathology (chronic pancreatitis) or with healthy individuals, and could potentially be used for the detection of PC.

In the present study, the potential of PBMCs to exhibit ODC metabolism and their association with PC, was investigated.

Materials and methods

Patients (cases). The present case-control study involved patients who were diagnosed with PAC. Mexican patients attending the Oncology Service, High Specialty Medical Unit, Western National Medical Center, Mexican Social Security Institute, Guadalajara, Mexico, between November 2019 and February 2021 (≥18 years of age) with histopathological diagnosis of PAC and without previous treatment were invited to participate in the present study.

Controls. Mexican, ≥18-year-old female and male healthy individuals or with controlled disease including hypertension, hypothyroidism and diabetes, with no tumor-associated complications were included in the control group.

The clinical and sociodemographic data of both groups were collected in medical consultation through an interview with the oncologist, and signed informed consent was obtained from all study participants.

Inclusion criteria. For patients, the inclusion criteria were: i) Histopathological diagnosis of PAC; and ii) no previous treatment. For controls, the inclusion criteria were: i) Health or controlled disease including hypertension, hypothyroidism and diabetes; ii) smoking (<5 cigarettes/day for <5 years; and iii) no recorded tumor-associated complications.

Exclusion criteria. The exclusion criteria for both groups were the following: i) Inadequate sample; and ii) incomplete clinical and/or laboratory data.

Samples. All samples were collected between November 2019 and February 2021 by the clinical staff at the Oncology Service, Specialty Hospital (Guadalajara, Unites Mexican States) before any treatment. Samples were correctly labeled in closed containers with double packaging and transported to the Division of Immunology.

Blood samples (5 ml) were obtained by venipuncture and collected in protease-free tubes with ethylenediaminetetraacetic acid (EDTA) as anticoagulant and diluted with PBS (1:1; cat. no. 10010023; Gibco; Thermo Fisher Scientific, Inc.). This mix was gently placed in Ficoll-Hypaque (density, 1.077; cat. no. 10771; Sigma-Aldrich; Merck KGaA) to obtain PBMCs.

Trypan blue was used to determine the number of viable cells in a cell suspension. A total of $1.3x10^6$ cells/ml were resuspended in fetal bovine serum (cat. no. 10437; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 5% of dimethylsulphoxide (cat. no. D8418; Merck KGaA) for cryopreservation until determination.

CA 19-9 and CEA determination using enzyme-linked immunosorbent assay (ELISA). Determination of CA 19-9 (CA 19-9 Accubind ELISA kit; cat. no. 3925-300A; Monobind, Inc.) and CEA (CEA Next Generation Accubind ELISA kit; cat. no. 4625-300A; Monobind, Inc.) was carried out according to the manufacturer's instructions. Briefly, 25 μl standard or samples from both study groups (controls and patients) were placed in the appropriate well, and 100 μl buffer solution was added, mixed and incubated at 37˚C for 90 min. The plates were washed five times. A total of 100 μl either CA 19-9- or CEA-labeled antibody was added to each corresponding well, mixed for 20-30 sec, covered and incubated for 60 min at room temperature (RT). A total of 350 μl wash buffer was added twice, and 100 μl working substrate solution was added, incubated again for 15 min at RT, thereafter adding 50 μl stop solution, mixing and reading in the multi-detection microplate reader (620-630 nm; Bio Tek Synergy HTX Multimode Reader; Agilent Technologies, Inc.). Results were expressed as U/ml.

Polyamine quantification by high-performance liquid chromatography (HPLC)

Sample collection and treatment. All samples were collected by the clinical staff in the Oncology Service prior to any treatments. Samples correctly labeled in closed containers with double packaging were transported to the Division of Immunology, Mexican Social Security Institute (Guadalajara, United Mexican States) for research and stored at -80˚C until polyamine determination.

Plasma. A total of 5-10 ml peripheral blood with EDTA was collected, centrifuged at 1,368 x g, for 15 min at RT (20-25˚C) and the plasma was separated and mixed with 5% perchloric acid (cat. no. 244252; Sigma-Aldrich; Merck KGaA) at a 1:1 ratio. In order to precipitate the proteins, a second centrifugation step at 7,267 x g for 10 min at RT was carried out. The acidic extract was recovered and stored at -80˚C until further analysis (29).

Urine. Urine was recovered in a sterile flask; 0.5 ml urine was mixed with perchloric acid and treated as plasma.

Saliva. Saliva was also recovered in a sterile flask and processed as the other aforementioned samples.

Table I. Elution gradient for the determination of polyamines.

Time, min	A, %	B, %	Flow, ml/min
0	0	100	1.0
2	25	75	1.0
3	25	75	1.0
5	50	50	1.0
6	60	40	1.0
8	90	10	1.0
10	90	10	1.0
12	0	100	1.0

A, 40 mM sodium phosphate buffer/acetonitrile (5:95 ratio); B, 80:20.

Polyamine determination. An analytical HPLC method for the quantitation of polyamines was developed and validated in different samples, including plasma, urine and saliva of patients with PAC. The concentration of polyamines was calculated from three calibration curves using a quaternary system (cat. no. G1310; Agilent series 1260; Agilent Technologies, Inc.), online degasser (cat. no. G1379), autosampler (cat. no. G1329), fluorescence detector (cat. no. G1365) and thermostat (cat. no. G1316).

Polyamine separation was carried out through a Phenomenex Luna C_{18} column, (100x4.6 mm; 5 μm). Elution was conducted through a gradient: Mobile phase consisted of a gradient of solvent A circumvention, sodium phosphate buffer (40 mM Na_2HPO_4)/acetonitrile (Tedia AS 1122001) at a ratio of 5:95, and B (80:20) at a ratio A/B of 0:100 at time 0, reversing the ratio in 10 min, with a running time of 12 min and a flow of 1.0 ml/min, according to the description in Table I. Three polyamines were determined simultaneously in the biofluids of patients with PAC at different stages and compared with those of the controls.

Detection of polyamines was carried out at an excitation length of 360 nm and at an emission of 560 nm. All of the elusion components were previously degassed, and the samples were filtered using Durapore membranes (0.22-μm; cat. no. GWP01300; MilliporeSigma).

PBMC recovery. Blood samples from patients and controls were obtained by venipuncture in 6-ml EDTA tubes, and blood was gently placed on Hystopaque®-1.077 (1.077-100 ml; Sigma-Aldrich; Merch KGaA) at 1:1 ratio; next, samples were centrifuged at 1,368 x g for 25 min at RT. The interface contained PBMCs, which were collected, washed twice with PBS for complete removal of Hystopaque and resuspended in 3 ml PBS. Cells were counted using a hemocytometer and viability was determined with the exclusion dye trypan blue.

Western blotting. Western blotting was performed as previously described by Cruz-Gálvez *et al* (30). Briefly, $3x10^6$ PBMCs from patients with PAC were thawed for 10 min at room temperature; after centrifugation (235 x g for 7 min at RT), the medium was gently discarded. The cell pellet was resuspended, washed twice with PBS at room temperature, lysed with radioimmunoprecipitation assay (RIPA) buffer

(0.5% deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl; cat. no. sc 24948; Santa Cruz Biotechnology, Inc.), and maintained on ice with protease/phosphatase inhibitors (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail; Sigma-Aldrich; Merck KGaA) for 30 min. Cells were resuspended in RIPA buffer in an ice bath during 30 min. Subsequently, cells were homogenized in a bio-disruptor by hydrodynamic agitation (15 pulses, 50% amplitude), ensuring that the temperature did not increase and cells were incubated on ice for 30 min. The total number of lysed cells was transferred into microcentrifuge tubes and centrifuged at 11,355 x g for 13 min at 4˚C.

Protein concentrations were determined using the DC Protein Kit (Bio-Rad Laboratories, Inc.). A total of 60 μg protein sample was subjected to electrophoresis using a 10% SDS/PAGE gel. Subsequently, the proteins were transferred onto Immobilon-P PVDF membranes (MilliporeSigma) and these were incubated with the Odyssey® Blocking Buffer reagent for 2 h, at RT with gentle agitation. Immunodetection of ODC was performed using a mouse monoclonal anti-ODC antibody (243-272 aa; human anti-ODC specific to the internal region of human ODC; cat. no. SC-398116; Santa Cruz Biotechnology, Inc.) diluted at 1:500 in blocking buffer and 0.1% Tween-20 at 4˚C overnight. β-actin (human anti-β actin; cat. no. SC-47778; Santa Cruz Biotechnology, Inc.) was used as an internal control. After incubation with a fluorescently-labeled secondary antibody (IRDye® 680 Donkey Anti-Mouse IgG; LI-COR Biosciences, Ltd; cat. no. 926-32212) diluted at 1:15,000 in PBS + 0.1% Tween-20, and 0.1% SDS, the ODC protein was visualized using the Odyssey® XF Imaging System (LI-COR Biosciences, Ltd.). The results were normalized for all experiments by the mean optical density of the gel background and zeroing with a dark gel spot.

Statistical analysis. Clinical and sociodemographic data are expressed as percentages and means were compared between the control and patient groups using the non-parametric Mann-Whitney U test and the Wilcoxon signed-rank test. Clinical parameters in patients were expressed as frequencies and percentages. Polyamines are expressed as mean ± standard deviation and compared using unpaired Student's t-test. $P<0.05$ and $P<0.01$ were considered to indicate a statistically significant difference. ODC expression was calculated by relative expression, evaluated by an increase in fold change, and analyzed by an unpaired Student's t-test. Survival of the patients was analyzed using the Kaplan-Meier method. All statistical analyses were performed using GraphPad Prism (version 9.5.0; Dotmatics).

Results

Patients. A total of 15 patients with a diagnosis of PAC attended an appointment for the first time at the Gastrointestinal Tumor Clinic of the Medical Oncology Service at the Western National Medical Center, IMSS, between December 2019 and December 2020. Patients who accepted to voluntarily participate in the present study (n=11) proceeded to sign an informed consent. Clinical and sociodemographic data, and blood samples were then collected.

Control group. The control group (n=11) included male volunteers (36.4%) and female volunteers (63.6%) who accepted to participate in the current study. They all signed an informed consent and donated a blood sample. Controls of similar age to that of the patient group were selected; some controls also had some risk factors, and were individuals with no recorded tumor complication, and with only one well-controlled chronic disease as follows: Two individuals had type 2 diabetes mellitus (DM2; 18.18%), two individuals had arterial hypertension (18.18%), one individual had hyperthyroidism (9.09%) and one individual had pancreatitis (9.09%). All controls provided their sociodemographic information.

Clinical and sociodemographic data. Patients (n=15) had a mean ± SD age of 54.80±8.54 years (range, 42-73 years) compared with that of the controls, who had a mean age of 47.40±10.24 years (range, 27-65 years). The patient group was consisted of 46.7 and 53.3% males and females, respectively, while the control group was consisted of 36.4 and 63.6% males and females, respectively (Table II).

When the risk factors of the studied groups were compared, the following was observed: Although the presence of DM2 did not reveal significant differences, 9.1 and 37.3% of the control and patient groups, respectively, were stratified by time of disease evolution, and 6.7% of the patients had >10 years of disease evolution compared with 13.3% of patients with a recent diagnosis (<1 year).

Smoking was reported in 27.3 and 40% of controls and patients, respectively, with 6.7% of patients being smokers for >5 years. Regarding smoking intensity, 50% were moderate smokers and 50% were heavy smokers. Smoking intensity appeared to be significant risk factor ($P<0.05$).

Alcohol was consumed by 36.4 and 26.7% of controls and patients, respectively. All controls who consumed alcohol indicated light consumption, while 75% of patients indicated high and excessive consumption according to the World Health Organization degrees of intensity of alcohol consumption (31).

Hereditary-family history (HFH) of cancer was more common in controls (54.5%), and it was homogenously distributed between grade 1 and 2, compared with 37.3% of patients with a predominant grade-1 antecedent.

Obesity was reported in 18.2 and 34.4% of patients and controls, respectively. Pancreatitis was only reported in 6.6% of controls. No significance was observed in these parameters when they were compared between the control and patient groups.

The investigation of the symptoms of PAC (Table III) indicated that abdominal pain was the principal symptom (46%), with all of the reported symptoms fulfilling the chronicity criteria (>3 months with abdominal pain that do not respond to treatment without pain control evaluated in retrospective) (32). All patients received symptomatic treatment and 10/11 patients had >3 months of first-symptom evolution. Although the patients presented secondary symptoms, imaging was not carried out.

General laboratory tests were requested by 4/11 patients. The most common symptoms of PAC included icteric skin, weight loss and ascites in 66.6, 20.0 and 6.6% of patients.

Distribution by age. In terms of age distribution, in patients with PAC aged between 42 and 73 years, the highest risk was found between the age of 50 and 60 years ($P<0.05$).

Table II. Demographical characteristics of patients (n=15) and controls (n=11).

Demographical characteristics	Patients	Controls	P-value
Age, years	54.80±8.54	47.40±10.24	0.60
Range	42-73	27-65	0.04[a]
Sex, %			
Male	46.7	36.4	0.60
Female	53.3	63.6	0.60
Risk factors, %			
DM2	37.3	9.1	0.78
DM2 recent diagnosis	6.6	0.0	0.43
Smoking	27.0	40.0	0.61
Heavy smoking	20.0	0.0	0.03[a]
Alcohol consumption	26.7	36.4	0.16
Obesity	18.2	34.4	0.27
Hereditary-family history of cancer	37.3	54.5	0.34
Chronic pancreatitis	0.0	6.6	0.35

[a]P<0.05. DM2, diabetes mellitus type 2. Data are presented as mean ± SD.

Table III. Early signs and symptoms in patients with pancreatic adenocarcinoma.

Sign or symptoms	n (%)
Abdominal pain	7 (46.0)
Anorexia	1 (6.6)
Reflux	1 (6.6)
Dyspepsia	1 (6.6)
Singultus	1 (6.6)
Type 2 diabetes mellitus	1 (6.6)
Icterus	10 (66)
Weight loss	3 (20.0)
Ascitis	1 (6.6)

Diagnosis and associated complications. Histological diagnosis was performed using a surgical piece of the tumor resection in three patients who were programed for surgery with curative intention (20%). For the remainder of the patients, evidence of malignancy was obtained by open biopsy (40%), endoscopy retrograde cholangiopancreatography (ERCP; 26.6%) and imagery-guided biopsy (6.6%), while there was a sample obtained by endoscopy from one patient who had metastasis in the gastric cavity and bleeding in the upper digestive tract.

ERCP was performed in 10/15 patients; in all cases, this was required for the treatment of obstruction in the bile ducts. However, the histopathological analysis of ductal aspiration and brushing was conclusive for PAC in only two patients. On the other hand, among six patients with open biopsy, three presented associated complications, including bleeding (n=1) and infection of the surgical wound (n=2). Finally, histology in all cases confirmed ductal PAC diagnosis.

Clinical stage (CS). The predominant CS at diagnosis was IV (40%), conditioned by hepatic and peritoneal metastasis, followed by CS IIIB (33.3%), IIB (6.6%), IB (13.3%) and IIIA (6.6%), according to the American Joint Committee on Cancer version 8 (33).

Treatment. The approaches used in the treatment of the majority of cases (38.4%) included treatment with FOLFIRINOX oxaliplatin (85 mg/m^2), irinotecan (150 mg/m^2), leucovorin (200 mg/m^2) and 5-fluoracil infusion (2,400 mg/m^2) on day 1 by continuous infusion over 46 h, every 14 days for 12 cycles. A total of 7.6% of patients received a double treatment scheme such as gemcitabine (1,000 mg/m^2)-cisplatin (25 mg/m^2) on day 1 and 8 or gemcitabine (1,000 mg/m^2)-capecitabine (100 mg orally for 14 days), while 23% of patients were treated with monotherapy and either gemcitabine or capecitabine (1,600 mg/m^2/day in two doses, from day 1 to 5).

Survival. Treatments and treatment intentions were variable. Three patients underwent tumor resection; at first, all patients (n=3) received adjuvant chemotherapy; one of them was programmed for radiotherapy due to compromised margins, while a change in the initial treatment intention from adjuvant to palliative treatment was planned for a second patient due to hepatic metastasis documented prior to the initiation of systemic treatment. One patient continued neoadjuvant treatment until the end of the study (February 2021). Among the 11 patients with non-resectable and metastatic disease, six were not candidates for cytotoxic treatment and received supportive care, while five patients received the same scheme of chemotherapy (FOLFIRINOX) with palliative intention. At cutoff, the death of five patients was recorded due to PC-related complications (n=4) and sepsis (n=1; Fig. 1).

Tumor markers CA 19-9 and CEA. As shown in Table IV, the CA 19-9 levels in patients with PAC (1,273.32±1,258.25 U/ml)

Table IV. Tumor markers.

Marker, U/ml	Patients	Controls	P-value
CA 19-9	1273.32±1258.25	7.51±9.88	0.002[b]
CEA	69.81±59.50	1.35±0.32	0.03[a]

[a]P<0.05, [b]P<0.01. CEA, carcinoembryonic antigen.

Table V. PBMC recovery.

	Patients	Controls	P-value
x10^6 PBMCs/5 ml blood	3.69±1.04	6.25±2.36	0.01[a]

[a]P<0.05. PBMCs, peripheral blood mononuclear cells.

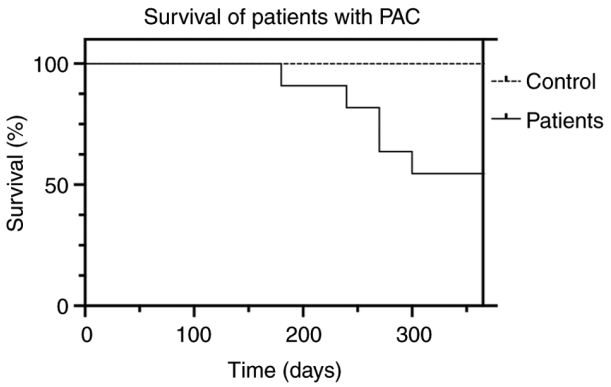

Figure 1. Survival of patients with PAC. Graph shows the survival of patients with PAC at 1 year (n=11). At cutoff, the death of five patients was recorded due to pancreatic cancer (n=4) and sepsis (n=1). Accumulated survival at this time was 63% at 365 days of observation. *P<0.01 compared with the control group. PAC, pancreatic adenocarcinoma.

were significantly increased compared with those in the control group (7.51±9.88 U/ml; P<0.01). Meanwhile, the levels of CEA were also significantly increased in patients compared with those in controls (69.81±59.50 vs. 1.35±0.32 U/ml, respectively; *P<0.05).

Polyamine quantification. Regarding the levels of plasma polyamines, no significant difference in putrescine concentration was observed between the control group (4.475±1.7403 μg/ml) and the patient group (5.0510±0.4494 μg/ml; Fig. 2A). The same observation was made for spermine concentration between the control (2.7130±2.3371 μg/ml) and the patient group (1.8520±1.4451 μg/ml; Fig. 2B).

Putrescine exhibited a significant increase in the urine of patients compared with the control group (*P<0.05; Fig. 2C), while spermine was significantly decreased in the patient group compared with the control group (**P<0.01; Fig. 2D).

Polyamine levels in the saliva of patients were also compared with those in the controls (Fig. 2E). Putrescine did not vary significantly between the two groups, while spermine increased significantly in the patient group (*P<0.05; Fig. 2F). Spermidine levels were <0.001 μg/ml in the three fluids: Plasma, saliva and urine.

ODC expression. The ODC expression in PBMCs from patients with PAC and in controls was investigated using western blotting. PBMC recovery is depicted in Table V. A total of 3.69±1.04x10^6 PBMCs were recovered from patients, while in the control group, we recovered 6.25±2.36x10^6 PBMCs (**P<0.01).

In Fig. 3A, the expression of ODC (53 kDa) in patients with PAC (top) and in controls (middle) was investigated using western blotting. Relative expression was normalized using gel background, β-actin (bottom) is shown as the internal control. In Fig. 3B, a 2-6-fold increase in ODC expression was noted (range, 0.4-6.3) in patients compared with that in controls. In Fig. 3C, the mean of fold change is shown in the control group (0.7333) compared with that in the patient group (2.0000), with differences shown as mean ± standard error of the mean (1.2670±0.6071), with a 95% confidence interval (**P<0.0001).

In Table VI, the area under the curve (AUC), Se, Sp, PPV, NPV, Youden's Index and the cut-off value of every parameter are presented: CEA, CA 19-9, polyamines in different biofluids and ODC expression.

The cut-off value was obtained by means of the minimal distance from the upper-left corner of the unit square of the receiver operating characteristic curves. It was observed that the most used biomarkers were CEA and CA 19-9, which demonstrated an AUC of 0.80. In addition, urine putrescine, saliva spermine and ODC expression were within the range 0.73-0.82. It was shown that plasma putrescine and spermine showed 70% of Se with low Sp (50 and 40%, respectively), while putrescine in urine (70% Se; 81.6% Sp) and spermine in saliva (80% Se; 80% Sp) exhibited higher values.

The accuracy of all parameters was 18.16% (ODC) and 23.2% (CA 19-9).

Discussion

PAC is a condition with increased incidence, characterized by late diagnosis due to the appearance of signs and symptoms at advanced disease stages, and these are usually non-specific contributing to a delay in diagnosis (34).

Despite notable advances in diagnostic imaging techniques and the improvement in treatment options in the last decades, at present, patients with PAC continue have a poor prognosis, with an estimated 5-year survival of 10-15% for resectable tumors and ~0% for locally advanced, non-resectable and metastatic stages (3).

A marked window of opportunity for improving the outcomes for these patients is the early diagnosis of PC, and the investigation of useful biomarkers to achieve this is urgent. Polyamines and ODC have been considered as possible biomarkers in colon, breast, lung and PC (35,36). To the best of our knowledge, no strategies to date have been implemented to evaluate their diagnostic usefulness. The aim of the present study was to evaluate the expression of ODC as a hallmark of PC in patients attending an outpatient clinic of the Medical Oncology Service at the CMNO.

Table VI. Sensitivity and specificity of biomarkers of pancreatic adenocarcinoma.

Biomarker	AUC[a]	Sensitivity[a], %	Specificity[a],%	PPV, %	NPV, %	YI	Cut-off value	ACC
CEA	0.800	70.0	90.9	60.00	90.90	0.5090	1.91	22.66
CA 19-9	0.800	50.0	100.0	50.00	100.00	0.5000	33.30	23.20
PPut	0.520	70.0	50.0	70.00	45.00	0.1540	4.81	19.71
PSpm	0.360	70.0	40	70.00	36.35	0.06300	1.11	20.57
UPut	0.764	70.0	81.6	70.00	81.81	0.5180	4.76	20.28
USpm	0.0810	11.1	18.2	80.00	100.00	0.8000	15.14	21.37
SPut	0.409	0.0	81.8	100.00	20.00	0.2000	2.85	20.20
SSpm	0.820	80.0	80	100.00	72.00	0.5272	1.08	21.00
ODC	0.735	67.0	77.8	66.66	87.50	0.5400	1.05	18.16

[a]Calculated by SPSS. Se, (TP/TNP)*100; Sp, (TN/TNC)*100; Youden's Index, [(Se+Sp)/100]-1; ACC, TP+TN/TP+TN+FP+FN. TN, true negative; CEA, carcinoembryonic antigen; AUC, area under the curve; PPV, positive predictive value; TP, true positive, TNP, total number of patients; NPV, negative predictive value; TNC, true number of controls; YI, Youden's index; PPut, plasma putrescine; PSpm; plasma spermine; UPut, urine putrescine; USpm; urine spermine; SPut, saliva putrescine; SSpm, salive spermine; ACC, accuracy; FP, false positive; FN, false negative; ODC, ornithine decarboxylase.

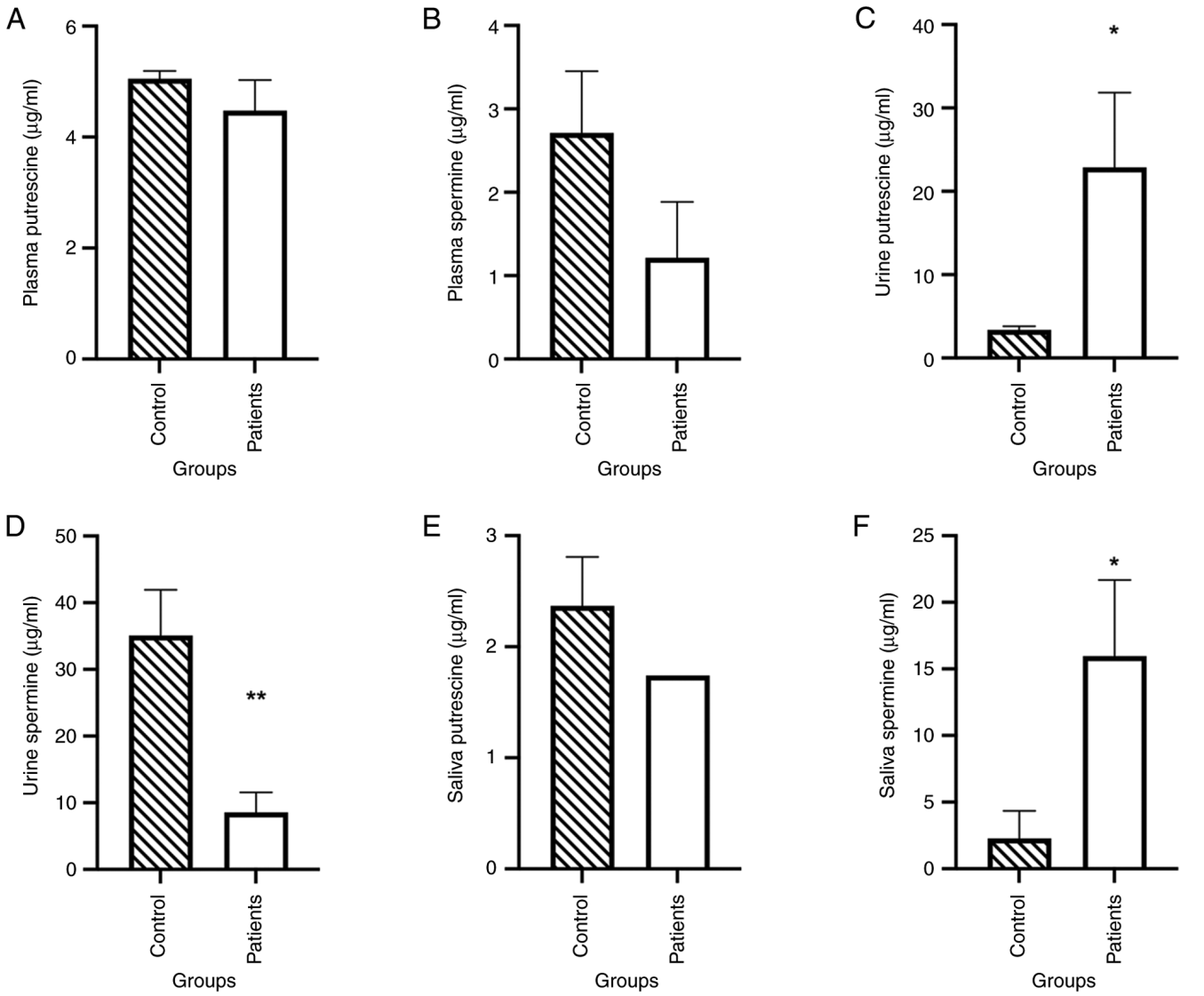

Figure 2. Plasma levels of polyamines. Polyamines were quantified using high performance liquid chromatography with fluorescence detection in the control and patient groups. (A) Plasma putrescine (μg/ml), (B) plasma spermine (μg/ml), (C) urine putrescine (μg/ml), (D) urine spermine (μg/ml), (E) saliva putrescine (μg/ml) and (F) saliva spermine (μg/ml). Data are expressed as mean ± SD. Unpaired Student's t-test was used for analysis. *P<0.05 and **P<0.01.

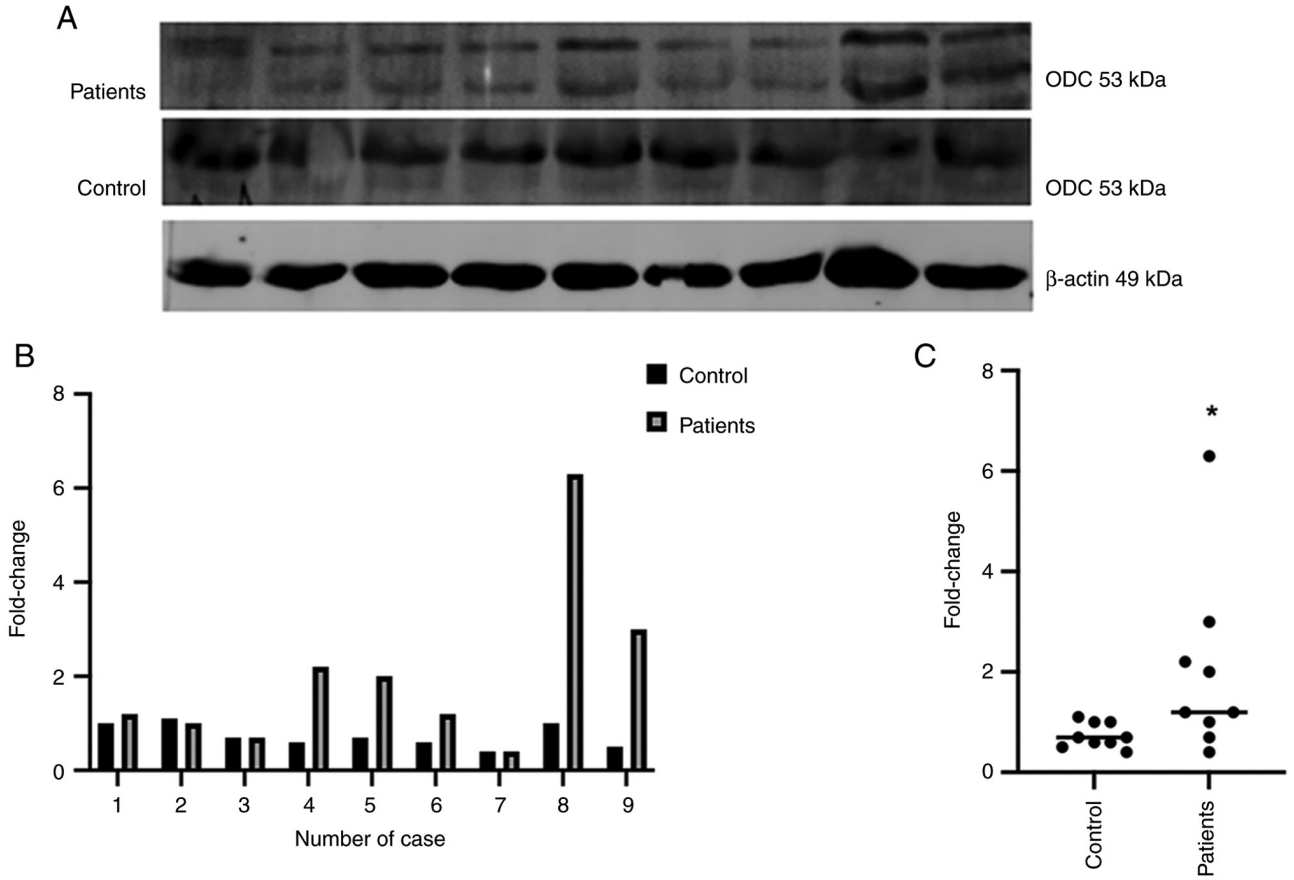

Figure 3. ODC expression in peripheral blood mononuclear cells from controls and patients with pancreatic adenocarcinoma was evaluated using western blotting. (A) Fold change was normalized with gel background. (B) Mean fold change of the control group (0.7333) and of the patient group (2.000). (C) Differences between means (B-A ± standard error of the mean; 1.2670±0.6071), 95% confidence interval with *P<0.0001. *Patient 10 was not evaluated due to insufficient sample. ODC, ornithine decarboxylase.

Regarding risk factors, it is noteworthy that the average age between the two groups with no statistical differences indicated that both groups were homogeneous. A total of 6.6% of patients with PAC were also recently diagnosed with DM2, but this was not associated with the expression of ODC or polyamine levels in biofluids. A difference in the intensity of smoking between the groups was also investigated since it has been reported that heavy smoking is a high risk factor: Other risk factors were consistent with other published studies, such as smoking, obesity, alcohol consumption and HFH cancer in the patient group (37).

The most commonly used biomarkers reported in literature include CA 19-9 and CEA (3,38). However, these biomarkers have some limitations, such as low Se (50 and 70%, respectively) but high Sp (100 and 90%, respectively) as observed in the present study. It was observed that there were patients with both high and normal levels of the aforementioned markers in the current study. This observation highlighted the need to identify novel biomarkers with higher Se and Sp. Polyamines and ODC have been described as possessing important pleiotropic effects on cancer cells, because they contributed to different processes that drive the progression of PC and can be detected in tissue and biofluids with good precision, which allows differentiation between controls and patients with PAC (6).

Regarding Se, good values for plasma putrescine and spermine, urine putrescine, and saliva spermine and ODC were observed; in addition, good Sp was observed for urine putrescine, and for saliva spermine and putrescine.

It is noteworthy that it was possible to establish the cut-off points for both ODC expression in PBMCs as well as for the levels of polyamines in the different biofluids. The aforementioned observations would enable the identification of patients at early stages of the disease through accessible and minimally invasive tests.

In the current study, ODC expression in the PBMCs of patients with PAC was increased by ≥2-fold compared with that of controls (67% Se; 77.8% Sp). This finding could represent the first important evidence in the search for biomarkers for diagnostic use, and it is pertinent to highlight that to the best of our knowledge this has not been reported before.

In the current study, the risk factors for PAC as well as the early signs and symptoms of the disease, diagnosis and treatment time, clinical stage at the time of diagnosis and treatments received were investigated in a Mexican cohort. These data could explain the poor prognosis of the disease and highlight the importance of identifying novel diagnostic biomarkers.

It has been established that the following combination of symptoms, including abdominal pain with poor response to symptomatic treatment for >4 months, weight loss and/or recently diagnosed DM with early evolution and out-of-context

metabolic syndrome, would be criteria for performing the evaluation of polyamines and ODC.

An important limitation of the present study was that the patients were enrolled from 2019 to 2020, and the total number of included cases was <30. It is noteworthy that although the current study is a pilot study and a larger cohort size is necessary for future studies, it contributed to innovative basic knowledge due to the investigation of ODC expression in the PBMCs of patients with PAC, as well as its diagnostic use, which to the best of our knowledge has not been investigated before in literature.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

LRRB and TDPR carried out clinical diagnosis, patient recruitment, consent letter application, treatments, and biofluid sample collection and transport. GHF acquired and analyzed data, wrote and reviewed the manuscript, and provided financial support for the western blotting experiments. PCOL developed the methodology used, wrote, reviewed and edited the manuscript, and provided financial support. ABC wrote and edited the manuscript, analyzed data and provided financial support. AMML determined polyamine levels in biofluids and carried out data analysis. KJPS determined polyamine levels in biofluids. LAPM carried out western blotting of ODC in patients and controls. AAL conceptualized the study, and wrote, reviewed and edited the manuscript. LFJS used software, carried out data analysis, and wrote, reviewed and edited the manuscript. MMVG conceptualized the study, developed methodology, used software, carried out data analysis, and wrote, reviewed and edited the manuscript. LRRB, AMLL, GHF and MMVG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present work was registered with the Mexican Social Security Institute (IMSS) National Scientific Research Committee (approval no. R-2019-785-101), and was approved by the Ethics Committee of Health Research of the IMSS, Mexico, United Mexican States. All participants were kindly invited to participate and all of them provided written informed consent. A total of 5/15 patients did not agree to donate their biological samples, but they did allow the use of their clinical and sociodemographic data. The present study was carried out in compliance with the guidelines of the Declaration of Helsinki.

Patient consent for publication

All participants (controls and patients) agreed to participate in the present study, and to donate blood, urine and saliva, and they provided written informed consent.

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Competing interests

The authors declare that they have no competing interests.

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