Indirect cholinergic activation slows down pancreatic cancer growth and tumor-associated inflammation

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Abstract

Background: Nerve-cancer interactions are increasingly recognized to be of paramount importance for the emergence and progression of pancreatic cancer (PCa). Here, we investigated the role of indirect cholinergic activation on PCa progression through inhibition of acetylcholinesterase (AChE) via clinically available AChE-inhibitors, i.e. physostigmine and pyridostigmine.

Methods: We applied immunohistochemistry, immunoblotting, MTT-viability, invasion, flow-cytometric-cell-cycle-assays, phospho-kinase arrays, multiplex ELISA and xenografted mice to assess the impact of AChE inhibition on PCa cell growth and invasiveness, and tumor-associated inflammation. Survival analyses were performed in a novel genetically-induced, surgically-resectable mouse model of PCa under adjuvant treatment with gemcitabine+/-physostigmine/pyridostigmine (n=30 mice). Human PCa specimens (n=39) were analyzed for the impact of cancer AChE expression on tumor stage and survival.

Results: We discovered a strong expression of AChE in cancer cells of human PCa specimens. Inhibition of this cancer-cell-intrinsic AChE via pyridostigmine and physostigmine, or administration of acetylcholine (ACh), diminished PCa cell viability and invasion in vitro and in vivo via suppression of pERK signaling, and reduced tumor-associated macrophage (TAM) infiltration and serum pro-inflammatory cytokine levels. In the novel genetically-induced, surgically-resectable PCa mouse model, adjuvant co-therapy with AChE blockers had no impact on survival. Accordingly, survival of resected PCa patients did not differ based on tumor AChE expression levels. Patients with higher-stage PCa also exhibited loss of the ACh-synthesizing enzyme, choline-acetyltransferase (ChAT), in their nerves.

Conclusion: For future clinical trials of PCa, direct cholinergic stimulation of the muscarinic signaling, rather than indirect activation via AChE blockade, may be a more effective strategy.

Background

The autonomous nervous system has been recently discovered to impact on cancer growth and progression in several solid and hematological cancers [1]. In pancreatic cancer (PCa), surgical denervation via vagotomy or pharmacological suppression of the cholinergic signaling were shown to exert a cancer-promoting effect [2,3]. In genetically induced LSL-Kras<sup>+/G12D</sup>;Pdx1-Cre (KC) mouse model of PCa, subdiaphragmatic vagotomy led to accelerated cancer growth, and treatment with direct muscarinic agonists restored normal KC phenotype [3]. Here, cholinergic signaling was shown to suppress tumorigenesis and cancer stemness via muscarinic type 1 receptor (M<sub>1</sub>R) signaling [3].

However, activation of muscarinic receptors by its main direct agonist, i.e. acetylcholine (ACh), is a process that is not confined to the autonomous nervous system. In fact, non-neuronal cholinergic signaling is highly common in most cell types and has been shown to regulate basic cell functions, such as proliferation, differentiation and apoptosis [4-6]. In this context, the role of non-neuronal acetylcholine as a local signaling molecule is often disregarded [7,8]. Together with its corresponding degrading and synthesizing enzymes (acetylcholine esterase/AChE and choline acetyltransferase/ChAT), it is expressed
in many eukaryotic cell types and even in plants and primitive uni- and multicellular organisms with no autonomous nervous system [8]. Depending on the muscarinic receptor subtype (M₁R – M₅R) to which ACh binds, muscarinic signaling can result in diverse cellular functions. The most potent and relevant ACh receptors that mediate cell proliferation and cell growth are muscarinic receptor type 1 (M₁R) and type 3 (M₃R), both widely expressed in most human tissues and especially in gastrointestinal tissues [5,9]. Local availability of ACh for autocrine and paracrine stimulation of muscarinic receptors regulates not only various physiological cell functions, but has also been shown to critically contribute to tumorigenesis [4]. For instance, in colon, breast and liver cancer, muscarinic receptor activation increased cancer cell proliferation and contributed to cancer progression [10-12]. This effect was mainly attributed to M₃R signaling [13]. Interestingly, in PCa, M₁R signaling resulted in reduced tumor growth [3]. This effect was mainly attributed to neuronal cholinergic input, e.g. from the vagus nerve [3]. However, in the PCa microenvironment, there are though several other non-neuronal sources of acetylcholine, such as cancer-associated fibroblasts and pancreatic stellate cells (PSCs), which are thought to influence pancreatic exocrine function via ACh secretion [14].

In this study, we demonstrate that PCa cell growth can also be decelerated by non-neuronal, indirect cholinergic signaling. This observation suggests that cancer cells, especially pancreatic cancer cells (PCCs), may be largely independent of the autonomous nervous system in their reaction to acetylcholine availability in the tumor microenvironment. Indeed, we demonstrate that human PCCs express high amounts of AChE and that inhibition of non-neuronal AChE suppressed PCC viability and invasion in vitro and in vivo. Notably, this effect was induced without surgical vagotomy, but only through non-neuronal, tumor cell intrinsic AChE inhibition. However, survival in a novel genetic, R0-resectable PCa mouse model was not influenced by AChE inhibition in the adjuvant setting. Accordingly the survival of resected PCa patients did not differ based on tumor AChE expression levels. These data imply that direct cholinergic stimulation, rather than indirect activation via AChE blockade, may be a more effective therapeutic strategy in PCa.

Methods

Cell culture

The human pancreatic cancer cell lines Panc-1 and SU86.86, the colon carcinoma cell lines SW620 and DLD-1, and the glioblastoma cell line LN229 were purchased from the American Type Culture Collection (ATCC). The T3M-4 cell line was a kind gift by Dr. R. Metzgar (Durham, NC, USA). Cells were kept and cultured in RPMI-1640 supplemented with 10% fetal calf serum (FCS), 100U/ml penicillin and 100µg/ml streptomycin (Gibco, Invitrogen, Karlsruhe, Germany) in a 5% CO₂ humidified atmosphere at 37°C.

Matrigel invasion assay

5,000 PCC (SU.86.86) were placed into each chemotaxis chamber insert of a 24-well plate (BD Falcon® 8 micron, Heidelberg, Germany) and incubated overnight. After 22h, the inserts were removed, cleaned of
non-migrating cells, fixed in 4% paraformaldehyde, and stained with Vybrant CFDA SE Cell Tracker Kit (Life Technologies, Darmstadt, Germany), and scanned via an automated digital epifluorescence microscope (Keyence BioRevo BZ-9000, Neu-Isenburg, Germany). The number of stained (migrated) cells was counted via ImageJ (version 1.44p, NIH, USA).

**Heterotypic xenograft model**

Athymic nude mice (NMRI-Foxn1\(^{nu/nu}\)) of 4-5 weeks of age and weighing 15-20g were kept under standard conditions in sterile cages and given food and water *ad libitum*. Mice were injected subcutaneously (s.c.) in their neck and dorsum with 4\(\times\)10\(^5\) cells of the PCa cell line T3M-4, and the animals were divided into two groups for the subsequent treatment: Group I was classified as the “prophylactic” group and treated starting with the day of tumor cell inoculation. Group II were not treated until the 1\(^{st}\) week after tumor inoculation to allow the tumor to reach a palpable size. Mice were treated with subcutaneous injections of the AChE inhibitors physostigmine or pyridostigmine as indirect parasympathomimetic agents. Physostigmine, which can cross the blood-brain-barrier (BBB), was administered at 0.1 \(\times\) LD\(_{50}\) and 0.3 \(\times\) LD\(_{50}\), and Pyridostigmine, which cannot cross the BBB, at 0.2 \(\times\) LD\(_{50}\) and 0.4 \(\times\) LD\(_{50}\). Solvent (saline) was injected to the control group. The animals received treatment 5 times a week for a period of 4 and 3 weeks for group I and II respectively. Animals were sacrificed by neck dislocation, and *tumor diameter (mm)* and *local invasive spread* (visible cell spread into neighbouring organs) of cancer cells were assessed.

**R0-resectable, electroporation induced transgenic mouse model of unilocal PCa**

Current oncogenic *Kras*-based mouse models of PCa develop multilocular tumors due to constitutive activation of the oncogene in the embryonic phase or due to its inducible activation in the adult age. This modality is not in harmony with human disease, which typically manifests as a single, i.e. unilocal, cancer in the pancreas. To address this discrepancy, Gürlevik et al. recently developed an R0-resectable, electroporation-induced genetic mouse model of unilocal PCa, which is induced upon injection and electroporation of plasmids containing the *Sleeping Beauty* (SB) transposase SB13, a *Kras*-G12V encoding transposon, and the Cre recombinase into the pancreatic tail of p53floxed mice (*p53*\(^{fl/fl}\)) [15]. Details on the plasmid constructs and the electroporation parameters have been described in the original publication [15]. Upon activation of the Cre recombinase, tumor formation was initiated in a local fashion (the “Pf” model), and three weeks after electroporation, the mice developed a unilocal tumor of the pancreatic tail that is amenable to surgical resection (Figure 5a-b). The model allows real-life-like performance of neoadjuvant and adjuvant therapy trials with these mice, which exhibit a strongly similar phenotype to the classical, oncogenic Kras-based mouse models of PCa such as *Ptf1a-Cre;LSL-Kras*\(^{G12D}\) (KC) and *Ptf1a-Cre;LSL-Kras*\(^{G12D,p53^{R172H}}\) (KPC) [16,17]. Using this model, we applied adjuvant chemotherapy combining gemcitabine with either physostigmine (at 0.2\(\times\)LD\(_{50}\), i.e. 160\(\mu\)g/kg, [http://datasheets.scbt.com/sc-252784.pdf](http://datasheets.scbt.com/sc-252784.pdf)), or with pyridostigmine (0.2\(\times\)LD\(_{50}\), i.e. 520\(\mu\)g/kg, [http://www.vetpharm.uzh.ch/reloader.htm?wir/00000015/5975_08.htm?wir/00000015/5975_00.htm](http://www.vetpharm.uzh.ch/reloader.htm?wir/00000015/5975_08.htm?wir/00000015/5975_00.htm)).
applied s.c. three times a week. Gemcitabine was administered as 6 repeats of weekly gemcitabine (100 mg/kg bodyweight diluted in physiological NaCl) intraperitoneally (i.p.), as shown previously [15].

**Multiplex enzyme-linked immunosorbent analysis (ELISA)**

Protein levels of IL-6, IL-10 and TNFalpha were measured in mouse serum via the Luminex® MAGPIX® multiplex ELISA system (Merck Millipore, Darmstadt, Germany) according to the instructions of the manufacturer.

**MTT viability assay**

To assess human PCa cell line growth, the MTT (3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide) assay was used. Cells were seeded at a density of 2,000 cells/well in a 96-well plate in serum-free RPMI-1640 medium. Treatment of cells with acetylcholine (Sigma-Aldrich, Taufkirchen, Germany), physostigmine, pyridostigmine or carbachol (all three provided by the internal pharmacy of the Klinikum rechts der Isar) began 12h after seeding at the concentration of 10, 20, 50, 100 or 300ng per well (in 100 µl) for physostigmine and pyridostigmine, at 100, 500 and 1000µM for acetylcholine and at 10, 25 and 50 mM for carbachol. The viability was measured at 0h, 24h, 48h and 72h after adding the MTT to each well (50µg/well) and allowed to incubate for 4 hours. The formazan products were solubilized with 100µl of propan-2-ol and the optical density was measured using a photometer at 570nm.

**Cell cycle analysis**

Upon reaching 90% of confluence, T3M-4 cells were treated with ACh at a concentration of 1000 mM, physostigmine and pyridostigmine at 30ng/µl each and the combined agents at 30 + 30ng/ µl. The PCC were then harvested, centrifuged at 200xg for 5 minutes and washed 2 times using phosphate-buffered saline (PBS). They were then resuspended in 1 ml of ice-cold PBS and added dropwise afterwards to ice-cold absolute ethanol for cell fixation. Cells were fixated for 24h at 4°C, recentrifuged and resuspended in 500µl Triton X-100 (Sigma) in PBS with added 100µg of DNAse-free RNase A (Sigma) propidium iodide (PI) at a concentration of 20µg/ml . Cells were then incubated for 15 minutes at 37°C and pipetted afterwards into 96-well plates protected from light for data acquisition. Forward and side scatter was measured using a Guava® easyCyte HT Sampling Flow Cytometer.

**Immunoblot analysis**

At 90% confluence, PCa cell lines were lysed and 30µg of protein was separated, electroblotted and the membrane was exposed to monoclonal and polyclonal antibodies (Table 1) at 4°C overnight. The equal loading of AChE-Blots was assured by re-probing with alpha-Tubulin antibody. The densitometric analysis of the Western Blot was performed via ImageJ (version 1.44p, NIH, USA).

**Phospho-Kinase profiling**
The Proteome Profiler Human Phospho-Kinase Array Kit (R&D Systems, Minneapolis, MN, USA) was used to obtain a semiquantitative comparison of the phosphorylation of 43 different human kinases in T3M4 cells that were either treated with 30 ng/µl physostigmine for 5 minutes or untreated, according to the instructions of the manufacturer.

**Patients and human tissue**

Tissue samples from patients undergoing pylorus-preserving Whipple's procedure for PCa of the pancreatic head were collected and processed as described before [18]. A total of 39 patients were included for the survival analyses (for patient characteristics, please see Table 2). A total of 20 patients were used for the correlation analysis between ChAT content in nerves and Union for International Cancer Control (UICC) stage (median age: 61, 11 male, 9 female, UICC stage distribution: IIA: 4 patients, IIB: 15 patients, III: 1 patient). All patients were informed, and written consent was obtained for tissue collection.

**Immunohistochemistry, immunofluorescence, semiquantitative analysis**

Consecutive 3µm sections from formalin-fixed human tissues were incubated with the corresponding antibodies (Table 1) overnight in a humid chamber at 4ºC. AChE IHC was detected with the Avidin Biotin Complex Peroxidase Standard Staining Kit (Thermo-Fischer, Waltham, USA). Histopathological analysis was performed by two independent observers (PLP, MJ) followed by resolution of any differences by joint review and consultation with a third observer (IED). Scores of 0-3 were given in 0.5 steps according to the amount of immunoreactivity in each tissue samples. For immunofluorescence staining, Alexa® Fluor 488 and 594 antibodies (Invitrogen, Germany, 1:250 concentration) in combination with 4’,6-diamidino-2-phenylindol (DAPI) nuclear stain were utilized.

**AChE activity assay**

For comparison of the AChE activity between the human PCa cell lines SU86.86 and T3M4, a colorimetric AChE activity assay kit (Sigma-Aldrich, Taufkirchen, Germany), which is based on the Ellman method, was applied according to the instructions of the manufacturer.

**Ethics approval**

All animal studies were conducted according to the national regulations and approved by the Regierung von Oberbayern (approval nr. ROB-55.2-2532.Vet_02-16-165). The study has been approved by the ethics committee of the Technische Universität München, Munich (approval nr. 154/20).

**Statistical analysis**

Results are expressed as mean ± SD. Two-group analyses were performed using the unpaired t-test for continuous values and with the Mann–Whitney U test for scores and indices. Linear regression was used for correlating tissue expression of AChE or ChAT with the UICC stages of PCa. Survival analyses were performed with the log-rank test and depicted as Kaplan-Meier curves. All tests were two-sided, and a p
value < 0.05 was considered to indicate statistical significance. All authors had access to the study data and had reviewed and approved the final manuscript

Results

Pancreatic cancer cells express high amounts of AChE

The role of ACh as a neurotransmitter in the parasympathetic nervous system (PSNS) is well-known, but its non-neuronal function as a local signaling molecule that influences basic cell functions such as apoptosis and proliferation, is often underestimated [9]. The cholinergic system comprising ACh and its synthesizing enzyme, choline acetyltransferase (ChAT), as well as its degrading enzyme, acetylcholinesterase (AChE), have been detected in several cancer entities, such as colon, liver and lung cancer [13,11,19]. However, the amount of AChE expression in PCa has not been genuinely analyzed before. By employing immunostaining against AChE, we demonstrated mild to weak staining in normal acinar cells, pancreatic islets and intrapancreatic nerves (Figures 1a-c). Notably, immunostaining increased in precursor lesions of PCa, i.e. pancreatic intraepithelial neoplasia (PanIN) lesions (Figures 1d-f). The strongest staining was found in areas of invasive ductal adenocarcinoma with prominent staining localized in the peri- and sub-membranous cell compartment of cancer cells (Figures 1g-i). Combined staining of AChE and the PCa cell marker Cytokeratin 19 (CK19) using double-immunofluorescence (IF) staining confirmed strong co-localization of CK19 with AChE (Figures 1g-l), underlining the specificity of AChE expression in cancer cells.

In order to further quantify the expression of AChE in PCa cells (PCC), we isolated dorsal root ganglia (DRG) from postnatal C57BL/6J mice. DRG neurons incorporate afferent neuronal signals of different qualities and express AChE [20]. Here, immunoblot analysis of commonly used human PCC lines, T3M-4, SU.86.86 and Panc-1, as well as colon carcinoma cell lines SW620 and DLD-1, and the glioblastoma cell line LN229, revealed high levels of AChE expression when compared to DRG of C57BL/6J mice (Figures 1m-n). Collectively, these results demonstrate that PCCs express high amounts of AChE in a specific manner.

AChE inhibition suppresses PCC growth in vitro

Next, we sought to explore the effect of AChE inhibition on cancer cell growth. Administration of physostigmine or pyridostigmine, two commonly used AChE inhibitor, led to significant inhibition of viability in T3M-4, but not in SU86.86 cells (T3M-4-physostigmine: 189±99% with 0.1ng/µl vs. 217±110% with solvent with 0.1ng/µl; T3M-4-pyridostigmine: 191±90% with 3ng/µl vs. 220±117% with solvent, Figure 2a-f). Similarly, following administration of acetylcholine (ACh), or of carbachol, a direct muscarinic receptor agonist, a dose-dependent growth reduction was evident for both cell lines (ACh-T3M-4: 242±165% with 1,000µM vs. 384±374% with solvent; ACh-SU86.86: 212±116% ng/µl with 1,000µM vs. 297±220% with solvent; Carbachol-T3M-4: 348±285% with 100µM vs. 511±391% with solvent, Carbachol-SU86.86: 260±145% with 100µM vs. 421±270% with solvent, Figures 2c&f-h). To exclude a difference in the basal AChE activity of the cell lines SU86.86 and T3M4, we performed a
colorimetric AChE activity assay based on the Ellman method, which revealed no difference in the basal AChE activity of the two cell lines (Figure 2i). These results confirmed that non-neuronal cholinergic signaling decelerates PCC growth in vitro, which was achieved through increasing ACh availability, through AChE inhibition, or through direct muscarinic stimulation.

**Cholinergic activation inhibits PCC invasion in vitro and in vivo**

In order to test the effect of AChE inhibition in PCCs on their invasive potential, we performed Matrigel-based invasion assays with SU86.86 cells (Figure 2j-l). Also here, in a dose dependent manner, physostigmine inhibited invasion when applied at the concentration of 30ng/µL (70.4±21.6% of control, Figure 2i). Pyridostigmine inhibited PCC invasion at intermediate and high concentrations of 10ng/µL (71.4±23.4% of control) and 30ng/µL (45.5±13.0% of control), respectively (Figure 2k), and ACh limited invasion significantly at the higher concentration of 1000µM (43.2±26.6% of control, Figure 2l). Collectively, these findings indicated that both AChE inhibition and direct cholinergic activation through ACh inhibit PCa cell invasion in vitro.

In order to test whether indirect cholinergic activation also reduces pancreatic cancer growth in vivo in animals with intact vagal innervation, we utilized a xenograft mouse model, which was generated by injecting PCC into Crl:NMRI-Foxn1 nu/nu nude mice. The effect of AChE inhibition was investigated in a prophylactic and a therapeutic treatment group (Figure 3a). Both groups received daily subcutaneous injections of low- or high-dose physostigmine or pyridostigmine. The prophylactic treatment arm started simultaneously with tumor induction, whereas therapeutic treatment started 1 week after tumor induction. After a 4-week injection period, tumor size was assessed. A significant decrease in the tumor size was observed in animals that received physostigmine or pyridostigmine prophylactically (p) in a dose-dependent manner (Control /saline=22.0±5.7mm vs. p-physo-high=14.5±1.3mm vs. p-pyrido-high = 11.8±2.5mm, Figure 3b). However, therapeutic administration of these indirect parasympathomimetics to established xenograft tumors did not influence tumor size over the course of the treatment (Figure 3c). In order to evaluate the invasive potential of PCC in vivo, we assessed the proportion of xenografted mice that showed penetrating tumor growth into neighboring organs, i.e. kidneys and lungs, following therapeutic or prophylactic treatment with AChE-blockers. In animals in which AChE was blocked prophylactically, only 18% of the specimens showed penetrating tumor growth, whereas 80% of the control group showed tumor infiltration into neighboring organs (Figure 3d, Supplementary figure 1). These findings suggested a partially tumor-suppressing effect of non-neuronal cholinergic activation in vivo, yet only in the context of developing tumors.

**Indirect parasympathomimetic agents suppresses immune cell infiltration by tumor-associated macrophages (TAM) and reduce serum cytokine levels in xenografted PCa mice**

Non-neuronal cholinergic signaling is also involved in the regulation of the immune system as most immune cells express ACh, AChE, and muscarinic receptors [21]. In this context, we aimed to analyze if indirect cholinergic activation not only has a direct cancer-suppressive effect, but also modulates the
immune response in the tumor microenvironment. Therefore, we quantified tumor-associated macrophage (TAM) amounts in pancreatic tumors of the xenograft mouse model. Tumor-associated macrophages are a subpopulation of cytokine-secreting monocytes and have been implicated in playing an important role in the tumor microenvironment (TME). Upon activation, TAM differentiate into M1 or M2 polarized macrophages and release abundant cytokines [22]. Here, we performed double-IF for the murine macrophage marker f4/80 and CD45 (Figure 3e). Our analysis demonstrated a reduction of CD45+/f4/80+-TAM infiltration in murine tumors of physostigmine or pyridostigmine treatment groups (Saline-prophl.: 6.6±0.8 cells/m², Physostigmine-high: 2.7±0.4 cells/m², Pyridostigmine-high: 2.9±0.7 cells/m², Figure 3e). As cholinergic activation is known to exert a systemic anti-inflammatory effect (“the cholinergic anti-inflammatory pathway”), we then assessed the serum levels of the cytokines interleukin 6 (IL6), interleukin 10 (IL10) and tumor necrosis factor-alpha (TNFalpha) in the xenografted mice (Figure 3f). Here, we detected a massive suppression of the levels of all these cytokines in all treated groups, regardless of the dosage of treatment, when compared to saline-treated controls (IL6 control: 582.8±428.0 pg/ml, IL6 treated: 25.4±21.5.0 pg/ml; IL10 control: 488.2±371.6 pg/ml, IL10 treated: 19.4±26.1 pg/ml; TNFalpha control: 540.2±398.4 pg/ml, TNFalpha treated: 10.4±29.3 pg/ml, Figure 3F). Collectively, these data suggested a prominent suppression of tumor-associated local and systemic inflammation markers in the xenografted PCa mice upon physostigmine or pyridostigmine treatment.

**Cholinergic activation leads to intracellular p-ERK1/2 and p-p38 MAPK inhibition and induces cell cycle arrest**

In order to determine molecular mechanisms responsible for growth and invasion inhibition in PCC upon AChE inhibition, we performed a phospho-kinase antibody array for screening that enables the profiling of 43 different human kinases in two experimental arms (Figure 4a-b). Here, we compared intensity of phosphorylation of these multiple kinases in T3M4 cells treated with either physostigmine or left untreated, and used the clues from this initial screen for subsequent validation analyses. Among well described mitogen-activated protein kinases (MAPK) that are known to be widely expressed in PCa and involved in cell proliferation, invasion, cell-survival and cell cycling, we found extracellularly regulated kinase 1 and 2 (ERK1/2), p38, proto-oncogene tyrosine-kinase Src (Src) and 5’-AMP-activated protein kinase α (AMPKα) to be altered under the treatment (Figure 4a-c) [23]. In validation immunoblots with T3M-4 cells, we confirmed the decrease in phosphorylated ERK1/2 (pERK) levels, particularly after treatment with high-dose pyridostigmine (61.5±13.9% of control). This effect was more pronounced for SU86.86 cells, which, after treatment with physostigmine or pyridostigmine, exhibited even more obviously diminished pERK1/2 levels in a dose-dependent manner at both mid-level and high concentrations (physostigmine-mid: 80.5±2.6% of control, physostigmine-high: 69.2±7.9% of control, pyridostigmine-mid: 70.2±8.2% of control, pyridostigmine-high: 60.3±11.8% of control, Figure 4e). As an essential component of the MAPK signal transduction pathway, p38 reacts to extracellular stimuli and mediates cellular responses [24,25]. In our experiments, phosphorylation of p38 was abolished upon administration of low (43.7±8.2% of control), mid- (53.4±17.3% of control) and high (69.3±10.9% of control) physostigmine concentrations, but not via pyridostigmine (Figure 4f). However, following
treatment with either of these drugs, there was no significant change in the amount of intracellular p-Src nor p-AMPKα (Figure 4g-h). In summary, our experiments demonstrated that inhibition AChE reduced ERK phosphorylation.

To investigate whether cell cycle progression of PCC is also affected by AChE inhibition, we performed a propidium iodide-(PI-) based, flow cytometric cell cycle analysis (Figure 4i-j). After administering ACh, significantly more PCCs were observed in the G1/0 phase (ACh: 56.1±3.2% vs. Control: 47.8±0.9%) and fewer cells in the S-phase (ACh: 10.0±0.1% vs. Control: 13.0±0.8%, Figure 4i-j). Physostigmine did not alter G1/0-phase amount, but reduced S-Phase cell-count (8.4±1.7%), and pyridostigmine enhanced G1/0-phase count (55.3±1.5%), but did not alter the S-Phase cell count (Figure 4i-j). No significant difference in cell count was noted for cells in G2/M-Phases, however following all treatments a trend towards lower cell counts was observed. Overall, we thus detected a cell cycle arrest in G1/0 phase following AChE inhibition.

Adjuvant indirect cholinergic treatment does not impact survival in a resectable PCa mouse model

In order to translate our findings into a clinically relevant setting, we used a novel R0-resectable, genetically induced PCa mouse model [15]. In this model, plasmids containing the Sleeping Beauty (SB) transposase SB13, a Kras-G12V encoding transposon, and the Cre recombinase were injected and electroporated into the pancreatic tail of p53oxed mice (p53/−) via mini-laparotomy [15]. Upon activation of the Cre recombinase, tumor formation was initiated in a local fashion (the Pf model), which is in contrast with the multilocular tumor growth of classical genetically induced mouse models of PCa (Figure 5a-b). Three weeks after the tumor induction, the animals developed macroscopically visible tumors. After pancreatic tail resection, mice received adjuvant chemotherapy with gemcitabine. Here, mice of the Pf-genotype exhibited a median survival of 41 days (Figure 5c). When adjuvant gemcitabine treatment was combined with physostigmine, median survival was 32 days. Combinational therapy with gemcitabine and pyridostigmine was associated with a median survival to 39 days (Figure 5c-d). The most common reason for death was combined local and distant (hepatic or peritoneal) recurrence. Thus, the AChE inhibitors did not generate any additive survival benefit in this innovative, adjuvant therapy setting.

Correlation of tissue AChE and ChAT expression with clinicopathological variables in human PCa

Lastly, to compare our findings from this translational mouse model to human PCa, we analyzed the expression patterns of AChE and the ACh synthesizing enzyme ChAT in human PCa tissues (n=39) by semiquantitive immunostaining scores and correlated to clinicopathological variables of the corresponding patients. Furthermore, high vs. low scores of AChE immunostaining (separated by the median score) did not result in any difference in the overall survival rate of the PCa patients (Figure 5e). Accordingly, tissue expression scores of AChE did not associate with different UICC tumor stages (Figure 5f). Interestingly, higher tumor grades, i.e. a poor tumor differentiation, were associated with significantly lower AChE IHC scores (G1: 1.5±0.2, G2: 1.0±0.2, G3: 1.0±0.4, Figure 5g), suggesting a spontaneous loss
of AChE in increasingly aggressive PCa. This was surprising, as we originally hypothesized that high AChE expression, i.e. diminished cholinergic input, would be associated with worse survival and poor differentiation. As this was not the case, we quantified the expression level of choline acetyltransferase enzyme (ChAT), which catalyzes the formation of acetylcholine, in the nerves of these tissues via immunohistochemical scoring. Correlation of ChAT expression levels to tumor stage revealed that high expression levels ChAT were indeed associated with low tumor stages ($r^2$: 0.20, p=0.049, Figure 5h). Hence, we concluded that advanced tumor stages were characterized by low cholinergic input due to low ChAT expression, and yet also by suppression of the degrading enzyme AChE. This simultaneous suppression of the ACh-synthesizing and ACh-degrading intrinsic mechanisms in PCa may explain the lack of a prognostic effect of tumor AChE levels in established mouse and human PCa.

**Discussion**

The present study suggested an anti-proliferative and anti-invasive effect of non-neuronal cholinergic signaling in pancreatic cancer. Inhibition of endogenous, non-neuronal AChE decelerated PCC growth and invasiveness *in vitro* & *in vivo*, which was linked to intracellularly reduced MAPK phosphorylation and reduced downstream phosphorylation of ERK1/2 and p38. Furthermore, prophylactic cholinergic activation in PCa mouse models with intact vagal innervation reduced both tumor invasiveness *in vivo* and immune cell infiltration by tumor-associated macrophages. However, administration of parasympathomimetic agents as co-adjuvant therapy together with gemcitabine did not influence the overall survival of mice in a resectable, transgenic mouse model of unilocular genetic PCa. Accordingly, AChE did not correlate to survival in human PCa and was actually suppressed in parallel with ChAT in higher grade tumors. Therefore, our study suggests that for targeting PCa, *direct* cholinergic stimulation of the muscarinic signaling, rather than *indirect* activation via AChE blockade, may be a more effective therapeutic strategy.

Various studies have previously reported a cancer-promoting effect of the vagus nerve. In a mouse model of gastric cancer, surgical vagotomy decreased gastric mucosal thickness and cellular proliferation [26,27]. The effect was thought to be mediated via muscarinic receptor type 3 (M3R) signaling, since knock-out of the M3R suppressed gastric cancer. These studies led to the conclusion that vagal innervation promotes gastric cancer via muscarinic M3 receptor in a Wnt mediated pathway [28]. However, in PCa, Renz et al. demonstrated that ablation of the vagal nerve actually accelerated cancer progression [3]. Treatment with the muscarinic receptor agonist, bethanechol was able to reverse the accelerated cancer progression due to vagal ablation. Overall, this study along with others led to the general hypothesis that vagal innervation has a cancer-attenuating effect in the pancreas. In a wide-scale analysis of neural fiber quality in PCa specimens, we previously found a low parasympathetic fiber content of nerves that were invaded by pancreatic cancer cells [29]. In line with these previous studies, in the current study, we were able to demonstrate a cancer-cell-suppressive effect of AChE inhibition and thus *indirect* cholinergic activation *in vitro* and *in vivo*. However, this effect was obtained without directly interfering with the autonomous nervous system, and yet did also not translate into an improved clinical
outcome, i.e. survival, in mouse PCa. These findings are of major importance for all studies related the role of cholinergic / parasympathetic nervous system in cancer, since all components of the cholinergic system (ACh, acetylcholinesterase, muscarinic acetylcholine receptors, acetylcholine transferase) are not exclusively expressed by neurons but ubiquitously present in almost all mammalian cells, including non-neuronal cells [8].

In order to understand non-neuronal cholinergic signaling and its involvement in basic cellular functions, such as proliferation and differentiation [6], one has to consider the different subtypes of muscarinic receptors that initiate these diverse cellular outcomes. There are 5 different muscarinic receptor subtypes (M1R – M5R), all of which are G protein-coupled receptors but may lead to different intracellular cascades in order to exert different extracellular outcomes. Upon activation, odd-numbered muscarinic receptors couple to G proteins that activate phospholipase C-β to initiate the phosphatidylinositol trisphosphate cascade, whereas even numbered muscarinic receptors couple to G proteins that inhibit adenylyl cyclase activity [9]. This complexity explains in part why, for instance, activation of the M3R subtype has been shown to promote cancer cell proliferation in gastric cancer, whereas activation of M1R subtype has been shown to attenuated pancreatic cancer proliferation. Although the role of muscarinic receptors in colon cancer has been previously characterized, most studies only focused on one of the 5 different receptors [9,13]. Even though there has been extensive research about the tissue-specific expression of muscarinic receptors, a comprehensive overview about the role of muscarinic receptors and its ligands in different cancer entities is still missing.

The lack of basic research on non-neuronal cholinergic signaling is even more evident for PCa. Very little is known about the role of the different muscarinic receptor subtypes as well as other components of the cholinergic-signaling-machinery, such as ChAT, AChE or ACh expression in PCa.

Therefore, our study contributes to the attempts to understand the non-neuronal AChE in PCa. Here, we demonstrated mild to weak staining in premalignant lesions, with increasing staining in overt pancreatic cancer. Mammals express 3 different classes of AChE, which differ with regard to their subunits. Each type of AChE has a different 3’ RNA sequence with a corresponding C-terminal sequence, which encodes the respective subunit. The AChE_H subunit contains a hydrophobic C-terminal sequence forming amphilic monomers and dimers and incorporates a GPI [6]. It is therefore often found closely spaced to the cell membrane. This would explain the perimembranous staining found in our study.

Based on our findings, indirect activation of cholinergic signaling via AChE inhibition is not sufficient to achieve a survival benefit in PCa, although it resulted in a prominent suppression of the tumor-associated inflammation in the tumor, and a drop of serum cytokine levels. This conclusion, which is based on our findings from a translational mouse PCa model and from human PCa, underlines that increasing the cholinergic input for attenuating PCa progression and for improving patient survival will probably not be possible via administration of two widely used clinical drugs, i.e. phystostigmine and pyridostigmine. In contrast, Renz et al. made use of a direct activator of muscarinic cholinergic signaling. i.e. the bethanechol, which did result in improved survival in the KPC model of PCa [3]. In the present study, we
combined the indirect cholinergics with an older chemotherapeutic, i.e. gemcitabine, in the adjuvant and palliative treatment. It is imaginable that a combination with a more current regimen such as gemcitabine and nab-paclitaxel or with FOLFIRINOX may yield even more potent results. Nonetheless, we observed immunosuppressive effects of indirect cholinergic stimulation in our study. It is conceivable that in a more humanized model, this immunomodulatory effect of indirect cholinergic stimulation may have been much greater. The NMRI-Foxn1nu/nu model is deficient with regard to T cell function due to a thymus abnormality. For this reason, we primarily assessed macrophage distribution upon treatment with indirect cholinergic agents. As a recent report showed, cholinergic activity of the vagus nerve inhibits macrophage-derived tumor necrosis factor-α secretion via T-cell derived acetylcholine in the spleen [30]. Our data suggest that there may also be a T-cell-independent, immunosuppressive effect of cholinergic activation on macrophages.

Conclusion

The present study suggests that the growth-suppressive effects of inhibition of the non-neuronal, cancer-cell-intrinsic AChE via indirect parasympahomimetic drugs do not translate into improved survival in PCa. Therefore, targeting PCa over its nervous-cholinergic side should pursue the track of direct, rather than indirect, parasympathetic-cholinergic activation. Future clinical study designs should thus include novel, selective direct muscarinic agonists, rather than clinically available, widely used indirect agonists.

Abbreviations

Acetylcholine / ACh
Acetylcholine esterase / AChE
Choline acetyltransferase / ChAT
Extracellular signal-regulated kinase / ERK
Mitogen-activated protein kinase / MAPK
Muscarinic receptor type / M1R
Muscarinic receptor type / M3R
Pancreatic cancer / PCa
Pancreatic cancer cells / PCC

Declarations
Ethics approval and consent to participate: All animal studies were conducted according to the national regulations and approved by the Regierung von Oberbayern (approval nr. ROB-55.2-2532.Vet_02-16-165). The study has been approved by the ethics committee of the Technische Universität München, Munich (approval nr. 154/20). All patients were informed, and written consent was obtained for tissue collection.

Consent for publication was obtained from all authors.

Availability of data and material: All data are available from the Authors upon reasonable request.

Competing interests: none

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Authors' contributions: IED and GOC designed the study. PLP, KW, ED, EG, BFM, JB, LR, JGD, MJ, and ST performed the experiments. PLP, ST, CJ and IED analyzed the data. AH, RI and FK contributed substantial intellectual input. HF supervised the study. PLP, LF and IED wrote the first draft of the manuscript. All authors have agreed on the final version of the manuscript.

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**Tables**

**Table 1. Primary antibodies.** IF: immunofluorescence, WB: Western blot, IHC: immunohistochemistry

| Antibody                                | Clone   | Species | Company                                  | Concentration          |
|-----------------------------------------|---------|---------|------------------------------------------|------------------------|
| p44/42 MAPK (Erk1/2)                    | 137F5   | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) | D13.14.4E XP® | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Src                                     | 36D10   | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Phospho-Src (Ser17)                     | D7F2Q   | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| AMPKα                                   | D5A2    | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Phospho-AMPKα (Thr172)                  | 40H9    | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| p38α MAPK                               | 7D6     | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Phospho-p38 MAPK (Thr180/Tyr182)        | D3F9 XP® | Rabbit  | Cell Signaling, Leiden, The Netherlands  | 1:1000                 |
| Anti-Acetylcholine-esterase (AChE)      | pAb     | Rabbit  | Prestige Antibodies®, Sigma-Aldrich, Taufkirchen, Germany | 1:200 (IF), 1:1000 (WB), 1:400 (IHC) |
| Anti-alpha-Tubulin                      | Ab11034 | Rabbit  | Abcam, Cambridge, UK                     | 1:10.000               |
| F4/80                                   | Ab6640  | Rat     | Abcam, Cambridge, UK                     | 1:75                   |
| CD45                                    | Ab10558 | Rabbit  | Abcam, Cambridge, UK                     | 1:100                  |
| ChAT                                    | Polyclonal | Rabbit  | Kindly provided by Prof. M. Schemann, TU Munich | 1:1000                 |
Table 2. Patient characteristics. UICC: Union for International Cancer Control

|        | N   |
|--------|-----|
|        | 39  |

**Sex**

|       |     |
|-------|-----|
| Male  | 22 (56.4%) |
| Female| 17 (43.6%) |

**Age** (median; min-max)

| Age (median; min-max) | 67.4 (31-83) |

**UICC**

| UICC   |     |
|--------|-----|
| UICC Ia| 3 (7.7%) |
| UICC Ib| 8 (20.5%) |
| UICC IIa | 9 (23.1%) |
| UICC IIb | 7 (17.9%) |
| UICC III | 10 (25.7%) |
| UICC IV  | 2 (5.1%) |

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