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Distribution of TRPC3 and TRPC6 in the human exocrine and endocrine pancreas

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ABSTRACT

Background: Expression and function of TRPC3 and TRPC6 in the pancreas is a controversial topic. Investigation in human tissue is seldom. We aimed to provide here a detailed description of the distribution of TRPC3 and TRPC6 in the human exocrine and endocrine pancreas.
Methods: We collected healthy samples from cadavers ($n = 4$) and visceral surgery ($n = 4$) to investigate the respective expression profiles using immunohistochemical tracing with knockout-validated antibodies.
Results: TRPC3- and TRPC6-proteins were detected in different pancreatic structures including acinar cells, as well as epithelial ductal cells from intercalate, intralobular, and interlobular ducts. Respective connective tissue layers appeared unstained. Endocrine islets of Langerhans were clearly and homogenously immunolabeled by the anti-TRPC3 and anti-TRPC6 antibodies. Insular α, β, γ, and δ cells were conclusively stained, although no secure differentiation of cell types was performed.
Conclusions: Due to aforementioned antibody specificity verification, protein expression in the immunolabeled localizations can be accepted. Our study in human tissue supports previous investigations especially with respect to acinar and insular α and β cells, while other localizations are here reported for the first time to express TRPC3 and TRPC6, ultimately warranting further research.

1. Introduction

The pancreas is an exocrine and endocrine gland of the gastrointestinal tract that is critically involved in digestion and blood sugar homeostasis [30]. The exocrine system relies on the acinar backbone that comprises approximatively 80 % of the total pancreatic volume [4]. The corresponding serous acinar cells produce the pancreatic juice – an enzymatic cocktail containing representatives of the α-amylase, lipase, and protease (e.g., trypsinogen and chymotrypsinogen) families [30]. Human adult specimens physiologically produce one to two liters of pancreatic juice daily [30]. Importantly, the ductal cells modify the pancreatic juice by abundant secretion of sodium bicarbonate (~140 mmol HCO_3^-/liter) [28] to buffer the strong acidity of the imported gastric juice [30]. In contrary, the endocrine pancreas is widespread in form of so-called islets of Langerhans throughout the exocrine background. Each islet of Langerhans is built up of several different endocrine cell types including α, β, γ, and δ cells, that respectively secrete glucagon – a catabolic blood sugar-increasing hormone, insulin – its anabolic blood sugar-reducing pendant, pancreatic polypeptide, and somatostatin (Fig. 1) [30]. Meticulously regulated intracellular calcium concentration oscillations have been suggested to be mandatory for both exocrine and endocrine function with respect to acinar and β cells [21, 36]. Store-operated channels (SOC), that are activated by increased cytosolic calcium levels subsequent to inositol 1,4,5-triphosphate (IP_3)-triggered endoplasmic reticulum calcium release, prolong the signaling response of initially receptor-stimulated calcium increase thus enabling various cellular functions including sustained exocytosis [21, 35]. In this context, Kim *et al.* found out that TRPC3 is critically involved as SOC in calcium entry responsible for secretory function in acinar cells [21]. TRPC3 belongs to a subset of tetrameric non-selective cation channels referred to as transient receptor potential canonical (TRPC) channels – a subfamily of the transient receptor potential (TRP) family that accounts next to the canonical variation, also so-called melastatin, vanilloid, polycystin, mucolipin, no mechanoreceptor potential C, and

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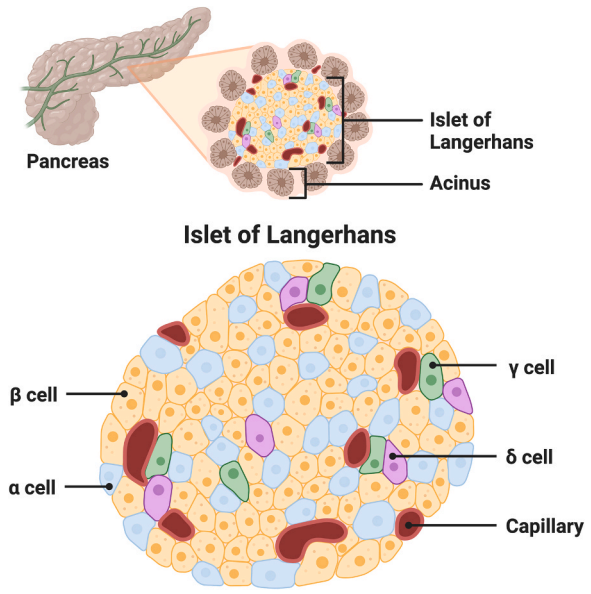

Fig. 1. The endocrine pancreas or the islet of Langerhans. About 80 % of insular cells are insulin-producing β cells. They are rather gathered at the islet's centre, while glucagon-secreting α cells are rather described to accumulate at the islets border. γ (pancreatic polypeptide) and δ (somatostatin) cells are also displayed. Created with BioRender.com (Agreement number: BF25ZPS83F; Toronto, Canada).

ankyrin subfamilies [34]. TRPC channels are expressed in different human tissues where they are suggested to be involved in a wide range of physiological functions (e.g., hearing [12], renal tubular calcium reabsorption [10]) and pathophysiological conditions (e.g., oncogenesis [10], cardiac hypertrophy [9]). As a matter of fact, their suggested involvement also extends to endocrine organs other than the pancreas such as the thyroid [3] or the parathyroid [23] gland for instance. Due to extended sequence homologies [34], TRPC6 – an interesting close subfamily member of TRPC3, has been reported to heteropolymerise with TRPC3 to ultimately increase the functional diversity of TRPC channels [24,48]. Especially TRPC3 has recently been suggested to be involved in insulin secretion in pancreatic β cells [36]. However, contradictory molecular investigations didn't support TRPC3-expression in β cells [32, 37], ultimately foregrounding the need to further investigate TRPC3 in the endocrine pancreas, as indicated by Rached *et al.* [36]. Another relevant aspect represents the aforementioned human exocrine pancreas, which has not been entirely submitted to TRPC channel-focused research yet, although a few studies investigating its serous acini appeared in the past.

To this end, and essentially inspired by Rached *et al.* [36] we investigated here in detail the expression of the non-selective cation channels TRPC3 and TRPC6 in rarely available healthy human pancreatic tissue. Finally, we support TRPC3- and TRPC6-protein expression in previously suggested localizations such as α, β, and acinar cells. Additionally, we provide clear evidence for the expression of these channels in different ductal epithelia, and also suggest their localization in γ and δ cells.

2. Materials and methods

2.1. Samples

Pancreas tissue was either obtained from human adults that decided to donate their bodies to education and science after death ($n = 4$) or from adult patients that had undergone visceral surgery ($n = 4$). All investigations were conducted in an anonymous manner, approved by the Ethics Committee of the Saarland Medical Association (163/20, 130/21 respectively), and performed in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from all involved patients. Body donors had been fixed within 72 hours post-mortem by injection of nitrite pickling salt-ethanol polyethylene glycol fixation (NEP) through the femoral artery [26]. The corresponding pancreatic samples had been removed by punch biopsy during dissection courses at the anatomical institute. Half of the body donors were female, half male. The mean age was 79 with a standard deviation of 3.5 years. The samples obtained by surgery had been fixed within the surgical schedule using 4 % buffered formalin. These pancreatic samples had been extracted in the context of three Whipple pancreaticoduodenectomies and one Traverso-longmire pylorus-preserving pancreaticoduodenectomy. Three of the patients were female, one male. The mean age was 68.5 with a standard deviation of 1.1 year at the surgery time-point. The patients were respectively operated because of an adenocarcinoma of ductal type, a neuroendocrine pancreas tumor, an adenocarcinoma of the distal choledochal duct, and a sessile tubular duodenal adenoma. The here considered samples were tumor free and age-appropriate, as evaluated and labeled by trained pathologists.

2.2. Tissue treatment and histology

Fixed samples were embedded in paraffin, sectioned at a thickness of 4 μm, and mounted on glass slides. Hematoxylin & Eosin (H&E)-stained sections were generated using routine techniques [5]. Briefly, samples were serially rehydrated by incubation in 100 % xylol (15 min; VWR International, Fontenay-sous-Bois, France) followed by decreasingly concentrated ethanol solutions (100 % [10 min], 90 % [5 min], 80 % [5 min]; Central Chemical Storage, Saarland University, Saarbrucken, Germany), stained in Ehrlich hematoxylin (8 min; Carl Roth GmbH & Co, KG, Karlsruhe, Germany), washed in distillated water and blued in fluent water (12 min). Staining in 0.1 % eosin (210 s; Central Chemical Storage, Saarland University, Saarbrucken, Germany) was adjusted in 90 % isopropanol, followed by final serial dehydration using 100 % isopropanol (10 min) and 100 % xylol (15 min). For immunohistochemical staining of TRPC3 and TRPC6 paraffin was removed, and antigen recovery was performed using citrate buffer (60 min, 95 °C). The primary antibody (polyclonal anti-TRPC3, lyophilized, ACC-016, 1:50, Alomone Labs, Jerusalem BioPark, Israel; polyclonal anti-TRPC6, lyophilized, ACC-017, 1:50, Alomone Labs, Jerusalem BioPark, Israel) was applied overnight and at room temperature. Instead of the primary antibody, 1:500 diluted rabbit serum that was kindly donated by Dr. Martin Jung was used for negative controls. A peroxidase labeled secondary antibody (HRP, Horseradish Peroxidase, anti-rabbit goat, A10547; Invitrogen AG, Carlsbad, CA, USA) and diaminobenzidine (DAB; incubation time = 5 min) tetrahydrochloride as chromogen (SK-4103 Vector Laboratories, Burlingame CA, USA) were added to detect the primary antibody. Nuclear counterstaining with hematoxylin (C. Roth, Karlsruhe, Germany) followed. According to the manufacturer's (Alomone Labs, Jerusalem BioPark, Israel) information, the anti-TRPC3 and anti-TRPC6 antibodies are knockout-validated and designed to detect the corresponding channels in mouse, rat, and human tissue. More specifically the anti-TRPC3 antibody (Peptide HKLSEKLNPSVLRC) detects the amino acid residues 822–835 of mouse TRPC3 that is localized to the intracellular COOH (carboxy-)terminus and the anti-TRPC6 antibody (Peptide [C]RRNESQDYLLMDELG) recognizes the amino acid residues 24–38 of the mouse TRPC6 channel that are localized to the intracellular N-terminus of the first transmembrane segment. Detailed description of the molecular structure of these channels is provided elsewhere [43]. Knockout-validation of each of these antibodies was also provided in other previously published reports, making the here presented results trustable [13,15,50].

2.3. Evaluation

Finally, the slides were digitalized using the Nano Zoomer S210

(Hamamatsu, Japan). Microphotographs were taken using the image viewing software NDP.view2 from Hamamatsu (U12388–01, Hamamatsu, Japan). No digital image editing applied.

3. Results

The cadaveric samples we first investigated featured advanced autolysis impeding islet of Langerhans identification. Therefore, healthy samples from visceral surgery were apprehended. The H&E staining (Fig. 2) displayed peripancreatic fat tissue, hemorrhages (following surgery), a lobar archi- and lobular parenchymatous subarchitecture (not shown). Higher magnification allows clear recognition of the parenchymatous structure involving serous acini (Fig. 2A), their draining "intercalate" ducts (Fig. 2B and C), as well as larger intralobular (Fig. 2C, D, and E), and interlobular ducts (Fig. 2F). The major pancreatic duct was unfortunately not displayed. Islets of Langerhans, in turn, are well recognizable (Fig. 2G and H). Nevertheless, tissue quality was different among the samples. We show here microphotographs (Figs. 2,3, and 4) from the sample with less advanced autolysis – if at all – that was surgically obtained due to a sessile tubular duodenal adenoma. The following description includes all samples, unless differently mentioned.

Anti-TRPC3 immunohistochemical staining was detected in exocrine acini (Fig. 3A and B) and in corresponding intercalate ducts (Fig. 3C). In most cases, the entire acinar cells (plasma membrane and cytoplasm) of the section plane were labeled by the chromogen, although intracellular staining intensity was variable. Certain acinar cells featured indeed stronger intracellular staining loci mimicking cell organelles (Fig. 3B).

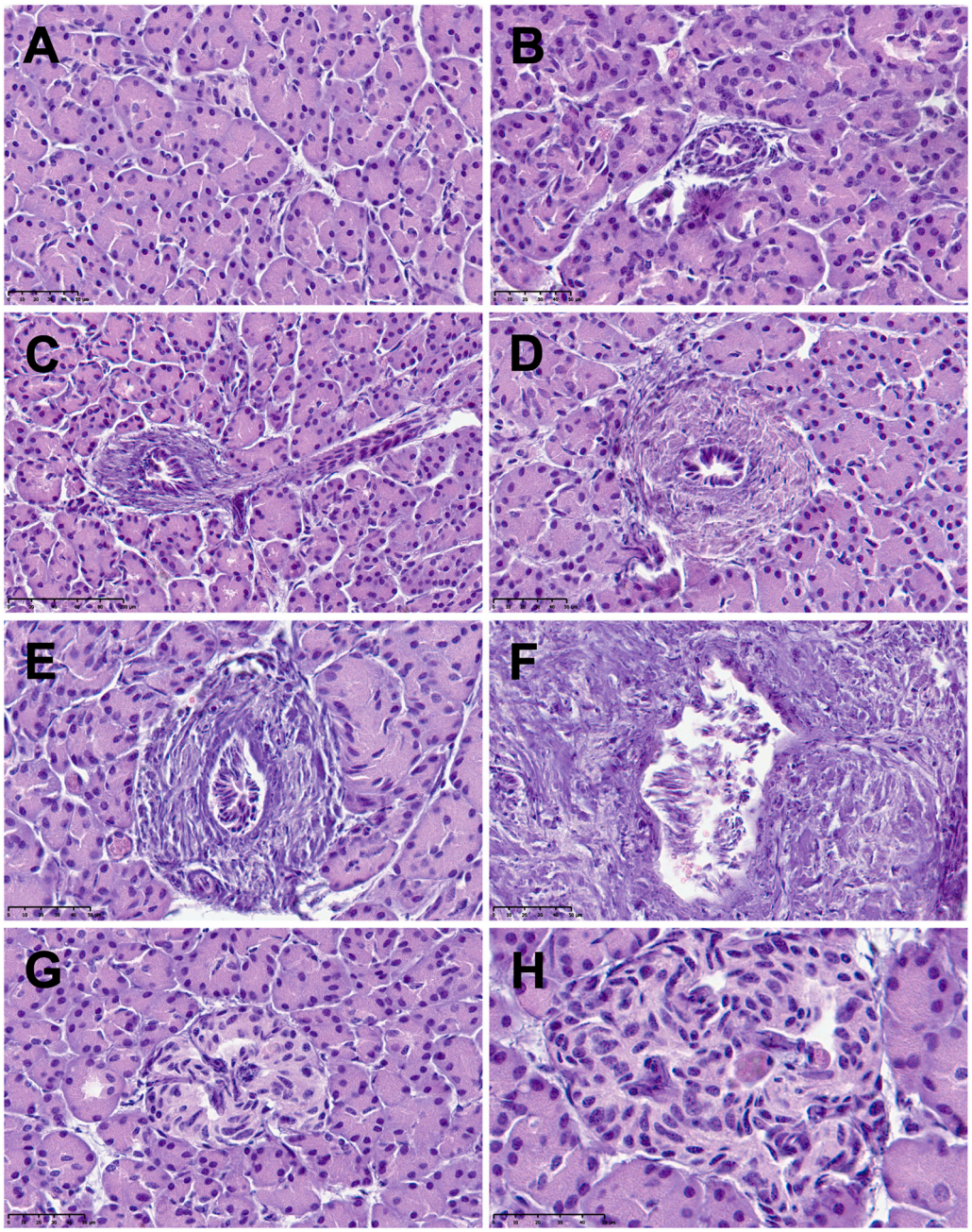

Fig. 2. Hematoxylin & Eosin staining of the pancreas. Exocrine acinous tissue (A). Intercalate duct surrounded by acini (B). An intercalate duct flows into an intralobular analogue (C). Intralobular duct surrounded by acini (D, E). Interlobular duct (F). Islet of Langerhans surrounded by acini (G). Islet of Langerhans surrounded by acini (higher magnification; H).

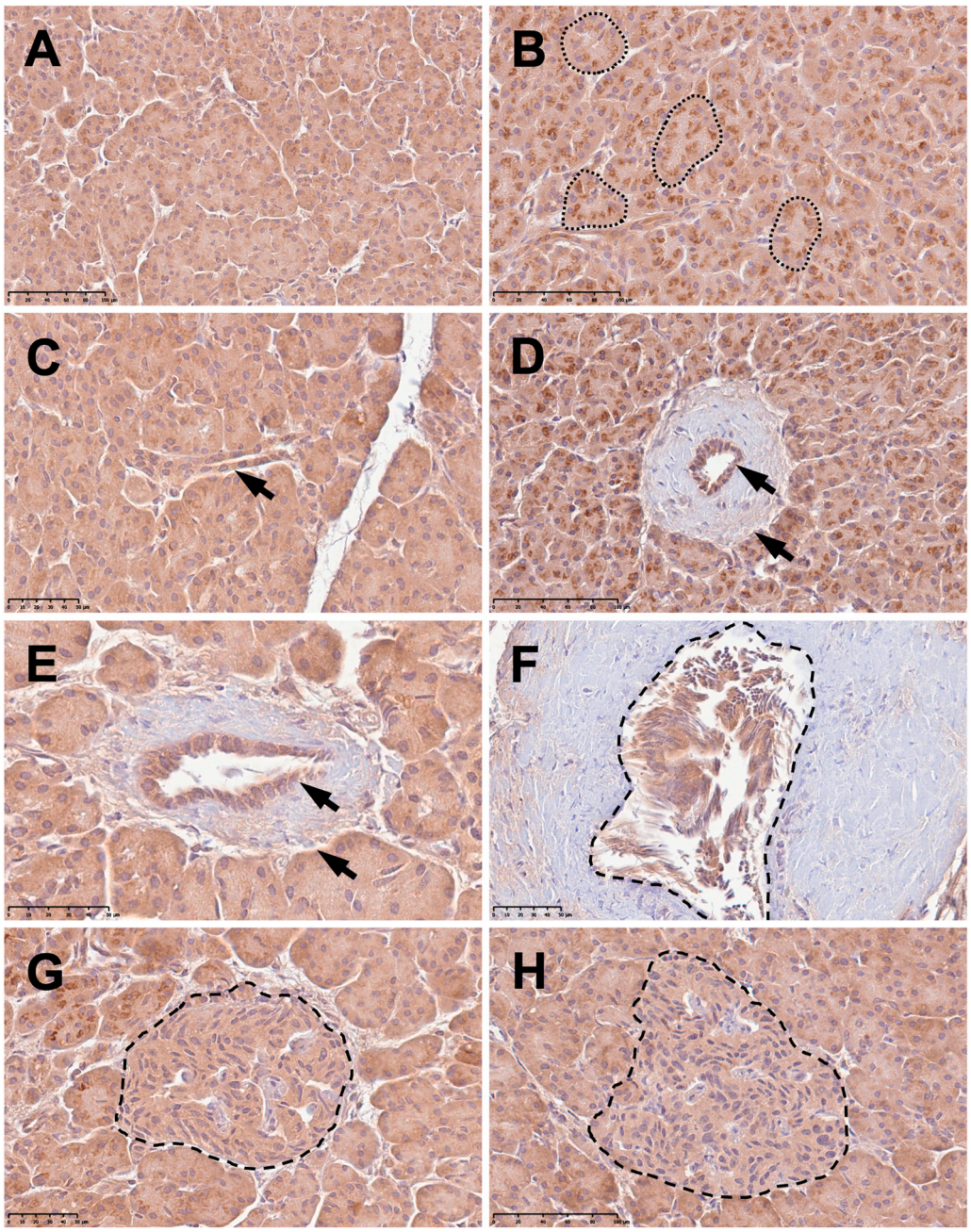

Fig. 3. TRPC3-protein expression in the human pancreas. Pyramid-shaped serous acinar cells (A). Pyramid-shaped serous acinar cells with increased isolated intracellular staining (B). Intercalate duct (arrow) surrounded by acini. Two different lobules are distinguishable (C). Intralobular duct surrounded by acinous tissue as observed in microphotograph B. Inner arrow indicates the ductal epithelium. Outer arrow represents the connective tissue layer (D, E). Interlobular duct. Ductal epithelium is recognizable inside the demarcation, and connective tissue layer outside (F). Islets of Langerhans in lower (G) and higher (H) magnification.

The respective appearances were largely neighbored. However, this staining pattern was essentially observed in the here depicted sample, which may be attributable to varying autolysis in the three remaining samples, that was howsoever noticeably less than in cadaveric tissue (not shown). Intralobular ducts feature highly prismatic epithelial cells that were immunoreactive suggesting TRPC3-protein expression (Fig. 3D and E). In contrast, the surrounding connective tissue layer was immunolabeling-deficient (Fig. 3D and E). Interlobular ducts were seldom, but similarly to intralobular analogues stained with respect to the luminal epithelium (Fig. 3F). The islets of Langerhans were immunoreactive and featured a homogenic signal distribution, thus not indicating clear contrasts among the insular cells with respect to TRPC3-expression. Insular capillaries were distinguished but remained unstained (Fig. 3G and H). Negative controls did not display any DAB-specific brown coloration (not shown).

As a matter of fact, a similar staining distribution was observed when using the anti-TRPC6 antibody (Fig. 4). Indeed, exocrine acini were stained. Again, in most cases the entire acinar cells (plasma membrane and cytoplasm) of the section plane were labeled by the chromogen (Fig. 4A and B). Interestingly, the anti-TRPC3-staining pattern mimicking cell organelles, was not observed with anti-TRPC6-staining in the same sample. The cuboidal cells of the intercalate ducts appeared clearly stained (Fig. 4C). Highly prismatic epithelia of intralobular (Fig. 4D and E) and interlobular (Fig. 4F) ducts were similarly stained, whereas their connective tissue layers were mostly lacking immunolabeling. The endocrine islets of Langerhans were homogenously stained

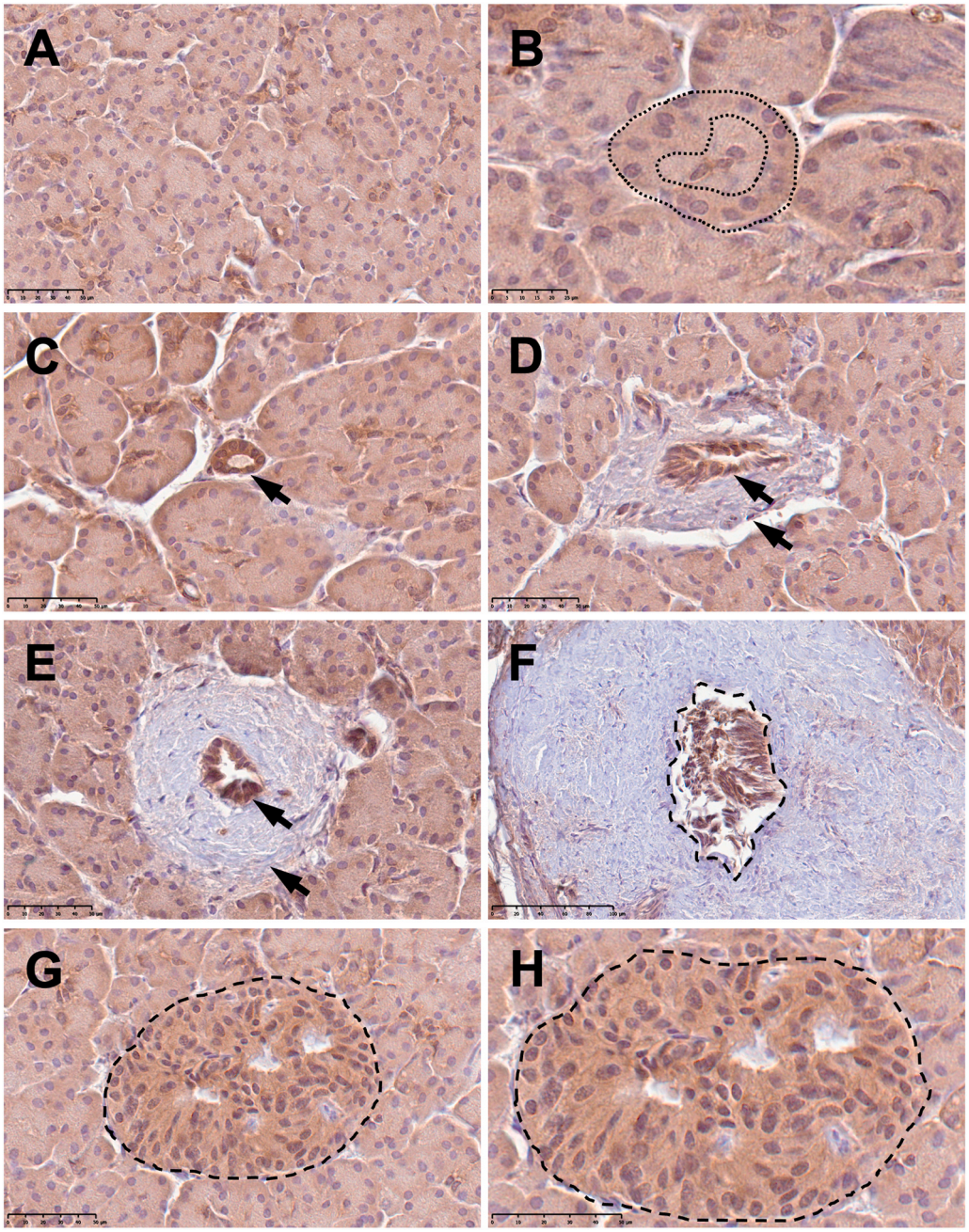

Fig. 4. TRPC6-protein expression in the human pancreas. Pyramid-shaped serous acinar cells in lower (A) and higher magnification (B). The demarcations delimit the acinar cells in the plane. Centroacinar cells are recognizable (B). Intercalate duct (arrow) surrounded by acini (C). Intralobular duct (oblique sectioned plane: D; transversal sectioned plane: E) surrounded by acinous tissue. Inner arrow indicates the ductal epithelium. Outer arrow represents the connective tissue layer (D, E). Interlobular duct. Ductal epithelium is recognizable inside the demarcation, and connective tissue layer outside (F). Islets of Langerhans in lower (G) and higher (H) magnification.

and seemed to tower above the exocrine backbone with respect to staining intensity. Again, unstained insular capillaries were detected (Fig. 4G and H). Negative controls did not display any DAB-specific brown coloration (not shown).

4. Discussion

We provide here evidence that suggests TRPC3- and TRPC6-expression in both the human exocrine and endocrine pancreas. Immunohistochemical protein detection is highly sensitive and can detect small channel amounts. Besides, it enables cellular and subcellular localization of the protein in contrary to other detection methods. Importantly, the use of knockout-validated antibodies as in the present study is a critical factor for results trustability. Such immunohistochemical investigations on the human pancreas, as performed here, are rare, since commonly available pancreatic tissue from cadavers is often impaired in quality. Manifestations such as tissue liquefaction, protein degradation, and other artefacts are known to occur [17,52]. Instead, samples need to be gained from visceral surgery, as previously done [36], which is concomitant with further discussions concerning the integrity and healthiness of the considered tissue. As mentioned above the tissue was quality-checked by trained pathologists and labeled as physiological and age-appropriate. Of course, pancreas tissue from older patients cannot reflect conditions in younger specimens, which restricts

the generalizability of any conclusions. Further, tumors, especially malignant entities can affect protein expression in neighbored healthy tissue. However, since not all of our samples were obtained from cancer-affected organs, and similar patterns were observed independently of the surgery indication, we assumed a good translationability of our results. Also, similar approaches have been chosen previously to investigate protein expression not only in pancreatic islets of Langerhans [36], but also in other organs such as the kidneys for instance [7,11,25]. When interpretating the findings, it has to be kept in mind that our small case number can lead to missing detection of potential variances in the pancreatic protein expression profile. Thus, further studies involving higher case numbers will be needed to verify the here presented results.

We detected TRPC3- and TRPC6-immunostaining throughout all pancreatic samples. The exocrine tissue was widely stained, although certain lobules or lobular segments of the best-preserved sample tended to display noticeable intracellular signals following anti-TRPC3-staining. Indeed, staining of both proteins was in most cases detected in different cellular sublocalizations that included the plasma membrane and the cytoplasm. As a matter of fact, conventional light microscopy does not allow differentiation of cell organelles other than the nucleus [19]. It is however conceivable, that organelles responsible for membrane proteins (e.g., TRPC3 and TRPC6) synthetization, sorting, and trafficking such as the endoplasmic reticulum and the Golgi apparatus conditioned the observed cytoplasmic immunoreaction [39]. Kokubun *et al.* discussed similar staining sources after having described TRPV1 and 2 channel-associated immunoreactivity that was rather localized to the cytoplasm than to the plasma membrane [27]. In addition, further studies reported the cytoplasmic localization of TRP channels in association with their functional mode [1,14,41]. That the anti-TRPC3 immunohistochemical signals were accentuated in certain lobules or lobular segments could be ascribed to differing oxygen supplies that ultimately dictate cellular metabolism [44]. Hepatocytes, for instance, are known to display an alterable metabolism and thus a protein expression depending on oxygen supply [49]. Why this phenomenon was only observed with respect to TRPC3- and not TRPC6-staining is unclear, although TRPC6-distribution was previously suggested not to be affected by altered oxygen supply in the liver [47]. This pattern was essentially observed in the best-preserved sample, which can be ascribed to in relation advanced autolysis in the other samples that renders such cellular or subcellular considerations mechanistically impossible. Of course, fixation artefacts as reason for differing representations cannot be excluded either. Evidently, this rather mimics a case-report, and further studies will be needed to address verification of these findings.

The relevance of these channels in acinar cells was established by Kim *et al.* who first revealed acinar expression of the TRPC3- and TRPC6-proteins in the murine pancreas [20]. Later, they demonstrated TRPC3 to be relevant for physiological exocytosis of pancreatic digestive enzymes by regulating the frequency of physiological cytosolic calcium level oscillations [21]. TRPC3-deletion, that was employed in this study, also reduced stimulated-pathological sustained cytosolic calcium levels, decreased intracellular trypsin activation, and subsequently pancreatitis severity [21]. Beyond, tools including TRPC3-knockout and pharmacological inhibition (TRPC3-inhibitor pyrazole 3 [Pyr3]) were later used in experimentally-induced acute pancreatitis, to suggest that TRPC3 is a critical influx-player in calcium-mediated toxicity and that its inhibition can attenuate the SOC-associated pancreatitis [22]. The study from Du *et al.* supported the relevance of TRPC3 and TRPC6 in acute pancreatitis, by showing that microRNA-26a (miR-26a) targets the SOCs TRPC3 and TRPC6, thus alleviating aforementioned physiological calcium oscillations and pathological sustained calcium elevations in acinar cells [8]. On one hand, miR-26a-deletion, led to increased SOC-expression, ultimately exacerbating acute pancreatitis, while on the other hand miR-26a-upregulation remarkably attenuated the histopathological manifestations [8]. Together with our detailed histological studies in human pancreas tissue, there is clear morphological and experimental evidence for the benefit of TRPC3- and possibly TRPC6-inhibition as therapeutic tool in patients suffering from pancreatitis.

Investigations of pancreatic duct cells with respect to TRPC3- or TRPC6-expression are significantly less compared to acinar cell studies, and probably even not existent to the authors knowledge. Since, we detected immunohistochemical signals in diverse pancreatic ducts, questions arise concerning their function in these localizations. In tissue analogy, functional TRPC3 was detected in salivary ductal cells [6]. Again, morphological and experimental studies will be needed to verify our results by higher case numbers and to explore the role of TRPC3 but also of TRPC6 in these eagerly bicarbonate-secreting cells. Besides the aforementioned exocrine, endocrine, and ductal cells, pancreatic stellate cells represent approximatively 7 % of all pancreatic cells, and have been shown to be implied in pancreas pathologies [18]. These stellate cells are commonly detected using specific immunohistochemical targets (e.g., vimentin, desmin, neural growth factor) [2,18], which has not been performed here. However, there are studies that suggest relevance of TRPC3 and TRPC6 in these cells. For instance, TRPC6 was shown to modulate the hypoxia-response [33], while TRPC3 was recognized to be relevant for cell migration especially promoting tissue fibrosis and thus disease progression in the context of pancreatic ductal adenocarcinoma, in which the channel was upregulated [31,40].

As mentioned above TRPC3-expression is less than clear in the endocrine pancreas. While certain studies didn't identify TRPC3 in human pancreatic β cells [32,37], others observed that TRPC3-blockade neither altered basal nor stimulated-insulin secretion in rat insulinoma cell lines INS-1E [38]. In the same time period, however, both TRPC3 and TRPC6 were proven to be involved in α and β cell proliferation [16], while only TRPC3 was shown to be implicated in PLC (phospholipase C)/PKC (protein kinase C)/GPR40 (G protein-coupled receptor 40) pathways resulting in cytosolic [Ca^{2+}]-elevation leading to insulin secretion in rat β cells [51]. Recently, Rached *et al.* demonstrated through immunofluorescence colocalization of TRPC3 and insulin in human pancreatic islets, ultimately suggesting TRPC3-expression in human β cells [36]. Similar patterns were observed in mice. Since anti-TRPC3-signature was also detected at the islet's borders, TRPC3-expression was indicated in human α cells, as supported by our study [36]. Functional relevance in these cells was also conceivable, since glucagon secretion is similarly calcium-dependent, although differently regulated than insulin secretion in β cells [45]. In this context, Takatani *et al.* provided evidence supporting the involvement of *trpc3* gene in insulin-induced and IRS1 (insulin receptor substrate 1)-mediated glucagon regulation in α cells [42]. Morphological aspects such as the respective islet localization of α and β cells and the centrifugal insular blood flow play here an essential role [29,30]. Beside presenting morphological findings, that are deepened by our results, Rached *et al.* further identified TRPC3 to be essentially involved in glucose-stimulated insulin secretion [36], which contrasts the aforementioned results from Sabourin *et al.* [38]. Rached *et al.* [36] tried to explain these discrepancies by use of suboptimal pharmacological inhibition and cell line in the reported publication [38]. Altogether, these experimental studies highlight the relevance of TRPC3 and/or TRPC6 in the endocrine system as well as its interest as therapeutic target. However, some of these studies didn't refer to protein detection, and only few were based on human tissue. From this point of view, our study in human tissue supports most of the corresponding studies suggesting TRPC3- and TRPC6-expression in islets of Langerhans. As a matter of fact, no relevant staining differences were observed among the insular cells in our study, suggesting a wide expression quite certainly involving α and β cells due to their high insular proportion and possibly also γ and δ cells, in which these cation channels haven't been investigated yet to our best knowledge.

In conclusion, we verified here the wide expression pattern of TRPC3 and TRPC6 in the human pancreas, after their relevance was suggested in different compartments including exocrine acinar, as well as endocrine α and β cells. Distinct localizations that include pancreatic ductal cells, as well as possibly endocrine γ and δ cells warrant experimental

research with respect to TRPC channel function.

Ultimately our study supports the concept of therapeutical TRPC3 and/or TRPC6 channel activation/modulation/inhibition [46] in exocrine (e.g., pancreatitis) and endocrine (e.g., diabetes mellitus) pancreatic conditions, that nevertheless needs to be further investigated.

Ethics statement

All investigations were implemented anonymously, approved by the Ethics Committee of the Saarland Medical Association (163/20, 130/21), and performed in accordance with the guidelines of the Declaration of Helsinki. Informed consent was obtained from all involved patients.

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None.

CRediT authorship contribution statement

Thomas Tschernig: Writing – review & editing, Supervision, Resources, Project administration, Methodology, Conceptualization. **Mathias Wagner:** Writing – review & editing, Resources. **Coline Diebolt:** Writing – review & editing, Methodology. **Emilie Kirstein:** Writing – review & editing, Methodology, Investigation. **Colya Englisch:** Writing – original draft, Visualization, Supervision, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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