MEDICAL SCIENCES PROCEEDINGS OF THE SHEVCHENKO SCIENTIFIC SOCIETY
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ISSN 2708-8634
ISSN 2708-8642
Founded in 1898
№2
2024

Proceedings of the Shevchenko Scientific Society. Medical Sciences **2024**, *2 (76)*. https://doi.org/10.25040/ntsh www.mspsss.org.ua

DOI: 10.25040/ntsh2024.02.13

For correspondence: Department of Surgery 2, Educational and Scientific Institute of Postgraduate Education, Zaporizhzhia State Medical and Pharmaceutical University, 26 Maiakovskyi Ave., Zaporizhzhia, 69035
E-mail: mikheev.u.a@gmail.com

Received: 12 Nov, 2024
Accepted: 15 Dec, 2024
Published: 27 Dec, 2024

ORCID IDs

Iurii Mikheiev:
https://orcid.org/0000-0002-0305-1570

Vasyl Skrypko:
https://orcid.org/0000-0002-1555-2030

Andrii Pasko:
https://orcid.org/0000-0002-6688-7666

Oksana Popadynets:
https://orcid.org/0000-0002-2093-5984

Disclosures: The authors declared no conflict of interest.

Author Contributions:

Conceptualization: Iurii Mikheiev;

Results of study: Vasyl Skrypko, Andrii Pasko;

Writing: Iurii Mikheiev, Vasyl Skrypko, Andrii Pasko;

Review & Editing: Iurii Mikheiev, Oksana Popadynets.

Ethical approval: Approved at the meeting of the Academic Council of the Zaporizhzhia Medical Academy of Postgraduate Education, Ministry of Health of Ukraine (Protocol No. 8, dated December 27, 2016). All patients provided informed consent to participate in this study. During the research, bioethical standards of both national and international legislation were adhered to, including the provisions of the WMA Declaration of Helsinki.

Funding: The work is carried out according to the research plan of the Department of Surgery and Minimally Invasive Technologies of the Zaporizhzhia Medical Academy of Postgraduate Education, Ministry of Health of Ukraine "Improvement of Modern Methods of Diagnosis and Treatment of Inflammatory and Chronic Diseases of the Hepatopancreatobiliary System" (State Registration Number 0118U003969).

Original research: Clinical sciences

PATHOMORPHOLOGICAL FEATURES OF THE PANCREAS DEPENDING ON CHRONIC PANCREATITIS DURATION

Iurii Mikheiev[1], Vasyl Skrypko[2], Andrii Pasko[2], Oksana Popadynets[2]

[1]**Zaporizhzhia State Medical and Pharmaceutical University, Zaporizhzhia, Ukraine**

[2]**Ivano-Frankivsk National Medical University, Ivano-Frankivsk, Ukraine**

Introduction: Chronic pancreatitis (CP) presents clinically as an inflammatory process that leads to complex morphological changes, resulting in the replacement of pancreatic parenchyma with connective tissue and the development of various complications. Defining an optimal surgical strategy remains a relevant issue today.

The purpose of the study was to assess the morphological and immunohistochemical characteristics of the pancreatic parenchyma depending on CP duration.

Methods: A total of 147 (57.1%) patients were examined retrospectively. A prospective comparative study involving 257 patients analyzed the short and long-term outcomes to evaluate the effectiveness of the "early surgery" strategy developed at the clinic and the surgical management methods for CP. The study conducted a morphological and immunohistochemical assessment of the pancreatic parenchyma, analyzing the long-term outcomes of the primary pathogenetic surgical treatments for CP.

Results: Morpho-functional changes in the pancreatic parenchyma, observed more than three years (median 5.85 years) after the onset of CP clinical manifestations, were characterized by progressive fibrosis due to increased expression of type I collagen and fibronectin, which resulted in dense cuff-like perineural and perivascular fibrosis and stenosis of the pancreatic ducts around tubular complexes, clinically corresponding to the presence of intense pain syndrome.

Conclusions: An essential role of pancreatic stellate cells in developing pancreatic parenchymal fibrosis in CP has been established. Fibrotic lesions of the pancreas are irreversible, and the resulting morphological and structural changes lead to both exocrine and endocrine dysfunction. More than three years after the onset of clinical manifestations of CP, type I collagen expression was observed in the acinar tissue, with thin strands detected within the islets of Langerhans. Intraepithelial ductal neoplasia was also identified, which may later progress to ductal adenocarcinoma of the pancreas. It has been demonstrated that the improvement in surgical outcomes for complicated forms of CP with biliary and ductal hypertension is achieved through planned surgery within the first three years of CP development, before the onset of irreversible pathological changes, including the potential for oncological transformation of the pancreas.

Keywords: pancreas, chronic pancreatitis, morphological changes, fibrosis.

Introduction

Pain is the most common symptom of chronic pancreatitis (CP), affecting between 50% and 85% of patients [1]. The mechanism of pain in CP is multifactorial and poorly understood. Several hypotheses have been proposed to explain pain in CP, including pancreatic ductal hypertension or increased pressure in the pancreatic parenchyma, cyst formation, hypertrophy of intrapancreatic nerve fibers, neuropathic changes, and tissue damage due to oxidative stress [2]. Pain in CP is thought to result from a pathogenic interplay among the pancreatic parenchyma, immune cells, and the peripheral nervous system [3].

The complex pathogenesis of pain in CP presents a significant clinical challenge in modern pancreatology, and an understanding of its mechanisms is essential for selecting effective treatments for this patient population [4].

A pathological characteristic of CP and ductal adenocarcinoma is the progressive fibrosis mediated by pancreatic stellate cells (PSCs). One of the earliest cellular mechanisms of fibrosis involves the activation of PSCs through autocrine and paracrine signaling, suggesting that the effects of PSC activation, including the initial inflammation and subsequent fibrosis, can progress even after removing the primary damaging factor.

PSCs play a crucial role in the development of fibrosis in CP. They are directly activated by toxic factors such as alcohol or chemokines, transforming growth factor beta (TGF-beta), and platelet-derived growth factor (PDGF), which are released during necrotic inflammation of the pancreas, leading to the deposition of collagen and other extracellular matrix proteins in the interstitial space and resulting in damage to and deformation of pancreatic acinar and ductal cells [5]. Ultimately, this leads to the loss of the pancreatic lobular architecture, deformation of the main pancreatic ducts, and significant changes in the islet apparatus [6]. Fibrotic damage to the pancreas is irreversible, and the resulting morphological and structural changes lead to both exocrine and endocrine dysfunction, ultimately resulting in pancreatic insufficiency and the onset of diabetes mellitus. The pancreas is typically affected diffusely, although variations in the severity of changes may present as a mosaic pattern. The cardinal morphological features in CP are the triad of fibrosis, loss of acinar tissue (atrophy), and duct changes (narrowing and dilation) [7]. Macroscopically, the pancreas is firm and shrunken with irregular contours; the main pancreatic duct (MPD) and smaller ducts are dilated, with the potential presence of calculi within the ducts. The normal macroscopic lobulation of the parenchyma may be markedly altered or lost due to fibrotic scarring.

On microscopy, the lobular arrangement of ducts remains intact, but interlobular and intralobular fibrosis is evident, accompanied by the loss of acinar cells. The ducts are tortuous and dilated, often containing protein plugs (lamellar eosinophilic masses) and calculi; periductal fibrosis, accompanied by destruction of the duct epithelium, is frequently observed surrounding the ducts. Ductular proliferation may also be observed within the lobules. Changes in the ducts may lead to fibrosis and/or result from fibrosis. In response to recurrent episodes of inflammation, necrosis, and reparative changes, fibrosis results in strictures and ductal obstruction [8]. Other signs of CP include the presence of islet cell conglomerates.

Currently, there is no reproducible and universally accepted histological grading system for the severity of CP. The terms “mild,” “moderate,” and “severe” are commonly used, but they can be challenging to differentiate in practice. Most research studies use the fibrosis scoring system proposed in 1991 [9], combined with an assessment of pancreatic function [10].

Regarding current trends in the surgical treatment of CP, better relief control and preservation of exocrine pancreatic function are observed with early surgery. The generally accepted definition of early surgery is three or fewer years from the onset of the disease [11]

The purpose of the study was to assess the morphological and immunohistochemical characteristics of the pancreatic parenchyma depending on CP duration.

Patients and Methods

Morpho-histochemical changes in the pancreas in CP were analyzed using pancreatic tissue biopsy samples obtained during surgery (in most cases, histological material was taken from the head of the pancreas). Tissue samples were collected and processed following standard protocols, fixed in a 10% neutral formalin solution, embedded in paraffin, and stained with hematoxylin and eosin. Histological sections, four μm sick, were prepared using a rotary microtome (Microm HM-340E, Thermo Fischer Scientific Inc., MA, USA) and placed on adhesive glass slides (SuperFrost Plus; Menzel-Gläser, Braunschweig, Germany) for immunohistochemical (IHC) analysis. IHC analysis was conducted following standard protocols using monoclonal mouse antibodies: smooth muscle actin (Clone 1A4) targeting alpha-smooth muscle actin (α-SMA) and desmin (Clone D33) targeting desmin (DAKO, USA); rabbit

monoclonal antibodies (Clone RAH C11) against type I collagen (Imtek, Russia); and rabbit polyclonal antibodies against fibronectin (DAKO, USA). After deparaffinization, high-temperature antigen retrieval in Tris-EDTA buffer (pH=9.0), and blocking of endogenous peroxidase activity with 3% hydrogen peroxide, incubation with primary antibodies was performed according to the manufacturer's instructions, followed by visualization of the IHC reaction with diaminobenzidine (DAKO, USA). The results of the IHC reaction were evaluated using an Axioplan 2 microscope (Carl Zeiss MicroImaging GmbH, Germany) and a C5060WZ digital camera (Olympus, Japan). For the photomorphometric study, ten fields of view were photographed for each case. To standardize the data, photographs were taken under controlled conditions: a microscope magnification of ×200, warm white light at 3200 K, camera settings of F3.2 (aperture), 1/400 (shutter speed), ISO 100 (sensitivity), and manual white balance adjustment. Quantitative analysis of the intensity levels and relative area of expression of the investigated stromal markers was conducted using ImageJ v.1.48 digital image processing software with the built-in color deconvolution plugin and the H-DAB (hematoxylin + diaminobenzidine) staining analysis scheme to measure the surface area of structures in IHC preparations and the optical (densitometric) immunostaining intensity, following a patented method (Patent No. 99314, Ukraine, IPC 2015: G01N 21/00, G06K 9/00, Method of Photo Digital Morphometry of Immunohistochemical Preparations). An automatically generated histogram of the 8-bit grayscale image from the filtered DAB channel was used for quantitative morphometric analysis of the components, along with software-based calculations of optical density and standard deviation. For further morphometric calculation of the relative surface area in the grayscale image, a standard sensitivity threshold (Threshold tool) was applied, with the Default setting used for calculating the expression of α-SMA and type I collagen, and Rényi entropy applied for calculating the expression of desmin and fibronectin.

Results

The results of comprehensive pathohistological and IHC studies of pancreatic tissue samples obtained during surgeries for CP and pancreatic and biliary hypertension in 72 patients were evaluated.

The entire sample was divided into two study groups: Group I ("early surgery") included 32 patients diagnosed with CP less than three years before surgery, and Group 2 ("late surgery") comprised 40 patients diagnosed with CP more than three years before.

According to the METAVIR scoring system, the severity of fibrosis in Group I ("early surgery") was as follows: twelve (37.5%) patients had mild fibrosis (F1), sixteen (50.0%) patients had moderate fibrosis (F2), three (9.3%) patients had severe fibrosis (F3), and one (3.2%) patient had cirrhosis (F4). In Group II ("late surgery"), moderate fibrosis (F2) was observed in three (7.5%) patients, severe fibrosis (F3) in twenty-eight (70.0%) patients, and cirrhosis (F4) in nine (22.5%) patients. Three patients with CP and F3 fibrosis and one with F4 fibrosis were excluded from Group I, while three patients with CP and F2 fibrosis were excluded from Group II.

Histological examination (hematoxylin and eosin staining) of tissue samples from Group I patients with F1 fibrosis revealed mild interlobular fibrosis. The interlobular spaces exhibited slight expansion with no notable histological remodeling or tubule destruction, and the acinar tissue remained intact (Fig.1-A).

Parallel histochemical analysis using Mallory's trichrome and Van Gieson's staining techniques demonstrated the presence of thin collagen fibers in the interlobular septa surrounding individual lobules.

Histological examination of tissue samples revealed inflammatory infiltration by lymphohistiocytic cells (lymphocytes, macrophages, plasma cells) in the fibrotic areas at the early stages of CP with pancreatic and biliary hypertension.

Comprehensive pathohistological analysis of Group I patients with CP, pancreatic and biliary hypertension, and F2 fibrosis revealed histoarchitectural remodeling of the pancreas, affecting both the stromal and glandular-ductal patterns. Significant perilobular and diffuse interlobular fibrosis were observed around pancreatic lobules. The preparations showed cuff-like periductal fibrosis surrounding the distorted and dilated interlobular ducts.

The interlobular space and septa were enlarged, and inflammatory cell infiltration was observed in the stromal pattern, ranging from mild to moderate intensity.

A notable distinction between CP with F2 fibrosis and CP with F1 fibrosis is the presence of dense fibrosis surrounding the nerve fibers, which, according to clinical findings, may be associated with the development of pain syndrome. However, in both F1 fibrosis and F2 fibrosis, the pancreatic acinar tissue remains intact. In all patients of the "early surgery" group, fibrotic changes were characterized by pronounced perilobular fibrosis affecting all

lobules, widespread intralobular fibrosis, and prominent periductal fibrosis surrounding large, sharply narrowed, and cystically dilated ducts (Fig. 1-B).

Parallel IHC analysis using α-SMA on tissue samples from Group I patients with CP and mild to moderate fibrosis revealed that PSCs, typically quiescent around the MPD and other ducts, were activated and produced collagen and other extracellular matrix proteins. PSCs and α-SMA are central to developing dense fibrosis in CP, synthesizing abundant extracellular matrix proteins, including fibronectin and types I, III, and IV collagen, and intermediate filament proteins, such as desmin and vimentin.

Additionally, α-SMA-positive cells included fibroblasts, which formed the primary fibrotic mass in the pancreas in CP. Marker expression appeared as diffuse membrane and cytoplasmic staining in PSCs and fibroblasts. In the "early surgery" group, the expression level of α-SMA (Fig. 1-C) was (39.73±1.96) optical density units (ODU), and the relative area of α-SMA-positive stromal cells was (15.84±0.61)% (Fig. 2).

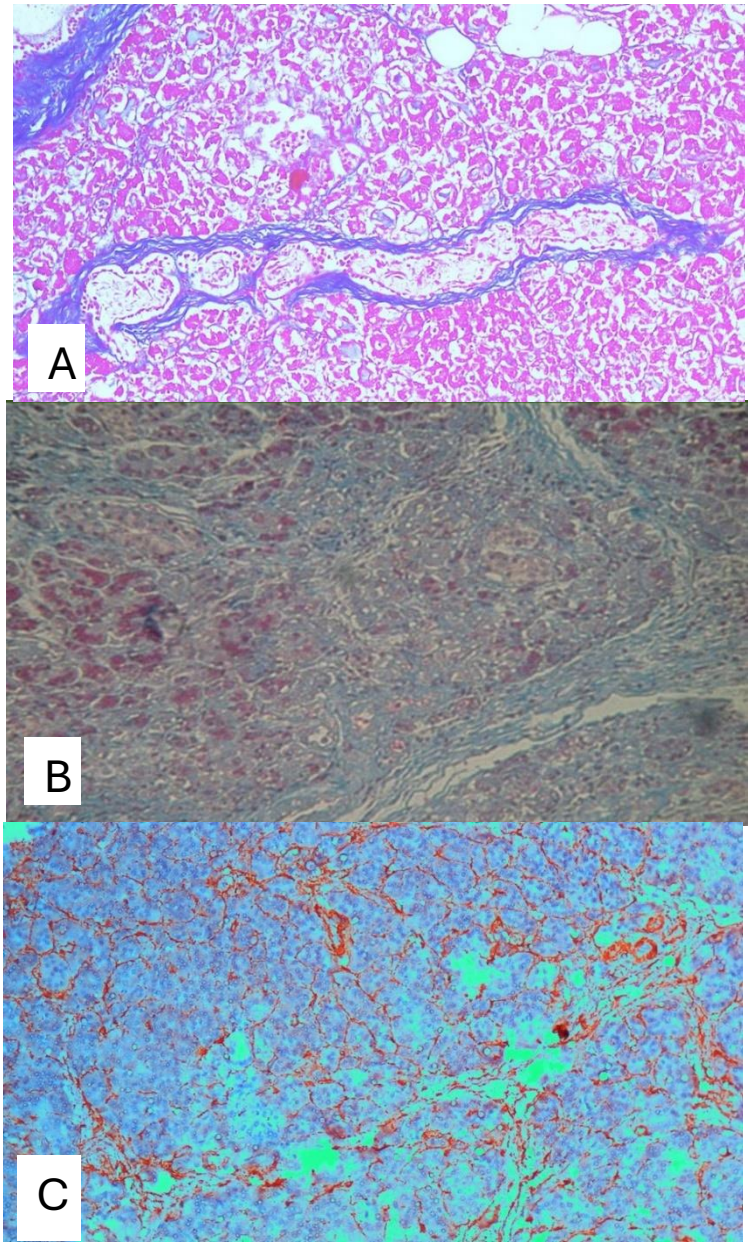

Figure 1. A-Pancreas of a Group I patient ("early surgery") with F1 fibrosis, showing minimal changes, including mild interlobular fibrosis. Masson's trichrome staining, magnification ×400; B- Pancreas of a Group I patient ("early surgery") showing signs of fibrotic transformation. Perilobular fibrosis with connective tissue strands extending into the pancreatic lobules, distorting the typical acinar structure. Mallory's trichrome staining, magnification ×400; C-Immunohistochemical reaction with Mo a-Hu Smooth Muscle Actin Alpha antibodies in Group I ("early surgery"). Magnification ×200. Weak staining of pancreatic stellate cells and their processes for α-SMA.

IHC analysis of tissue samples using type I collagen revealed diffuse membrane and cytoplasmic staining in spindle-liked cells, constituting the stromal pattern in CP. Marker expression appeared as thin strands. The relative area of positively stained "fibrillar" fibers was low, measuring (22.70±1.4)%. In contrast, the marker expression level was moderate, reaching (84.11±2.24) ODU (Fig. 2).

Correlation analysis revealed an inverse correlation between α-SMA+ cells and the expression level of type I collagen ($r=-0.19$, $p<0.05$). A direct moderate correlation was observed between the relative areas of α-SMA+ cells and type I collagen ($r=0.42$, $p<0.05$).

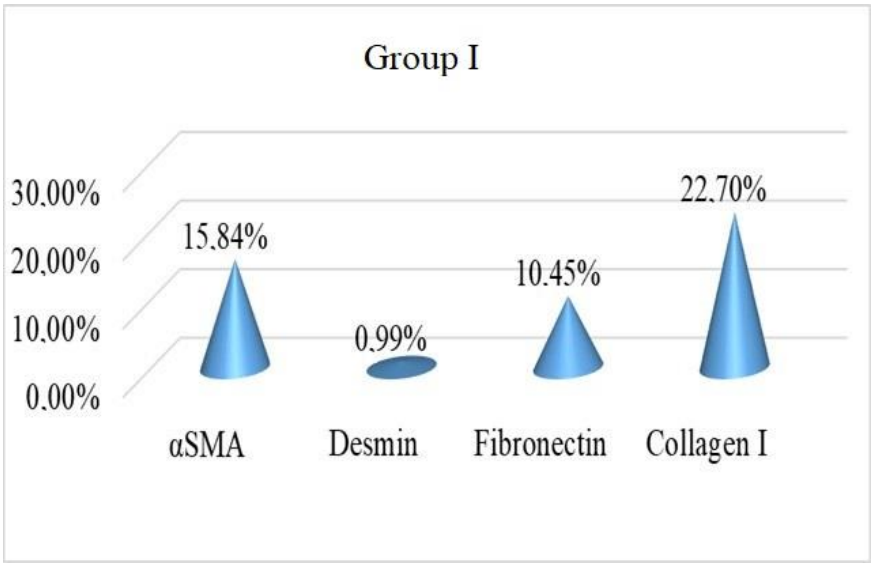

Figure 2. Median values of the relative area of mesenchymal marker expression in Group I ("early surgery").

The stromal marker fibronectin was used, which binds to fibronectin synthesized as an insoluble fibrillar network by the parenchymal and stromal cells of the pancreas. Its primary localization is the cell surface and extracellular matrix. Expression appeared as diffuse brown staining in the membrane and cytoplasm of spindle-shaped stromal cells forming fibrous sheaths around nerve fibers and ducts. Fibronectin expression was also observed as diffuse brown membrane staining in acinar-to-ductal metaplasia and in transformed acinar cells forming tubular complexes. In the "early surgery" group, a moderate level of fibronectin expression was observed, with an average of (29.43±2.69) ODU. The area of fibronectin-positive cells was (10.45±0.89)%. The IHC reaction with desmin in the stromal pattern closely resembled the expression of α-SMA (Fig. 3).

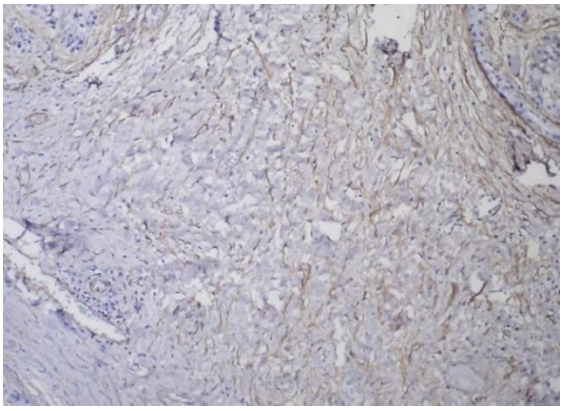

Figure 3. Immunohistochemical reaction using desmin in Group I ("early surgery"). Magnification ×200. Weak desmin expression in the connective tissue.

The similarity between the IHC reaction with desmin and α-SMA expression is that desmin is synthesized by both extracellular matrix cells and PSCs. Desmin expression was observed in both the stroma and acinar tissue. In Group

I, desmin expression was characterized by weak diffuse membrane and cytoplasmic staining, with an average of (3.72±0.24) ODU. The relative area of desmin expression was (0.99±0.05)%.

Significant changes in the pancreatic architecture in CP with pancreatic and biliary hypertension were observed in F3 and F4 fibrosis, using hematoxylin and eosin staining, Mallory's trichrome method, and Van Gieson's staining. The stromal pattern prevailed over the parenchymal pattern. In 18 (45%) Group II patients, combined fibrosis was observed, including interlobular, perilobular, intralobular, perineural, and perivascular fibrosis. Additionally, perineural infiltration was found in the specimens, potentially exacerbating the pain response (Fig.4 -A, B).

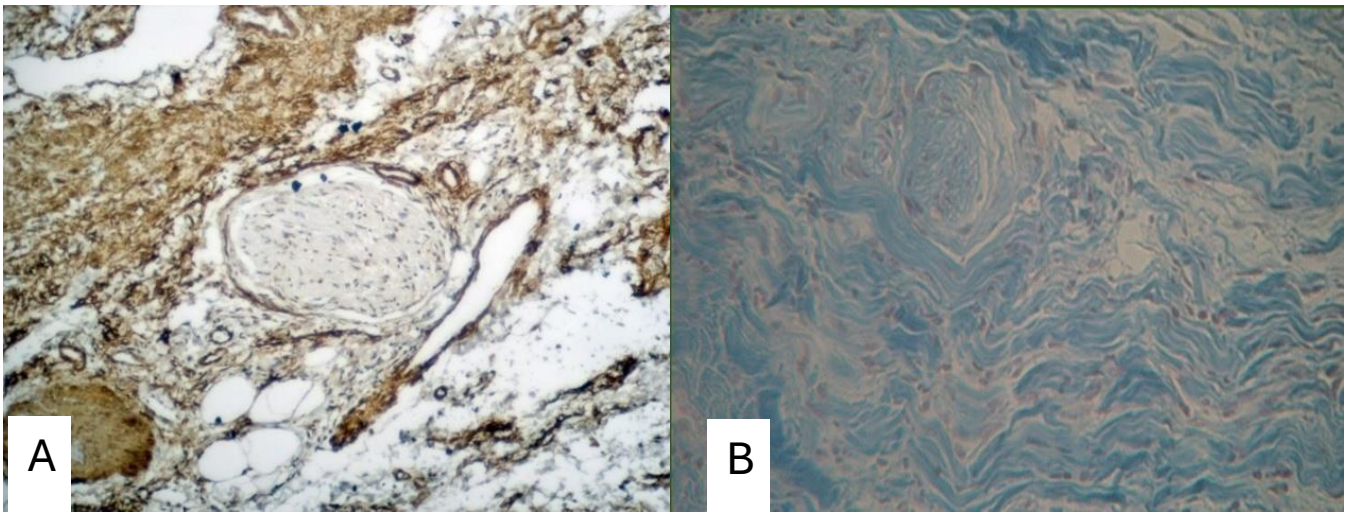

Figure 4.A- Immunohistochemical reaction with Mo a-Hu Fibronectin antibodies. Magnification ×200. Perineural fibrosis of the pancreas in a Group II patient ("late surgery"); B-Perineural fibrosis of the pancreas in a Group II patient ("late surgery"). Mallory's trichrome staining, magnification ×200

A fibrous sheath was also observed around the distorted, cystically dilated interlobular ducts. Extensive fibrous collagen strands of connective tissue replaced the acinar tissue. The preparations showed tortuosity, distortion, and dilation of the ducts with retention cysts, which were not observed in Group I patients with mild to moderate fibrosis. In addition to the exocrine portion of the pancreas, CP with severe fibrosis and cirrhosis exhibited both preservation (Fig. 5-A) and complete involvement of the endocrine portion, specifically the islets of Langerhans. The latter was affected by fibrosis, with nearly complete replacement of the acinar tissue of the pancreas by connective tissue, preserving the small ducts and narrowing the main ducts (Fig. 5-B).

Foci of compensatory acinar-to-ductal metaplasia were observed, where individual acinar cells transformed into ductal epithelial cells. These foci formed small tubular structures lined by a single layer of cuboidal epithelium with a narrow lumen. The nuclei were normochromic, the cytoplasm was eosinophilic, and mitotic figures were absent.

Tubular structures formed foci of small tubular complexes. The interlobular ducts exhibited significant dilation, with protein plugs observed in the lumens. Morphological analysis revealed an increase in the number of duct branches, characterized by ductal enlargement into two main patterns typical of fibrosis in other parenchymal organs: elongation of ducts and the formation of numerous parallel branches of small and medium diameter.

The ductal epithelium exhibited foci of grade 1 to grade 2 pancreatic intraepithelial neoplasia resulting from ductal obstruction by protein plugs and strictures due to pancreatic fibrosis.

The IHC reaction with the α-SMA marker in tissue samples from the "late surgery" group showed that α-SMA expression in PSCs exhibited greater intensity and a larger relative staining area in standardized fields of view (Fig. 5-C). In both study groups, α-SMA expression was observed as diffuse membrane and cytoplasmic staining in PSCs and fibroblasts.

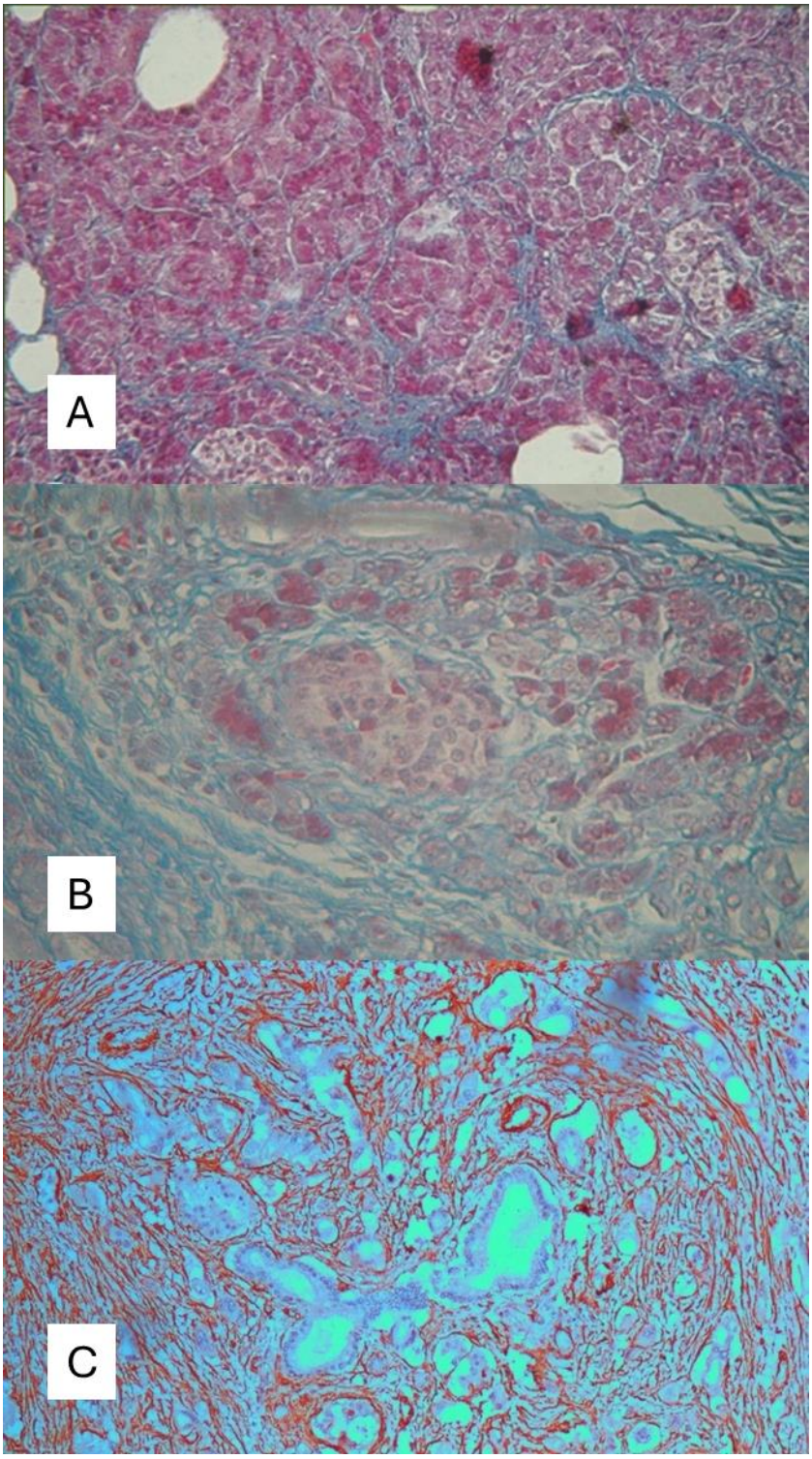

Figure 5. A- Interlobular fibrosis and disruption of the acinar architecture, with intact islets of Langerhans, in a Group II patient ("late surgery"). Mallory's trichrome staining, magnification ×200; B- Connective tissue infiltration of the islets at the site of a destroyed lobule in a Group II patient ("late surgery"). Mallory's trichrome staining, magnification ×400; C- Immunohistochemical reaction with Mo a-Hu Smooth Muscle Actin Alpha antibodies. Magnification ×200. Intensive staining of pancreatic stellate cells and their processes for α-SMA in Group II ("late surgery")

The expression level of α-SMA+PSCs was (47.17±3.52) ODU (Fig. 6), which was 1.18 times higher compared to the expression level in Group I, $p<0.05$.

In Group II, the relative area of α-SMA expression averaged (18.45±1.30)%, which was 0.86% higher than in Group I, $p<0.05$.

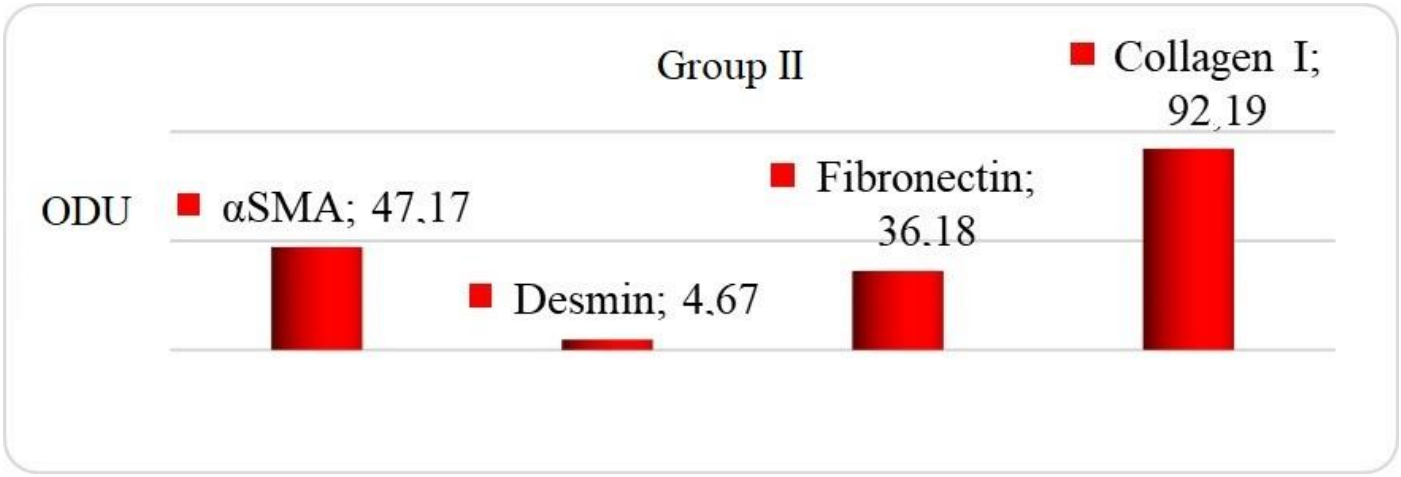

Figure 6. Median expression levels of mesenchymal markers in tissue samples from Group II ("late surgery")

IHC staining of tissue samples for type I collagen revealed diffuse membrane and cytoplasmic staining in spindle-shaped, fibroblast-like cells, forming a dense, stone-like stromal pattern in CP with pancreatic and biliary hypertension. The marker expression level was moderate, averaging (92.19±4.14) ODU.

The comparison of the staining levels between the study groups revealed a 1.10-fold increase in the median in the "late surgery" group ($p<0.05$). The relative area of type I collagen and "fibrillar" fibers showed a moderate level, averaging (27.96±1.99)%. The comparison of the relative areas of type I collagen expression between study groups revealed an increase in Group II ($p<0.05$).

As the histological severity of CP with pancreatic and biliary hypertension progressed, accompanied by severe fibrosis and cirrhosis of the pancreas, there was a notable increase in the intensity of type I collagen staining in the stroma, along with an expansion in the relative area of its expression. This resulted in dense, cuff-like fibrosis around nerve endings, clinically presenting as pain, along with peritubular fibrosis that narrowed the ductal lumens around the tubular complexes and perineural and perivascular fibrosis. Type I collagen expression was observed in the stromal pattern of CP and the acinar tissue as thin strands within the islets of Langerhans.

IHC staining of tissue samples with fibronectin showed diffuse brown membrane and cytoplasmic expression in stromal cells forming interlobular, perilobular, and intralobular fibrosis and fibrosis surrounding the ducts and ductal epithelium, which developed as a compensatory response from acinar cells (acinar-to-ductal metaplasia). In Group II, fibronectin expression was also moderate, as in Group I, but with a 1.23-fold increase, averaging (36.18±3.39) ODU ($p<0.05$). The area of fibronectin-positive cells in Group II was (13.05±1.01)%, which represented a statistically significant difference compared to Group I ($p<0.05$).

IHC analysis of tissue samples from the "late surgery" group revealed desmin expression in the stromal pattern around lobules, tubules, tubular complexes, perineurally, and in the acinar tissue cells. Desmin expression was characterized by a weak, diffuse membrane and cytoplasmic staining in cells, averaging (4.67±0.52) ODU. The relative area of desmin expression was low - (1.13±0.06)%.

Discussion and Conclusions

Our findings indicate that as pancreatic fibrosis progresses and the timing of surgical intervention in CP with pancreatic and biliary hypertension is delayed, there is a notable increase in the levels and relative area of expression of the relevant fibrosis markers. As fibrosis progresses, the stroma thickens, and the exocrine and endocrine portions of the pancreas are progressively replaced. Accordingly, direct surgical intervention on the pancreas within the early stages – up to three years from the onset of CP symptoms – is theoretically justified, as at this stage, the pancreatic parenchyma still retains its morpho-functional structure. Foci of pancreatic intraepithelial neoplasia observed in

patients with CP further support the notion that CP with pancreatic and biliary hypertension is a premalignant condition. Notably, the neoplastic process originates in the ductal epithelium, most frequently from the MPD. Subsequently, this can result in the development of ductal adenocarcinoma of the pancreas. This underscores the rationale for performing pancreatic duct resection in the surgical management of CP with pancreatic and biliary hypertension.

An essential role of PSCs in developing pancreatic parenchymal fibrosis in CP has been established. Fibrotic lesions of the pancreas are irreversible, and the resulting morphological and structural changes lead to both exocrine and endocrine dysfunction.

More than three years after the onset of clinical manifestations of CP, type I collagen expression was observed in the acinar tissue, with thin strands detected within the islets of Langerhans. Intraepithelial ductal neoplasia was also identified, which may later progress to ductal adenocarcinoma of the pancreas.

It has been demonstrated that the improvement in surgical outcomes for complicated forms of CP with biliary and ductal hypertension is achieved through planned surgery within the first three years of CP development, before the onset of irreversible pathological changes, including the potential for oncological transformation of the pancreas.

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