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Assessment of T- and B-lymphocytes and VEGF-A in acute pancreatitis

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Abstract

Aim: Highlighting certain characteristics of T- and B-lymphocytes, as well as of vascular endothelial growth factor-A (VEGF-A) as a prognosis factors for patients suffering from acute pancreatitis, with the scope of establishing diagnosis and therapy. *Materials and Methods*: Pancreatic tissue samples were analyzed, originating from 21 patients deceased due to acute pancreatitis, between the years 2015 and 2016. The study group was subdivided into two subgroups by pathology duration. As control group, pancreatic tissue fragments were sampled from eight patients, deceased due to non-pancreatic acute surgical abdomen (NPASA). *Results*: By analyzing the immunohistochemical expression of T-lymphocytes (immunomarked with the anti-CD3 antibody) and of B-lymphocytes (immunomarked with the anti-CD20 antibody), both on the tissue sections originating from the study patients, as well as the control group patients, we established that these were mostly present in the interstitium, following which the acini, less frequently in the islets, in general with B-lymphocytes being less present than T-lymphocytes. VEGF-A also tends to emerge in the acini periphery, at the border with the stromal connective tissue, while the islets are almost negative for VEGF-A. *Conclusions*: We can state that the decreased expression of T- and B-lymphocytes in normal pancreatic tissue, surrounding the necrosis areas, can be used as an aggravated prognosis factor for patients diagnosed with acute pancreatitis. Moreover, the increased immunohistochemical expression of VEGF-A can play an important role in tracking the evolution and pathology of acute pancreatitis.

Keywords: T-lymphocytes, B-lymphocytes, VEGF-A, acute pancreatitis.

Introduction

The retroperitoneal position of the pancreas and the presence of multiple receptors in its structure, which can trigger important vegetative phenomena, lead to marked difficulty and complexity in establishing the diagnosis of acute pancreatitis. Acute pancreatitis (AP) is considered a syndrome of glandular autodigestion, with multifactorial etiology, which creates a dramatic clinical picture, with severe evolution and high mortality rate [1].

In this context, the corroboration of clinical data with biochemical tests (hemoleucogram, enzymology methods, C-reactive protein, fibrinogen, urine trypsinogen) and imagistic assessment [computed tomography (CT), magnetic resonance imaging (MRI), ultrasonography] is imperative.

The extensive development of immunohistochemical techniques allowed the use of lymphocyte marking, especially in oncodiagnosis, prognosis and oncological therapy.

Extending the application of this technique to testing of chronic inflammation is being currently attempted [2]. The "classic" filiation of anti-inflammatory/anti-infectious cellular intervention affords lymphocyte populations a later participation in the phases of the processes mentioned. In this way, we might explain the insufficient study of lymphocyte subsets in acute inflammatory phenomena, such as acute necrotizing-hemorrhagic pancreatitis.

The aim of our research was chosen specifically, because of the scarcity of literature data referring to the characteristics of lymphocyte subsets, evidenced through immunohistochemical techniques, in acute pancreatitis these subsets being CD3 expressed by T-lymphocytes and CD20 represented by B-lymphocytes. Considering the important hemorrhagic component in the evolution of acute pancreatitis, vascular endothelial growth factor-A (VEGF-A) marking was also practiced.

These determinations can illustrate aspects of the microclimate in which the pathological phenomena take place, with impact both on establishing the diagnosis and prognosis as well as therapy.

Materials and Methods

In the present study, there were analyzed pancreatic tissue samples originating from 21 patients deceased due to acute pancreatitis, between the years 2015 and 2016. The study group was subdivided into two subgroups by the time of disease. Thus, if death occurred within two weeks from the clinical and paraclinical diagnosis of acute pancreatitis (abdominal pain, serum levels of pancreatic amylase and lipase and CT changes), the subgroup AP1 was formed (acute pancreatitis patients deceased within two weeks from diagnosis through multiple organ dysfunction syndrome – MODS), while patients deceased

in the following months since the diagnosis made up subgroup AP2 (acute pancreatitis patients deceased within months through extensive retroperitoneal necrosis and/or sepsis). As control group, pancreatic tissue fragments were sampled from eight patients, deceased due to non-pancreatic acute surgical abdomen (NPASA).

The patients' autopsies were performed in the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania. After revisiting the pathology, new slides were cut from selected blocks and processed for immunohistochemistry. Briefly, the cross-sections were deparaffinized and rehydrated in grading alcohol series, and then processed for automated immunohistochemistry, in a Leica Bond Max immunostainer (Leica Biosystems, Nussloch, Germany), which includes proprietary protocols for antigen retrieval, endogenous signal block, primary antibody incubation, amplification, detection and counter-staining with Hematoxylin.

As primary antibodies, we utilized primary mouse anti-human monoclonal antibodies directed against CD20 for T-lymphocytes (clone MJ1, Leica, PA0906, ready to use), CD3 for B-lymphocytes (clone LN10, Leica, PA0553, ready to use), and VEGF-A (clone VG1, Dako, M7273, diluted as 1:50). Finally, the slides were coverslipped with a permanent mounting medium.

To complete the demographic, clinical and biological data, we used both the clinical patient charts and histo-pathological bulletins.

The study was approved by the Ethics Committee of the University of Medicine and Pharmacy of Craiova and in each case we obtained an informed consent from the patient family.

The images were obtained with a Nikon 55i microscope (Apidrag, Bucharest, Romania), equipped with a 5 Mp camera with charge-coupled device (CCD) sensor, outfitted with 10×, 20×, 40× and 60× high-powered magnification objectives, which increased the detail level of the observed structures. The images were then further processed with the Image ProPlus AMS 7 software (Media Cybernetics, Rockville, MD, USA). As for the analysis of the immunohistochemical expression of VEGF-A, the images were analyzed from the perspective of both the signal area, as well as integrated optical density (IOD). Furthermore, the percentage of lymphocytes immunomarked with anti-CD3 and anti-CD20 antibodies was determined for the immunohistochemical expression of the above-mentioned lymphocytes.

Finally, all the data obtained with Image ProPlus AMS software were exported in Microsoft Office Excel (Microsoft Corporation, Redmond, Washington, USA), graphically represented and analyzed with SPSS software (IBM SPSS Statistics *ver.* 20.0) using Student's *t*-test and *Z*-test for proportions. All results were expressed as average ± standard deviation. We considered *p*-values <0.05 as being statistically significant in all cases.

Results

Our study included 42.85% women (n=9) and 57.15% men (n=12) (Figure 1). Taking into consideration the fact that population in Dolj County (Romania) is made up of around 48.8% men and 51.2% women [3], we can state that a statistically highly significant difference exists between these proportions and the proportions determined for the number of patients in the study group, the value of p being determined through the *Z*-test for proportions to the value of <0.001.

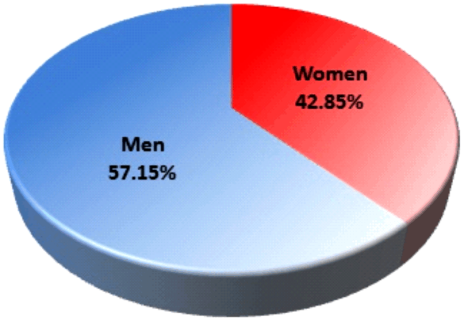

Figure 1 – ***Patients with acute pancreatitis: distribution by gender.***

In regards to the hemoleucogram data, it is to be mentioned that the level of lymphocytes on admission, was lower for patients in the AP1 subgroup compared to the AP2 subgroup, with a statistically significant difference (p=0.037), while for other hemoleucogram parameters no statistically significant differences were found. This data is summarized in Table 1.

Table 1 – ***Partial results of blood routine tests comparing AP1 and AP2 groups on admission***

Parameter	AP1	AP2	*P*-value[1]
HGB [g/dL]	12.73±1.62	12.94±1.55	0.471
HCT [%]	42.13±3.45	43.76±4.24	0.346
RBC [10^6/mm^3]	4.34±0.36	4.25±0.59	0.388
WBC [10^3/mm^3]	13.72±2.19	14.75±2.03	0.291
LYM [%]	19.40±2.23	25.33±3.68	0.037*
MON [%]	5.21±0.99	6.36±0.75	0.093
GRA [%]	72.39±2.94	68.53±3.89	0.089
PLT [10^3/mm^3]	286.43±116.01	318.19±66.18	0.349

[1]Student's *t*-test; *P<0.05, statistically significant; AP: Acute pancreatitis; HGB: Hemoglobin; HCT: Hematocrit; RBC: Red blood cells; WBC: White blood cells; LYM: Lymphocytes; MON: Monocytes; GRA: Granulocytes; PLA: Platelets.

The histopathological study of the pancreatic tissue samples of the 21 deceased patients, who had been diagnosed with acute pancreatitis, highlighted both areas of normal pancreatic tissue as well as areas of tissue necrosis, structured and unstructured (Figure 2, A–D). Areas of incipient cytosteatonecrosis with cellular ballooning and irreversible nuclear lesions, as well as areas where necrosis first appears to affect Langerhans islets and only afterwards pancreatic acini were also found.

By analyzing the immunohistochemical expression of T-lymphocytes (immunomarked with anti-CD3 antibody), both on tissue sections originating in study group patients as well as control group patients, we determined that they were relatively numerous, especially in the interstitium, followed by the acini and less frequently in islets (Figure 3).

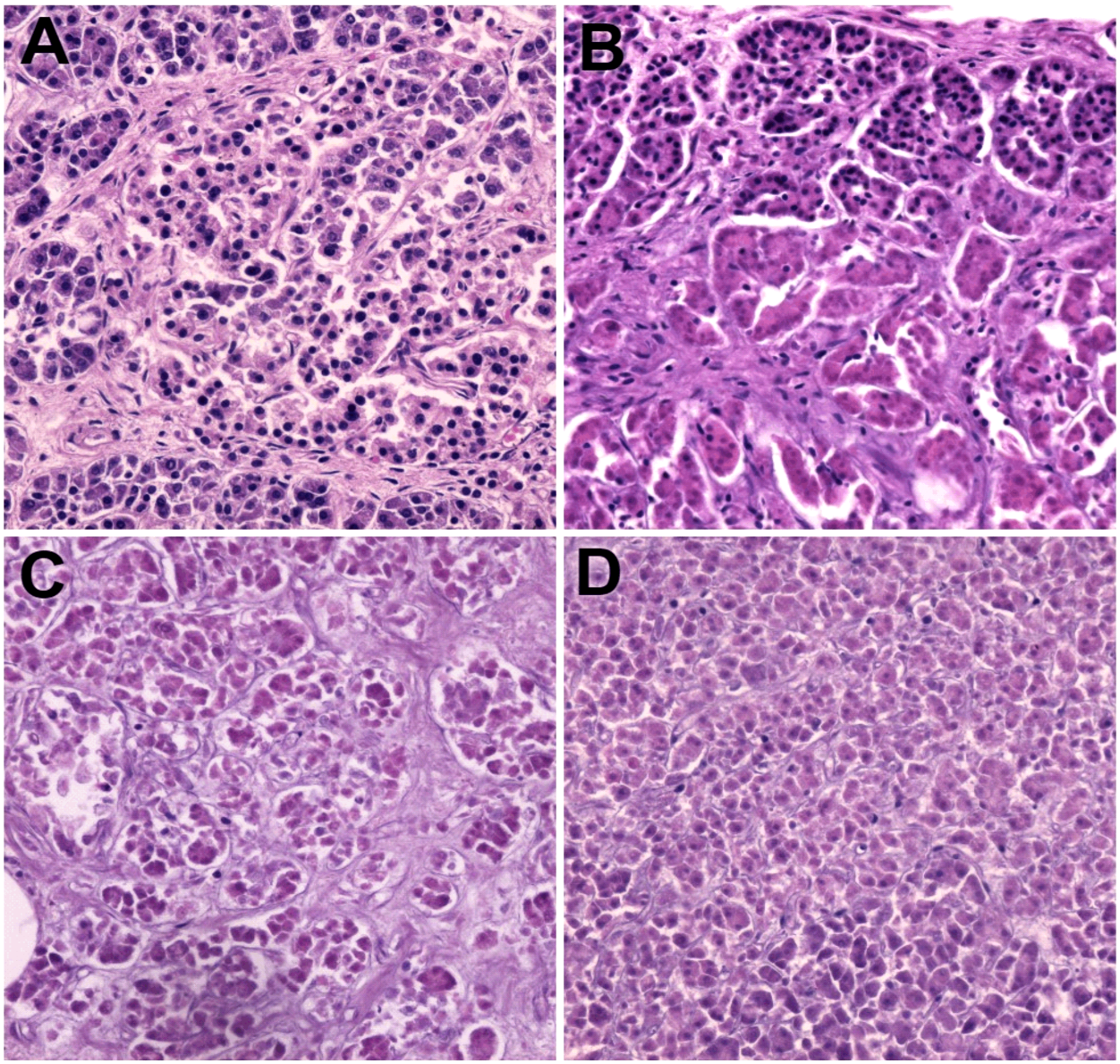

Figure 2 – ***Pancreatic tissue, HE staining, ×200: (A) Acini and normal islets; (B) Limited acinus necrosis with intact acinus; (C) Structured pancreatic necrosis; (D) Unstructured pancreatic cytosteatonecrosis.***

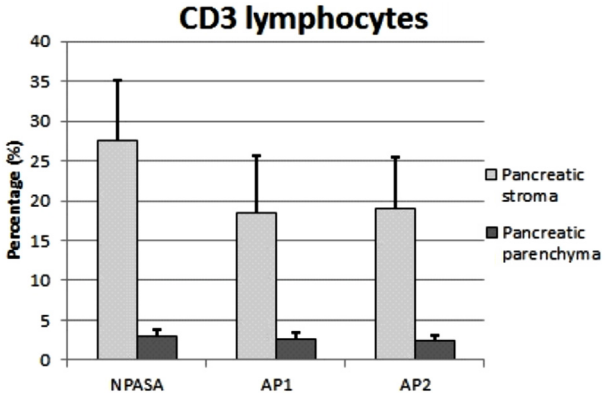

Figure 3 – ***CD3 lymphocytes expression in control group (NPASA) and in the two subgroups of patients with acute pancreatitis (AP). NPASA: Non-pancreatic acute surgical abdomen.***

The percentage of T-lymphocytes for patients from the control (NPASA) group was higher (27.53±7.6% for pancreatic stroma and 3.03±0.83% for pancreatic parenchyma) than in the case of subgroup AP1 patients (18.4±7.22% for pancreatic stroma and 2.67±0.7% for pancreatic parenchyma) or of subgroup AP2 patients (19.02±6.51% for pancreatic stroma and 2.48±0.54% for pancreatic parenchyma) (Figure 4). It is to be mentioned that significant statistical differences both between the control group and the AP1 subgroup (p=0.013) and the control group and the AP2 subgroup (p=0.015) were recorded for T-lymphocytes expression in the pancreatic stroma, while between the AP1 and AP2 subgroups no statistically significant differences were recorded (p=0.42). However, no statistically significant differences were recorded for T-lymphocyte expression in the pancreatic parenchyma, between neither the control group and the AP1 subgroup (p=0.181), the control group and the AP2 subgroup (p=0.079), nor between the AP1 and AP2 subgroups (p=0.277).

By analyzing the immunohistochemical expression of B-lymphocytes (immunomarked with anti-CD20 antibody) both on tissue sections originating in study group patients

as well as control group patients, we determined that they were less numerous than T-lymphocytes, while also appearing mostly in the interstitium and in the acini and less frequently in islets (Figure 5).

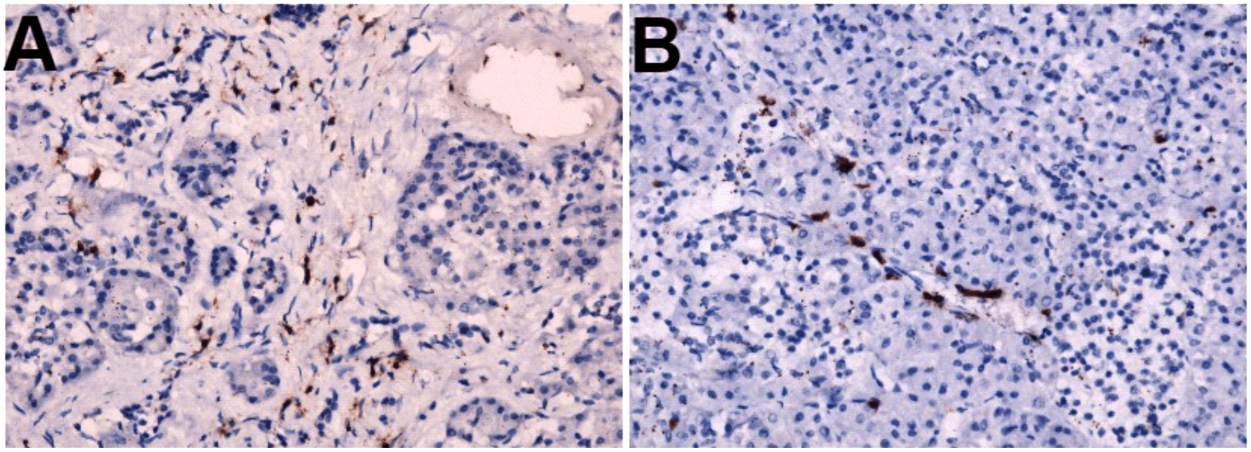

Figure 4 – ***CD3 immunostaining, ×200: (A) CD3 in pancreatic stroma; (B) Perivascular CD3 in pancreatic islets and acini.***

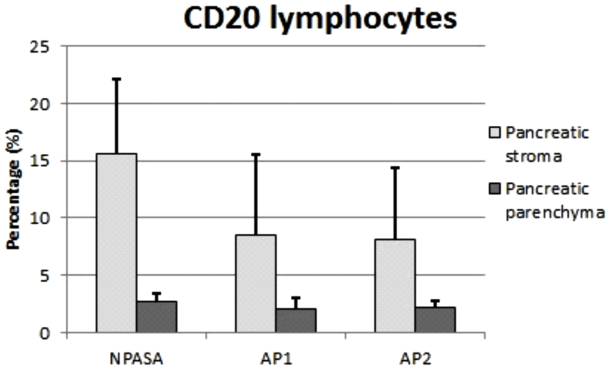

Figure 5 – ***CD20 lymphocytes expression in control group (NPASA) and in the two subgroups of patients with acute pancreatitis (AP). NPASA: Non-pancreatic acute surgical abdomen.***

The percentage of B-lymphocytes for patients from the control (NPASA) group was higher (15.66±6.51% for pancreatic stroma and 2.66±0.74% for pancreatic parenchyma) than in the case of subgroup AP1 patients (8.52±7.06% for pancreatic stroma and 2.08±0.89% for pancreatic parenchyma) or of subgroup AP2 patients (8.15±6.22% for pancreatic stroma and 2.23±0.58% for pancreatic parenchyma) (Figure 6). It is to be mentioned that significant statistical differences both between the control group and the AP1 subgroup (p=0.027) and the control group and the AP2 subgroup (p=0.016) were recorded for B-lymphocytes expression in the pancreatic stroma, while between the AP1 and AP2 subgroups no statistically significant differences were recorded (p=0.45). However, no statistically significant differences were recorded for B-lymphocyte expression in the pancreatic parenchyma, between neither the control group nor the AP1 subgroup (p=0.089), the control group and the AP2 subgroup (p=0.107) nor between the AP1 and AP2 subgroups (p=0.351).

By analyzing the expression of VEGF-A in the pancreatic tissue of control group patients, as well as the deceased acute pancreatitis patients, we can state that VEGF-A also tends to emerge in the acini, at its periphery with the stromal connective tissue, while the islets are almost negative for VEGF-A expression (Figure 7). As for the signal area for VEGF-A, we observed lower values for the control (NPASA) group (4140.63±943 μm^2) than the AP-group (4524.94±745 μm^2), a statistically significant difference was recorded in this case (p=0.042) (Figure 8). On the other hand, in regards to IOD for VEGF-A, we observed lower values for the control (NPASA) group (589937.2±65469.47) than the AP group (639784.9±63208.13), in this case a statistically significant difference (p=0.035) was recorded (Figure 9).

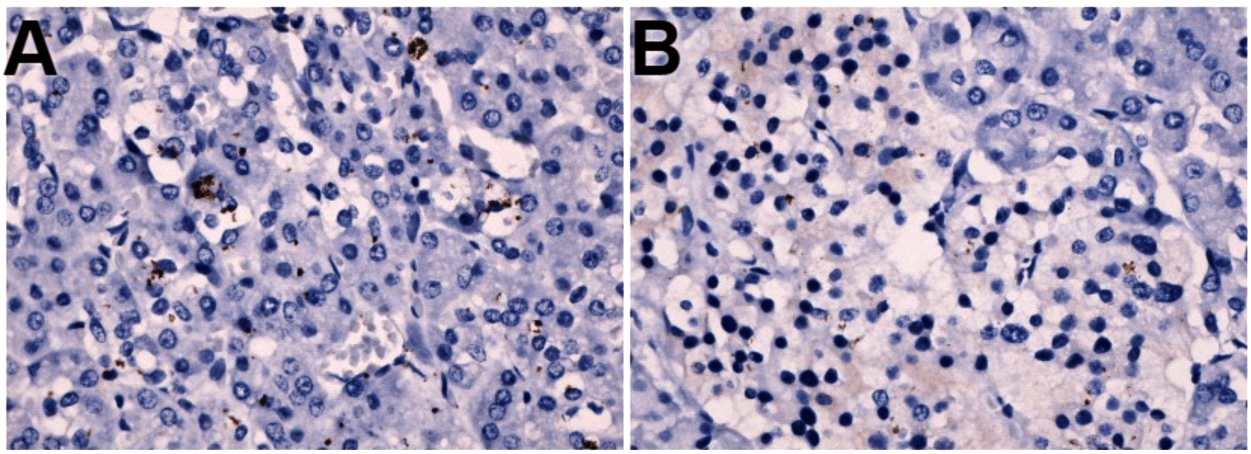

Figure 6 – ***CD20 immunostaining, ×400: (A) CD20 less frequent emerges in acini first; (B) CD20 negative islet.***

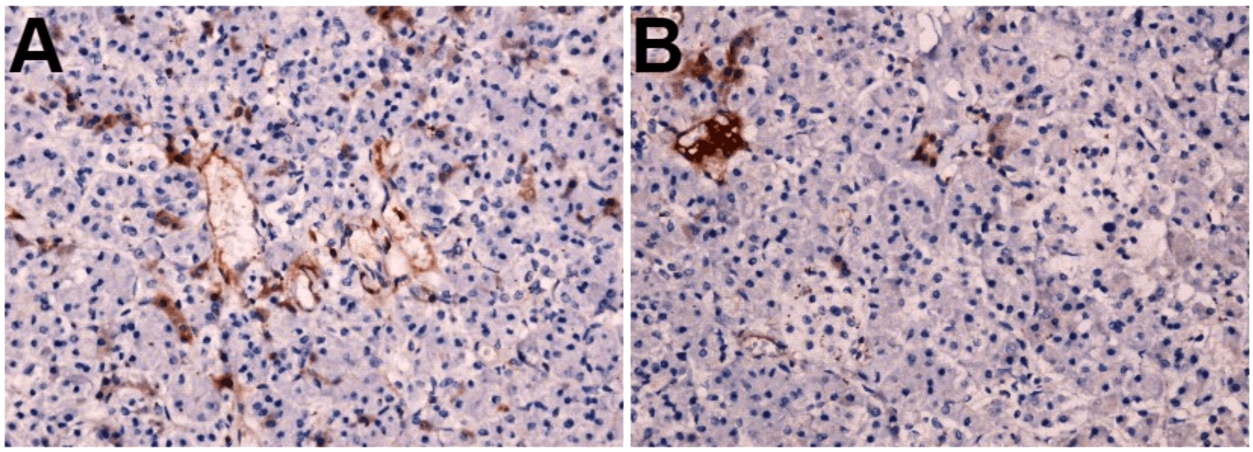

Figure 7 – *VEGF-A immunostaining, ×200: (A) VEGF-A in acini, towards periphery; (B) VEGF-A negative islets.*

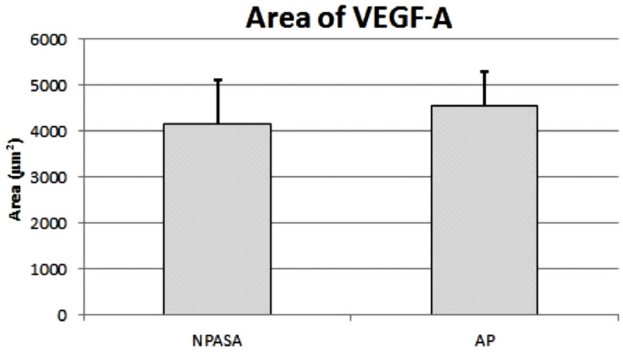

Figure 8 – *Area of the VEGF-A expression in control group (NPASA) and in group of patients with acute pancreatitis (AP). NPASA: Non-pancreatic acute surgical abdomen.*

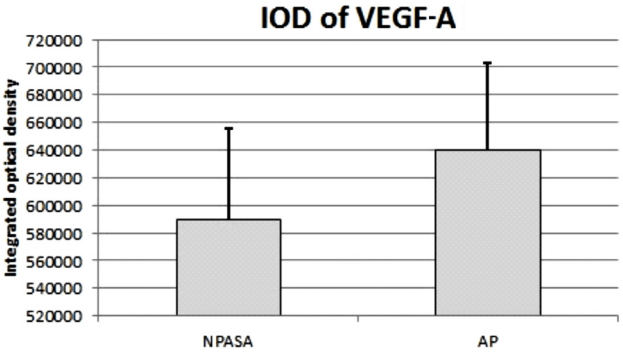

Figure 9 – *Integrated optical density (IOD) of the VEGF-A expression in control group (NPASA) and in group of patients with acute pancreatitis (AP). NPASA: Non-pancreatic acute surgical abdomen.*

It is to mention that these observations were made on the acini left intact around the necrosis areas, since the tissue necrotic destruction area was negative for all markers.

Discussion

Acute pancreatitis is defined as multi-etiological pancreatic inflammation, with a progressive clinical evolution from light form to severe, with the possible emergence of "multiple organ failure", with a mortality rate estimated at 20–30%. The pathogenic process is incompletely clarified, due to the considerable difficulty of diagnosis [4].

Recent research based on analyzing lymphocyte participation to the inflammatory pancreatic response, shows that persistent lymphocyte numerical decrease might be an independent marker of progressive inflammation, regulatory T-cells being closely linked to the severity of the incipient stage of pancreatic pathology, even as far as being a predictive factor for prognosis [5–7].

The motivation for our study lay in the literature data previously discussed, going in depth on the lymphocyte line research and highlighting the changes to some immunomarked lymphocyte subsets – with anti-CD3 antibodies for T-lymphocytes and anti-CD20 antibodies for B-lymphocytes.

By statistical calculation and processing the percentages of immunomarked cells, statistically significant differences were recorded for the presence of T-lymphocytes in the pancreatic stroma – when comparing the control group data with those of the two subgroups (p=0.013 for AP1 and p=0.015 for AP2, respectively). Statistical interpretation of the data comparison of the subgroups for marked T-lymphocytes present in stroma does not yield a statistically significant difference (p=0.420). Statistically significant differences were also recorded for anti-CD20 antibodies marked B-lymphocytes, when comparing the control group and the AP1 subgroup data (p=0.027) and the control group and AP2 subgroup data (p=0.015), respectively. There were no statistically significant differences between the two subgroups for immunomarked B-lymphocytes, similarly to the case of T-lymphocytes.

Literature data analysis of previous studies on the lymphocyte changes in acute pancreatitis show decrease of lymphocyte numbers in the incipient stages of AP [8, 9]. This confirms the unpublished partial results of our research group, which show a decrease in lymphocyte number in AP evolution – a statistically significant decrease between day 1 and 3 from patient hospitalization. Simultaneously, our current results show a decreased number of lymphocytes, a significant change when comparing the data of the two subgroups – in which the decrease of lymphocyte number was more significant in AP1 subgroup, with patients deaths in the first two weeks from the diagnosis, compared to AP2 subgroup, in which death occurred in months since establishing the existence of pancreatic pathology. This data allows us to consider lymphocyte evolution as an important prognosis factor,

in accordance with those mentioned by [10–14]. Taking into account the hemorrhagic nature of AP changes, we considered it important to study the presence of VEGF-A, through immunomarking, and the intervention of this factor in the angiogenesis of physiological and pathological processes (inflammation, cicatrization, tumor development and metastasis) [15, 16].

In our study, we observed a statistically significant (p=0.042) reduced presence of the VEGF-A marking area in the control group, in comparison with the AP patient groups.

As for the IOD examination of VEGF-A, a lower value was obtained in the control group, in comparison with the patient group, which was also statistically significant (p=0.035). The increase of VEGF-A in AP conditions is predictably, associated with the inflammatory process and endothelial damage. Our results could not be compared with literature data, because the majority of VEGF-A research was done in the oncological domain.

Our research must continue on an even larger number of patients, to achieve better statistical support. The reduced number of literature references explains the need to intensify research of lymphocyte changes in inflammatory pancreatic pathologies, since it can supply an important marker for diagnosis and most especially prognosis of the pathology.

Conclusions

We can state that the decrease in T- and B-lymphocytes expression in normal pancreatic tissue around necrosis areas can be used as an aggravated prognosis factor for acute pancreatitis patients. On the other hand, the increase in VEGF-A immunohistochemical expression can highlight an important role for it in tracking the evolution and pathogeny of acute pancreatitis. All these elements can constitute the focus of future therapeutic strategies, applied differentially to this extremely severe and difficult to prognosis pathology.

Conflict of interests

The authors declare that they have no conflict of interests.

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