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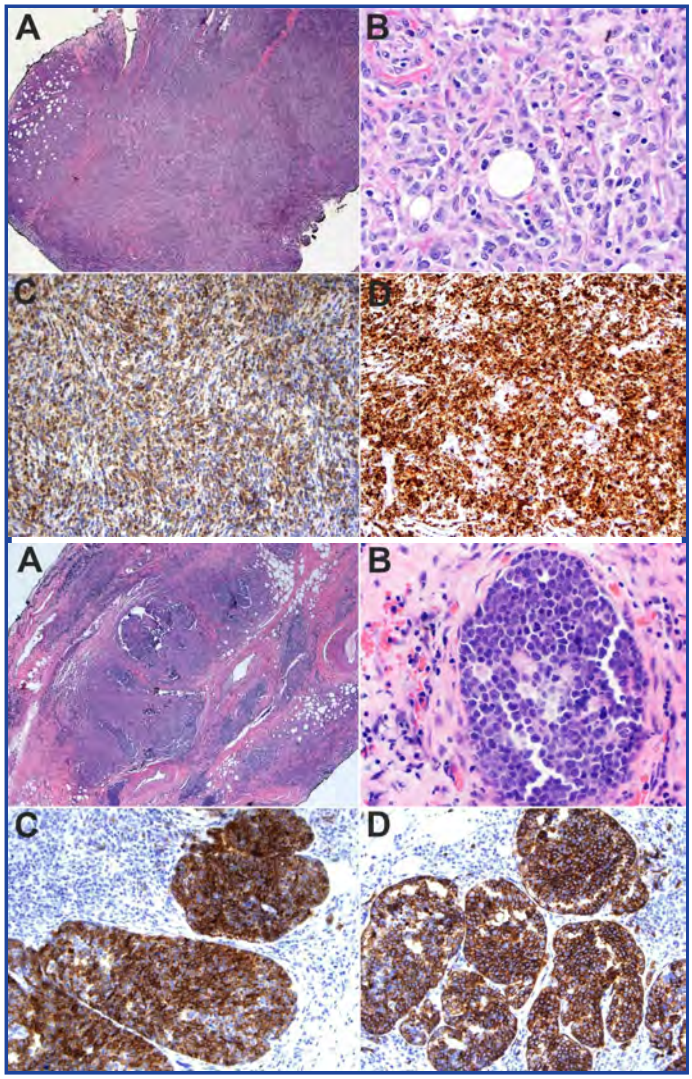

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RESEARCH ARTICLE

Endogenous hepatic toxicity and oxidative stress in the complications of gallstones associated with cholestatic jaundice

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ABSTRACT

Introduction. Cholestatic jaundice installed in gallstones is considered a multifactorial pathogenetic process. The correlation of endogenous hepatic intoxication with the reaction of the pro- and antioxidant system, characteristic of various complications of gallstones associated with cholestatic jaundice, remains an undefined subject.

Material and methods. The study included the investigation of 105 patients with complicated gallstones and associated cholestatic jaundice, and the control group – 35 patients with uncomplicated gallstones. The age of the patients varied between 51 and 72 years, with an F/M ratio of 3/1.

Results. The degree of endotoxemia, assessed at hospitalization by peptides with average mass values, was consistent with the inflammation present in groups 2 and 3, reporting a statistically significant difference compared to the control group [p c/2 < 0.001 (t = 11.1); p c/3 < 0.001 (t = 11.07)]. DAM, as the final product of lipid peroxidation, evaluated at hospitalization, shows double marked values in all groups compared to the control group [p < 0.001 (t = 10.7); (t = 10.9); (t = 16.5)]. The antioxidant activity, assessed at hospitalization, shows a statistically significant decrease in superoxide dismutase values [p < 0.001 (t = 6.4); (t = 4.1); (t = 5.7)], as well as catalase [p < 0.001 (t = 6.7); (t = 5.6); (t = 7.2)] in all groups, by 30-40% compared to the control group.

Conclusions. In established cholestatic jaundice, there is an obvious activation of lipoperoxidation processes, contributing to the increase of endogenous intoxication and early suppression of antioxidant activity. There is a direct relationship between the increase in prooxidant activity and the level of endogenous intoxication in all complications of gallstones associated with cholestatic jaundice, but it is more elevated in infectious complications, indicating a need for an early approach.

Keywords: oxidative stress, endogenous intoxication, cholestatic cholemia, cholestatic jaundice.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript

Correlation of systemic oxidative stress with endogenous intoxication characteristic for different complications of gallstones associated with cholestatic jaundice.

The research hypothesis

Assessment of oxidative stress deviations dependent on the level of hepatic endotoxemia present in complications of gallstones associated with cholestatic jaundice.

The novelty added by the manuscript to the already published scientific literature

A complex analysis of the systemic oxidative stress, in correlation with the endogenous intoxication present in various complications of gallstones associated with cholestatic jaundice, was carried out. This involved the evaluation of parameters related to the pro- and antioxidant systems, the level of peptides with average mass, and the biochemical parameters characterizing liver syndromes: cholestasis, liver cytolysis, liver synthesis function, and liver mesenchymal inflammation.

Introduction

Being part of the five most frequently encountered syndromes in surgical practice, cholestatic jaundice syndrome in the specialized literature is exposed in both benign and malignant pathologies. According to existing data in the literature, the presence of cholestatic jaundice in the complications of gallstones is reported in 10-20% of cases, with choledocholithiasis as the main cause but also being characteristic of acute biliary pancreatitis, acute cholecystitis, cholangitis [1-2]. Although significant progress has been made in recent years in the diagnosis and treatment of mechanical jaundice syndrome in gallstones, there are still many problems and challenges [3-5]. Considered a medical-surgical emergency, cholestatic jaundice syndrome requires a surgical approach as early as possible, but in some cases, biliary decompression, carried out both by laborious surgical interventions and by endoscopic methods, induces a worsening of the patient's condition with the installation of some severe evolutionary complications, caused by existing homeostatic disturbances [6].

In the current concepts of publications on the subject, cholestatic jaundice installed in gallstones is considered a multifactorial pathogenetic process. It is known that obstructive cholestasis induces serious functional disorders of homeostasis, causing the suppression of the metabolic activity of the liver, inhibiting the immune system and hemostasis mechanisms, reducing over time the excretory function of the kidneys, and limiting the barrier function of the digestive tract [7]. These data are an argument that the severity of the pathological condition is determined by a common link, consisting of the endogenous intoxication syndrome (EI) characteristic of the complications of gallstones and the occurrence in these conditions of systemic oxidative stress (OS). The level of "peptides with average mass" (PAM) is considered a universal biomarker of EI [8]. PAM are components with high biological activity and a polyfunctional spectrum of action that are formed upon the intensification of non-enzymatic proteolysis, including blood protein proteolysis. An essential feature of PAM is the increased biological capacity of the compounds in this group, the action of which further aggravates the existing metabolic disorders, inducing their continuous formation with the appearance of a "vicious circle". The severity of endotoxemia reflects the imbalance between the formation of endotoxins and the body's capacities for their transformation and biological elimination [8, 9]. In turn, OS develops because of the imbalance between the hyperproduction of active forms of oxygen and the insufficiency of the body's antioxidant capacity, which usually increases suddenly in various toxic states [10]. Based on existing studies aimed at the pathogenesis of the disturbances produced by cholestatic jaundice, the importance of the influence of free radicals that ultimately induce hepatocyte damage is proven [10-12].

However, the analysis of the literature reveals few studies with reference to the role of endogenous intoxication in the occurrence of systemic oxidative stress in cholestatic jaundice associated with gallstone complications. Additionaly, the available literature data do not contain information that aims at the deviations of oxidative stress depending on the degree of hepatic endogenous intoxication present in different complications of gallstones.

Material and methods

The clinical research was carried out within the *Nicolae Anestiadi* Department of Surgery of *Nicolae Testemițanu* State University of Medicine and Pharmacy. The study included the investigation of 105 patients with complicated gallstones and associated cholestatic jaundice, and the control group consisted of 35 patients with uncomplicated gallstones. Depending on the evolutionary complications present, the patients from the core investigation batch were divided into 3 homogeneous groups: group 1 – chronic gallstones and cholestatic cholemia (n = 35), group 2 – acute lithiasic cholecystitis, cholangitis, and cholestatic cholemia (n = 35), group 3 – acute biliary pancreatitis and cholestatic cholemia (n = 35). The age of the patients varied between 51 and 72 years. The female/male ratio was estimated to be 3/1. The criteria for including patients in the basic group were the presence of gallstones in the complication phase, associated with cholestatic jaundice, confirmed by diagnostic parameters:

- clinical – history of gallstones or biliary colic at onset, presence of skin jaundice;
- paraclinical – laboratory characteristic tests – elevated values of serum bilirubin, specifically the direct fraction, elevated values of alkaline phosphatase, elevated values of leukocytes, fibrinogen, serum urea, amylase, as well as marked values of ALAT; and imaging confirmation of gallstones and the diameter of the main bile duct > of 0.8 cm.

Cases that met the stated criteria were included in the study. Blood samples were collected during hospitalization until the start of treatment and after biliary decompression. Endogenous intoxication, ascertained by the values of peptides with average mass (PAM), was determined according to the procedure by Gabrielian N.I. *et al.* (1984) [13]. The

comparative evaluation of systemic OS in groups was performed by assessing the correlation in the blood serum between the intensity of peroxidic oxidation of lipids (POL) and the antioxidant potential (AP). The intensity of POL was determined by the values of the final product of lipoperoxidation – malonic dialdehyde (MDA), assessed according to the procedure modified by Gudumac V. *et al.* (2010) [14]. The antioxidant potential was expressed by superoxide dismutase (SOD) and catalase (CAT) values. SOD activity was estimated according to the procedures modified by Gudumac V., Tagadiuc O. *et al.* (2010) [15]. CAT activity was determined according to the method modified by Baciu E. and Nastas I. (1996) [16].

The data of each case were recorded in a standardized form and processed using the operative methods of statistical evaluation of the computer program Windows 2010 (GraphPad Software, Inc.) and Excel (Microsoft®, USA) 2010, Medcalc. The obtained results were compared and reported in relation to the values of the parameters of the control batch; also, the deviations of the values in the batches were compared. The statistical analysis was performed applying the Mean (M), Mean Error (m), Dispersion, Standard Deviation, Asymmetry (Skew), Coefficient of variation (%), and T-Student statistic test. The discrepancy with the margin of error <5% was considered statistically significant ($p < 0.05$). For paired group comparisons, the ANOVA test and the Tukey HSD test were used.

Results

The analysis of the examination results confirms the homogeneity of the groups depending on age and concomitant pathologies, with an insignificant difference in group II [p c/1 < 0.05 (t = 2.55); p c/2 > 0.05 (t = 1.10); p c/3 < 0.01 (t = 2.84)]. The homogeneity of the groups is also with insignificant deviations between groups I and II [p 1/2 > 0.05 (t = 1.64); p 1/3 < 0.01 (t = 4.55); p 2/3 < 0.01 (t = 3.31)]. An earlier hospitalization of patients was highlighted in groups II and III, caused by the presence in these patients of the painful, progressive inflammatory component, an important factor that contributes to the reduction of the duration of jaundice until hospitalization with data of medium-grade jaundice [p 1/2 < 0.001 (t = 3.5); p 1/3 < 0.001 (t = 9.6); p 2/3 < 0.001 (t = 8.6)]. Survey data and results of laboratory parameters in the study groups and the control group at hospitalization are presented in Table 1.

Table 1. Survey data and results of laboratory parameters at hospitalization.

Markers	Control group (n=35)	Group 1 (n=35)	Group 2 (n=35)	Group 3 (n=35)	p1/2	p1/3	p2/3
Age	59.1±1.12	64.2±1.65*	61.4±1.75 ****	53.1±1.79**	p>0.05 t=1.64	p<0.01 t=4.55	p<0.01 t=3.31
Duration of jaundice	0	4.44±0.38	3.19±0.19	1.48±0.06	p<0.001 (t=3.5)	p<0.001 (t=9.6)	p<0.001 (t=8.6)
Temperature (°C)	37±0.5	37±0.5****	38±0.5****	37±0.5****	p>0.05 (t=1.41)	p>0.05 (t=0)	p>0.05 (t=1.41)
Alkaline phosphatase (U/l)	149.8±12.4	881.14±78.3***	541.4±34.6***	760.0±83.0***	p<0.001 (t=3.9)	p>0.05 (t=1.06)	p<0.05 (t=2.4)
Total bilirubin (mmol/l)	12.1±0.79	119.31±8.7***	78.55±5.3***	80.28±4.9***	p<0.01 (t=4.0)	p<0.01 (t=3.9)	p>0.05 (t=0.2)
Direct bilirubin (mmol/l)	4.2±0.06	88.2±6.7***	56.2±4.2***	54.8±3.4***	p<0.01 (t=4.0)	p<0.01 (t=4.4)	p>0.05 (t=0.3)
ALAT (U/l)	19.57±0.97	128.7±8.2***	207.1±12.1***	289.62±9.4***	p<0.001 (t=5.3)	p<0.001 (t=12.9)	p<0.001 (t=5.4)
Blood amylase (U/l)	34.57±1.4	37.9±1.9****	43.1±2.3****	164.9±9.7***	p>0.05 (t=7.4)	p<0.001 (t=12.8)	p<0.001 (t=12.2)
Fibrinogen (U/L)	2.69±0.06	3.52±0.1***	4.21±0.1***	4.43±0.17***	p<0.01 (t=4.87)	p<0.01 (t=4.61)	p>0.05 (t=1.1)
Blood leukocytes (n)	6.23±0.23	7.51±0.31**	12.12±0.52***	10.24±0.43***	p<0.01 (t=7.61)	p<0.01 (t=5.15)	p<0.01 (t=2.78)
Neutrophil (n)	3.86±0.22	7.5±0.39***	13.1±0.43***	9.9±0.56***	p<0.01 (t=12.5)	p<0.001 (t=3.74)	p<0.01 (t=5.31)
Urea (U/l)	6.23±0.21	6.56±0.24****	7.0±0.21*	7.92±0.47**	p>0.05 (t=1.37)	p<0.05 (t=2.57)	p>0.05 (t=1.78)
Creatinine (U/l)	68.8±1.19	81.0±3.5**	86.58±3.2**	82.17±2.8**	p>0.05 (t=1.17)	p>0.05 (t=0.26)	p>0.05 (t=1.03)

Note: p<0.05*; p<0.01**; p<0.001***; p>0.05**** - compared to the values of the control group; Group1 - chronic gallstones and cholestatic cholemia; Group 2 - acute lithiasic cholecystitis, cholangitis and cholestatic cholemia; Group 3 - acute biliary pancreatitis and cholestatic cholemia; p1/2 - compared to the values of the group 1 to 2; p1/3 - compared to the values of the group 1 to 3; p2/3 - compared to the values of the group 2 to 3; ALAT – alanine aminotransferase; t - test used to compare the means of two independent samples.

Examination of the levels of biochemical markers of cholestasis reported a statistically significant difference in all study groups compared to the control group, confirming the existing homeostatic disturbances: alkaline phosphatase [p < 0.001 (t = 8.2); (t = 3.9); (t = 7.27)], direct bilirubin [p < 0.001 (t = 12.5); (t = 12.3); (t = 14.9)]. The comparative

examination of parameters in groups shows an insignificant difference of alkaline phosphatase in groups 1 and 3 [p > 0.05 (t = 1.06)] and direct bilirubin in groups 2 and 3 [p > 0.05 (t = 0.3)]. However, according to the examinations, the severity of the liver disease is correlated with the markers of the inflammation syndrome - elevated values of fibrinogen, blood leukocytosis, neutrophils with a significant statistical difference (p < 0.001), more evident in groups 2 and 3 (Fig.1).

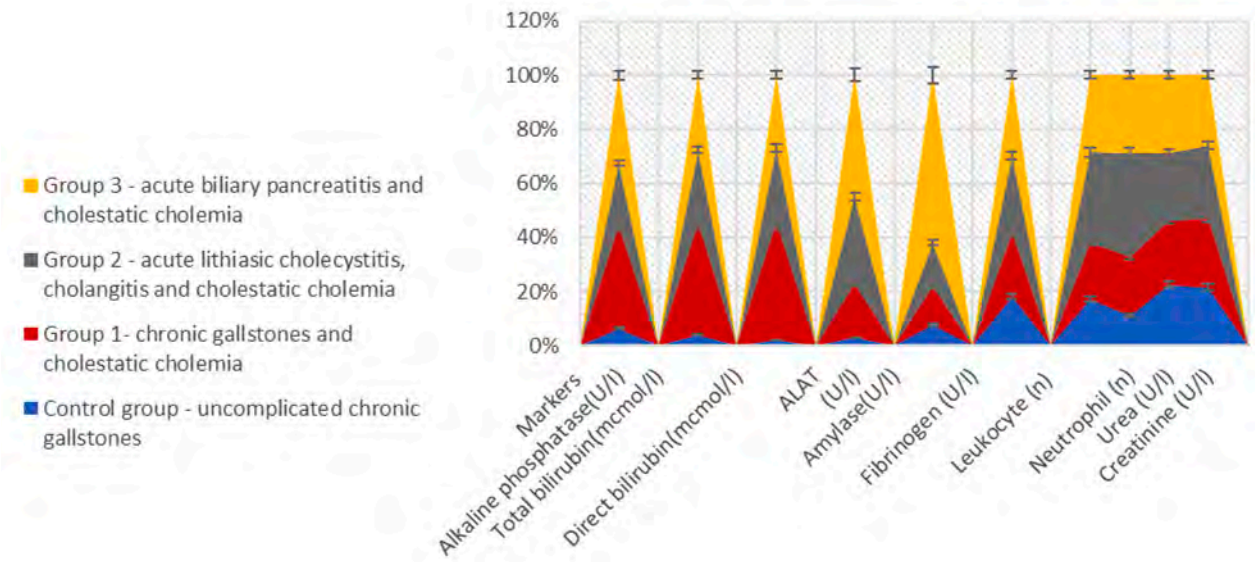

Fig. 1. Correlation of biochemical parameters in various complications of gallstones associated with cholestasis.

Note: ALAT – alanine aminotransferase.

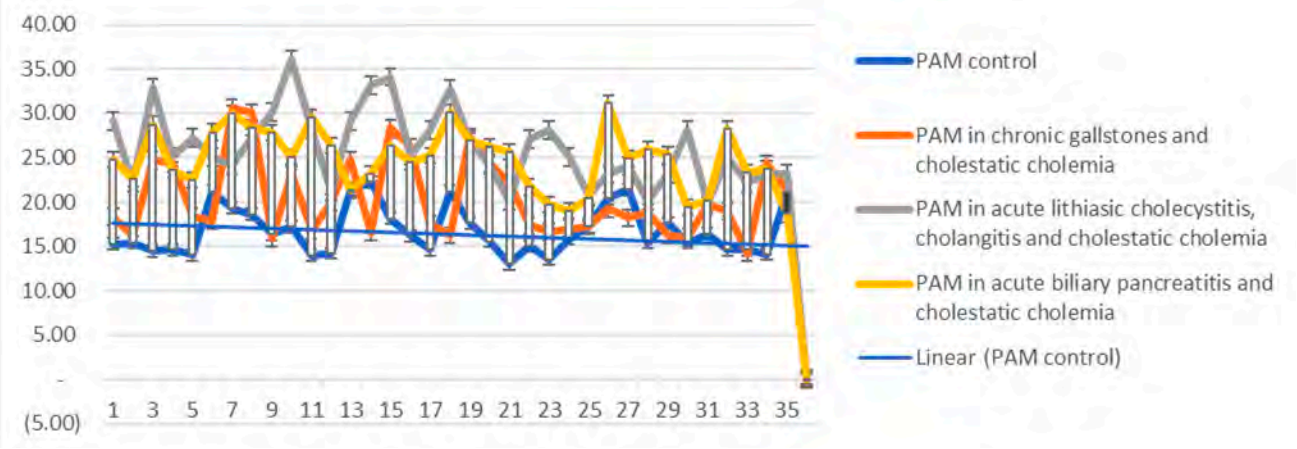

Fig. 2 Comparative evaluation of PAM values at hospitalization.

Note: PAM – peptides with average mass.

Also, the degree of endotoxemia assessed at hospitalization by PAM values was consistent with the inflammation present in groups 2 and 3, reporting a statistically significant difference compared to the control group [p c/2 < 0.001(t = 11.1); p c/3 < 0.001(t = 11.07)], regardless of the fact that the values of cholestasis markers in these groups were lower (Fig. 2).

Using the ANOVA test for the comparison of groups also confirmed a statistically significant difference with a p < 0.00001 for an F = 15.06988. The Tukey HSD test for pairwise group comparisons showed a negative Q with no statistical difference only in the comparison of group II and III, both with a marked inflammatory syndrome (Table 2).

MDA as the final product of lipid peroxidation, evaluated at hospitalization, shows double marked values in all groups, compared to the control group [p < 0.001 (t = 10.7); (t = 10.9); (t = 16.5)] (Fig. 3).

The ANOVA and Tukey HSD comparison test in paired groups reported that in the presence of a marked lipoperoxidation, regardless of the complication associated with gallstones, a negative Q is found without statistical difference in all study groups, with minimal value disproportions (Table 3).

Table 2. Pairwise Comparison of Endotoxemia (Tukey HSD Test).

Pairwise Comparison	$HSD_{.05}$ = 8.5102 $HSD_{.01}$ = 10.3447	$Q_{.05}$ = 3.6554 $Q_{.01}$ = 4.4434
C – Gr 1	21.31	Q = 9.39 (p = .00000)
C – Gr. 2	9.60	Q = 4.23 (p = .01595)
C – Gr.3	12.89	Q = 5.68 (p = .00044)
Gr.1 – Gr.2	11.71	Q = 5.16 (p = .00178)
Gr.1 – Gr.3	8.43	Q = 3.71 (p = .04488)
Gr.2 – Gr.3	3.29	Q = 1.45 (p = .73573)

Note: HSD – honestly significant difference; Q negative – blue color; C – control group; Gr.1 – chronic gallstones and cholestatic cholemia; Gr. 2 – acute lithiasic cholecystitis, cholangitis, and cholestatic cholemia; Gr.3 – acute biliary pancreatitis and cholestatic cholemia.

Table 3. Pairwise comparison of MAD in subgroups at hospitalization (Tukey HSD test).

Pairwise comparison	$HSD_{.05}$ = 13.0262 $HSD_{.01}$ = 15.8343	$Q_{.05}$ = 3.6554 $Q_{.01}$ = 4.4434
Gr.1 – Gr.2	5.03	Q = 1.41 (p = .75078)
Gr.1 – Gr.3	6.84	Q = 1.92 (p = .52697)
Gr.2 – Gr.3	1.81	Q = 0.51 (p = .98400)

Note: HSD – honestly significant difference; Q negative – blue color; Gr.1 – chronic gallstones and cholestatic cholemia; Gr. 2 – acute lithiasic cholecystitis, cholangitis, and cholestatic cholemia; Gr.3 – acute biliary pancreatitis and cholestatic cholemia.

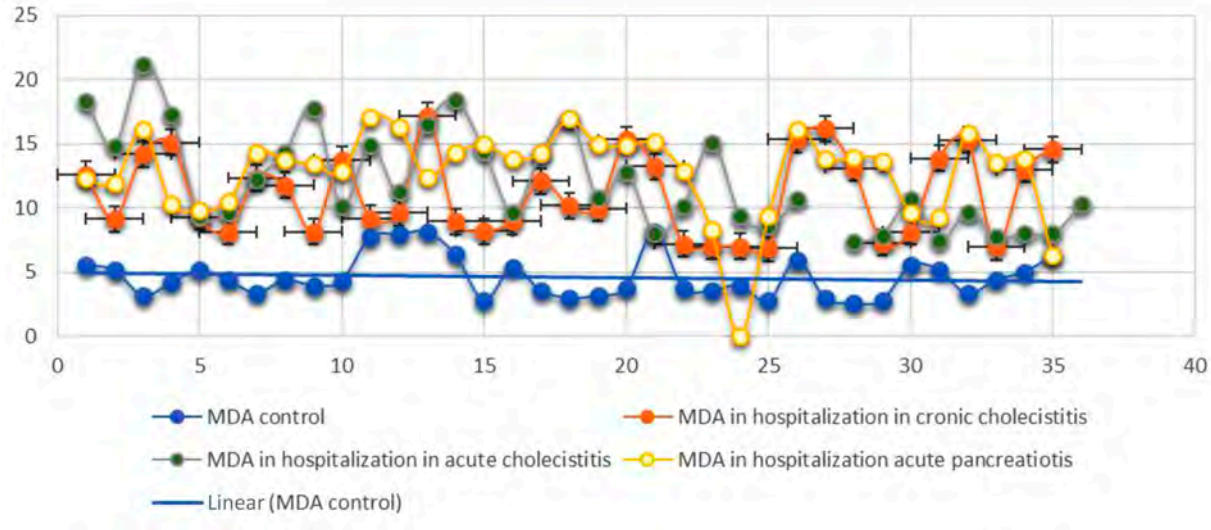

Fig. 3 Comparative evaluation of MDA values at hospitalization.

Note: MDA – malonic dialdehyde.

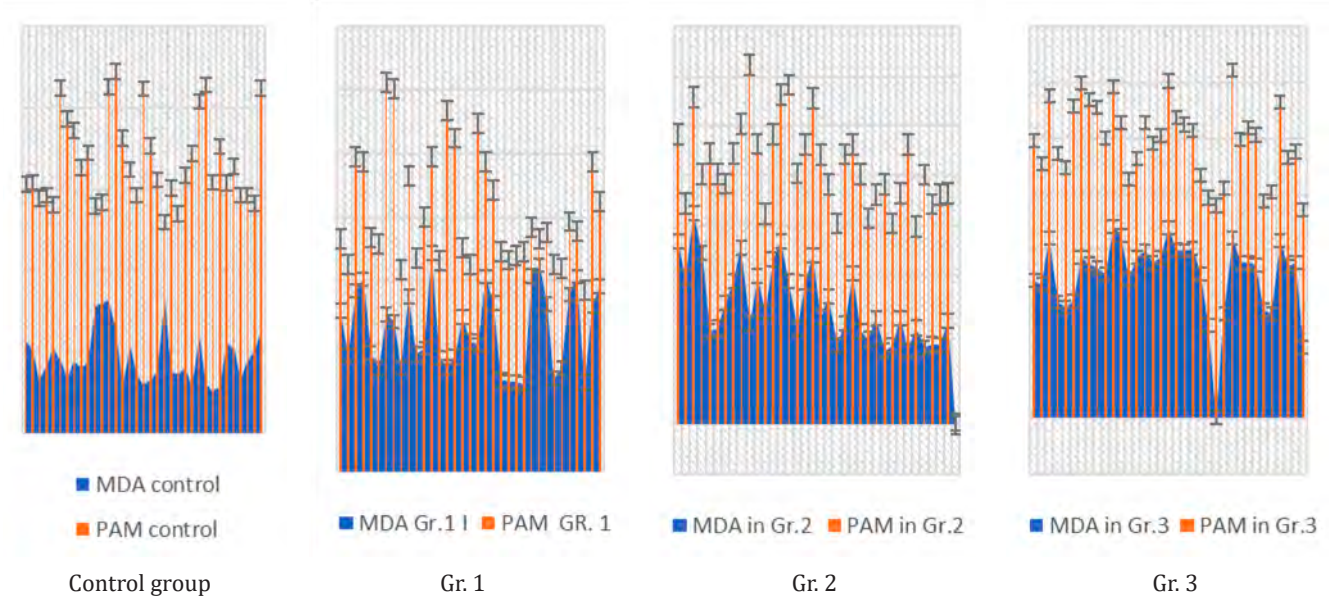

Fig. 4 Correlation of MDA and PAM values at hospitalization.

Note: MDA – malonic dialdehyde; PAM – peptides with average mass; Gr.1 – chronic gallstones and cholestatic cholemia; Gr. 2 – acute lithiasic cholecystitis, cholangitis, and cholestatic cholemia; Gr. 3 – acute biliary pancreatitis and cholestatic cholemia.

The assessment in the study of the correlation of lipid peroxidation with the degree of endogenous intoxication reported a direct dependence of the increase in PAM parameters with the elevation of DAM (Fig. 4).

Antioxidant activity, assessed at hospitalization by superoxide dismutase and catalase values, shows a statistically significant decrease in both SOD values [p < 0.001 (t = 6.4); (t = 4.1); (t = 5.7)] as well as CAT [p < 0.001 (t = 6.7); (t = 5.6); (t = 7.2)] in all groups, by 30-40% compared to the control group.

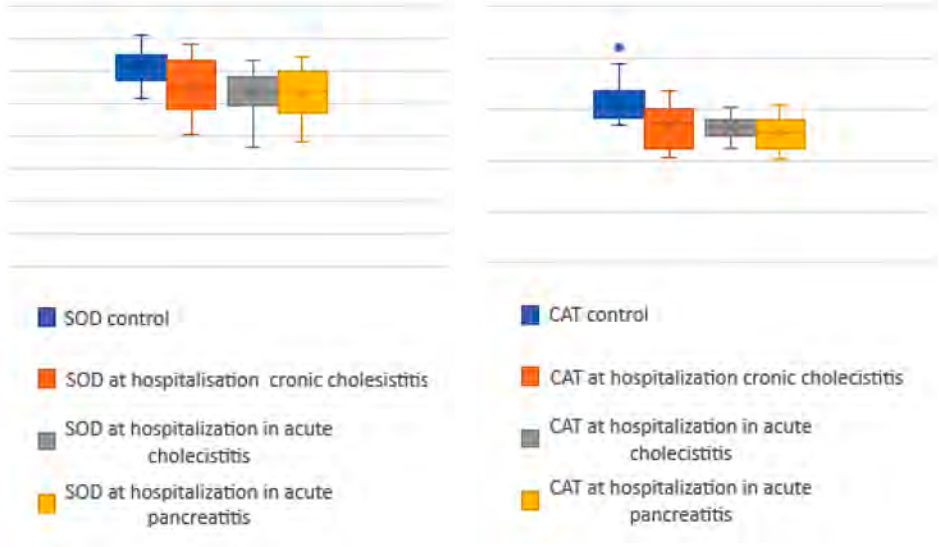

Fig. 5. SOD and CAT antioxidant activity at hospitalization.

Note: SOD – superoxide dismutase; CAT – catalase.

However, the ANOVA comparison test in paired groups and the Tukey HSD Test showed a negative Q with a statistically insignificant difference (p > 0.05), confirming that in all complications, the antioxidant system is compromised without major value differences (Table 4).

The correlation of endogenous liver intoxication with the reaction of the pro- and antioxidant system characteristic for various complications of gallstones associated with cholestatic jaundice is presented in numerical values in Table 5.

Table 4. Pairwise Comparison of SOD and CAT in groups at hospitalization (Tukey HSD Test).

Pairwise Comparison of SOD	$HSD_{.05}$ = 239.0962 $HSD_{.01}$ = 90.6385	$Q_{.05}$ = 3.6554 $Q_{.01}$ = 4.4434	Pairwise Comparison of CAT	$HSD_{.05}$ = 12.1089 $HSD_{.01}$ = 14.7192	$Q_{.05}$ = 3.6554 $Q_{.01}$ = 4.4434
Gr.1 – Gr.2	13.94	Q = 0.21 (*p* = .99878)	Gr.1 – Gr.2	0.47	Q = 0.14 (*p* = .99963)
Gr.1 – Gr.3	16.40	Q = 0.25 (*p* = .99802)	Gr.1 – Gr.3	0.47	Q = 0.14 (*p* = .99963)
Gr.2 – Gr.3	2.46	Q = 0.04 (*p* = .99999)	Gr.2 – Gr.3	0.01	Q = 0.01 (*p* = .00001)

Note: SOD – superoxide dismutase; CAT – catalase; HSD – honestly significant difference; Q negative – blue color; Gr.1 – chronic gallstones and cholestatic cholemia; Gr. 2 – acute lithiasic cholecystitis, cholangitis and cholestatic cholemia; Gr. 3 – acute biliary pancreatitis and cholestatic cholemia.

Table 5. Evaluation of the degree of endotoxemia and systemic oxidative stress in groups at hospitalization.

Markers	Control group (n = 35)	Group 1 (n = 35)	Group 2 (n = 35)	Group 3 (n = 35)	p1/2	p1/3	p2/3
MDA	4.62±0.27	11.12±0.53***	12.11 ± 0.64***	13.16±0.44***	p>0,05 (t=1.19)	p<0,01 (t=2.96)	p>0,05 (t=1.35)
SOD	1230.74±16.39	1096.97±27.4***	1060.91±21.05***	1057.44±25.2***	p>0,05 (t=1.04)	p>0,05 (t=1.06)	p>0,05 (t=0.11)
CAT	15.79±0.33	13.17±0.33***	13.27±0.14***	12.62±0.25***	p>0,05 (t=0.29)	p>0,05 (t=1.3)	p>0,05 (t=1.41)
PAM	16.77±0.45	20.4±0.75*** p<0.001 (t=4.1)	26.16±0.71** p<0.001 (t=11.1)	24.81±0.57** p<0.001 (t=11.07)	p<0,01 (t=5.5)	p<0,01 (t=4.68)	p>0,05 (t=1.48)

Note: *: p<0.05*; p<0.01**; p<0.001***; p>0.05**** – compared to the values of the control group; Gr.1 – chronic gallstones and cholestatic cholemia; Gr. 2 – acute lithiasic cholecystitis, cholangitis, and cholestatic cholemia; Gr. 3 – acute biliary pancreatitis and cholestatic cholemia; MDA – malonic dialdehyde; SOD – superoxide dismutase; CAT – catalase; PAM – peptides with average mass; p1/2 – compared to the values of the group 1 to 2; p1/3 – compared to the values of the group 1 to 3; p2/3 – compared to the values of the group 2 to 3; ALAT – alanine aminotransferase; t - test used to compare the means of two independent samples.

Discussions

The liver has a wide variety of metabolic functions, due to which liver dysfunction is associated with various clinical-pathological sequelae. Dysregulation of liver metabolism activates mechanisms that cause the accumulation of toxic products and the installation of variable endogenous intoxication in different pathologies [17]. The concept of "endogenous intoxication" for a long time did not have a biochemical substrate for research, which is why it could not be measured quantitatively. Currently, several homeostasis indicators and parameters are considered markers of endogenous intoxication: bilirubin, transaminases, proteases and their inhibitors, fibrinogen, haptoglobin, ceruloplasmin, C-reactive protein, creatinine, urea, total protein levels and parameters, proteinograms, ESR, cellular, humoral characteristics, etc. However, the values of these indicators are important only in complex with other criteria, which can vary depending on the etiology of toxemia. It is impossible to adequately assess the degree of intoxication based on one of them, and defining all of them is very laborious and difficult from an economic point of view [8, 18]. Recent studies have demonstrated that, as a universal biochemical marker of EI, necessary to objectify the toxicity of a certain environment, regardless of etiopathogenetic characteristics, the level of "average molecules" can be considered. There is an opinion that the level of PAM primarily reflects the degree of altered protein metabolism and correlates with clinical and laboratory prognostic criteria for metabolic disorders [19]. Characteristic for the mechanical jaundice syndrome, PAM is considered the main group of endotoxins that determine the development of endogenous intoxication. Possessing diverse biological activity and contributing to the inhibition of enzyme systems, the disturbance of ionic membrane permeability, and the fixation and suppression of vitally necessary protein synthesis processes, PAM induce the development of endogenous intoxication, which, in the end, can evolve into hepatocellular insufficiency with alterations in the functions of various systems and organs [9, 19].

According to the results of our own research, the PAM level in cholestatic cholemia, present in the complications of gallstones, was increased in all groups, being more evident in the groups with the presence of acute inflammation (group 2) with an increase of 26.7%, and the enzymatic component supporting inflammation (group 3) with a 22.5% increase. Regardless of the presence of higher bilirubin parameters in group 1, the PAM values reported a less significant increase - only by 5.78%. These results are consistent with existing literature data, which show a percentage of increase – 29.5-39.7%, but without a specification characteristic of different groups of complications [8, 19]. As a rule, in the available literary sources, we found only the fact of increasing the PAM level in the blood (plasma, erythrocytes) of patients with mechanical jaundice, without differentiation in different clinical groups.

Several studies report that the development of endogenous intoxication in liver diseases of different genesis is largely due to a change in the ratio between lipid peroxidation products and the enzymes that cause their deactivation [20]. The presence of an increased number of free radicals can be considered as one of the triggering pathogenetic factors in the progression of pathological changes in the liver in patients with cholestatic jaundice [12, 21].

The present study confirms massive lipoperoxidation in all benign complications of gallstones associated with cholestatic jaundice, showing an increase in MDA values as the end product of peroxidation in all groups, being more evident in the groups with the presence of the enzymatic component supporting inflammation (group 3) – a 2.84-fold increase compared to the values of the control group and in the presence of acute inflammation (group 2) – a 2.6-fold increase. MDA values in group I reported a 2.4-fold increase over control group values. Also, the study confirmed the direct dependence of the PAM and MDA ratio, which is higher in patients with cholestatic jaundice and associated infectious complications, being an indication for biliary decompression as early as possible in these patients.

In various pathological conditions, including cholestatic jaundice, the acceleration of the formation of free radicals induces an imbalance between the prooxidant factors and the protective antioxidant systems, in which the prooxidant factors constitute a specific aggravating factor. The antioxidant system (AS) has the function of limiting the excess of free radicals in the body, acting preventively and creating positions to stop the progression of the harmful effects of free radicals [11, 22]. AS acts by maintaining the regulation mechanisms of the pro- and antioxidant balance, favoring conditions for the body to fight effectively in various pathological situations, limiting injuries and their extension [10, 11, 22]. The balance between the prooxidant action of free radicals and the level of antioxidants is essential to life and characterizes the resistance capacity of an organism [23].

According to our own results, in gallstone cholestatic jaundice, regardless of the type of condition, the antioxidant system is mobilized from the first hours of the appearance of the complication and the association of jaundice, but prooxidant activity and endotoxemia are prevalent, contributing to the inhibition of the antioxidant system. Antioxidant activity, assessed at hospitalization by superoxide dismutase and catalase values, shows a statistically significant decrease in all groups: group 1 – CAT < 16.7%, SOD < 10.9%; group 2 – CAT < 15.9%, SOD < 13.7%; group 3 – CAT < 20.1%, SOD < 14.1%, compared to the control batch. The publications on the subject do not present a valuable exposition of the deviations of the investigated parameters in different complications of gallstones associated with cholestatic jaundice but mention the early inhibition of antioxidant activity in the mechanical jaundice syndrome [12, 24].

Conclusions

The syndromic evaluation of liver activity in the complications of gallstones associated with cholestatic jaundice reveals the elevation of the values of the parameter characteristic for cholestasis and liver mesenchymal inflammation syndromes, which have an impact on the evolution of liver

synthesis function and liver cytolytic disorders. In established cholestatic jaundice, there is an obvious activation of lipoperoxidation processes, which contributes to the increase of endogenous intoxication and early suppression of antioxidant activity. There is a direct linear relationship in the increase of prooxidant activity and the level of endogenous intoxication, in all complications of gallstones associated with cholestatic jaundice, but being more elevated in infectious complications, serving as an indication for an early solution.

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

The study was initiated before the creation of the Research Ethics Committee at *Nicolae Testemițanu* State University of Medicine and Pharmacy, so the study was approved by the scientific council and the patient's informed consent was obtained.

Authors' contributions

All the authors have contributed equally to the results presentation in the paper.

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RESEARCH ARTICLE

Model predicting the onset of antiepileptic drug resistance in women of reproductive age with epilepsy: analytical study

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ABSTRACT

Introduction. In attempt to find an answer regarding the possible scenarios of epilepsy evolution in women of reproductive age (e.g. worsening, remission, antiepileptic drug resistance, status epilepticus occurence), preferably - objective, based on simple, replicable, observable indicators that can be included in a mathematical probability estimation model, could significantly improve their quality of life and increase the effectiveness of prescribed treatments.

Materials and methods. Bidirectional, cohort, descriptive-analytical study, conducted between 2016-2020. Primary data were collected in the *Diomid Gherman* Institute of Neurology and Neurosurgery, the State Hospital of Republic of Moldova and the *Excellence* Private Medical Institution. Out of 366 unique parameters, which were recorded in the 159 patients enrolled in the study at each visit (total, 4 documentation visits over 5 years period), 10 parameters were selected for multivariate analysis, considered relevant for predicting clinically significant outcomes. Criteria for parameter relevance were: reaching $p \leq 0.1$ in univariate analysis, easy documentation. Subsequently, testing for multicollinearity (calculation of variance inflation factor) and the contribution of each parameter in the formula was performed using the Akaike informativeness criteria. The performance of the developed predictive models was expressed by the area under the ROC curve, positive and negative prognostic power. Statistical analysis: GraphPad Prism, v. 9 trial (Graph Pad Software, Boston, USA).

Results. Age at onset of the disease 14.0±6.3 years; age at first referral to specialist 24.0±7.2 years. The developed predictive model, based on 3 parameters (depressive state, annual frequency of seizures, presence of brain lesions on MRI) has a positive predictive value of 83%, negative of 62%, with an area under the ROC curve of 0.72 (95%CI = 0.56 to 0.88) and a probability of occurrence of 96%.

Conclusions. Depressed patients with documented structural lesions on MRI and a high frequency of epileptic seizures have a progressive, significant risk (an OR of 5.3-24.0) of developing resistance to antiepileptic drugs.

Keywords: resistance, antiepileptic drugs, epilepsy, women of reproductive age, predictive model.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript

There is a lack of reports in the scientific literature on the possibility of predicting the onset of resistance to antiepileptic medication. Our study was based on recorded data from women of reproductive age with epilepsy and their 5-year follow-up.

The research hypothesis

Certain clinically relevant parameters, selected from a list of available parameters, can be parameterized and included in a mathematical model predicting the onset of antiepileptic drug resistance in women of reproductive age with epilepsy.

The novelty added by manuscript to the already published scientific literature

The predictive model developed, based on 3 parameters (depressive state, annual seizure frequency, presence of brain lesions on MRI) has a positive predictive value of 83%, negative of 62%, with an area under the ROC curve of 0.72 (95%CI = 0.56 to 0.88) and a probability of 96%.

Introduction

Epilepsy is a fairly common disease (ranked 3rd after stroke and Alzheimer's disease) in the general population, affecting 1-3% of people [1-3]. The true prevalence of epilepsy is unknown and virtually impossible to estimate. In Republic of Moldova, according to data from the National Bureau of Statistics, 60,000 people are diagnosed with epilepsy, of which 13,500-15,000 are women [4]. Correspondingly, the estimated prevalence of epilepsy in Republic of Moldova is 16-19 persons per 1000 population (2015) [5]. As the disease is a stigmatizing one, especially in relation to women, epilepsy becomes an important public health and medico-social problem.

One of the most common requests from patients and their relatives in medical consultations is to provide an answer regarding the outcome and/or prognosis (chance of cure, recurrence, duration, likelihood of complications, success of the treatment, etc.). In order to give a plausible answer, doctors either rely on their own experience, on literature data, or apply various estimation and forecasting tools (scores, nomograms, mathematical models).

The limitations of each of the outcome prediction methods is different - from empirical estimation (intuition of the outcome), with 50/50 probability of error vs. success, based on practical experience, to a population approximation (based on probabilistic scores and models). Unfortunately, none of the methods, no matter how sophisticated, is able to provide a personalized prognosis of what the patient requires.

A compromise option would be to use probabilistic prediction models, based on clinical, instrumental and laboratory indicators, developed using logistic regression. The logistic regression method allows the likelihood of an event to be predicted, based in particular on binary events (presence or absence of crisis, abandonment or not of treatment, etc.). Among the advantages of using logistic regression in the present research are the ease of implementation, interpretation, and sufficient clinical accuracy, given simple and easily obtained parameters. Among the disadvantages should be mentioned the assumption of a linear relationship between the independent variable (the sought outcome) and the dependent variable(s) (which in the real world is not always linear) and the problem of (multi)collinearity. Contemporary statistical tools, however, allow the impact of these shortcomings to be minimized [6-8]. Treatment-resistant epilepsy is defined as the therapeutic failure of two or more antiepileptic drugs, correctly chosen and administered in appropriate doses, depending on the form of epilepsy. The mechanisms of this resistance are either pharmacogenetics in origin, the consequence of neuroplasticity following disease progression, or the consequences of the seizures themselves (e.g., hippocampal sclerosis).

About 7% to 20% of children with epilepsy have developed resistance to anti-epileptic drugs. Meanwhile, 30% to 40% of adult patients remain refractory to drug treatment. Numerous studies have touched on predictors associated with medical resistance in both children and adults. Confirmed risk factors for drug resistance are: early onset of the disorder, abnormal EEG findings and neurological deficit or mental retardation at the time of diagnosis, symptomatic etiology, high seizure frequency and lack of response to first anti-epileptic treatment [9].

In Xue-Ping W.'s (2019) meta-analysis [9] it was found that the prevalence of drug-resistant epilepsy was approximately 27% and the relative risk factors were: abnormal EEG (both slow wave and epileptiform discharges), status epilepticus, symptomatic etiology, febrile seizures and seizures of multiple types, positive outcome to short-term therapy, delayed neurodevelopment, and high frequency of primary seizures. Based on these risk factors, in clinical practice it would be useful for clinicians to predict the course of epilepsy within a short period after diagnosis and to early identify children at risk of intractable epilepsy; this is important both for counselling parents and for clinicians to consider alternative treatments.

However, despite the development of 20 new anticonvulsant drugs since the 1990s, the proportion of patients with drug-resistant epilepsy has remained stable (30%-40%) over the last 30 years. In addition, 80% of patients with epilepsy have been reported to have experienced adverse reactions related to their anticonvulsant medication and 30-40% have reported that adverse effects substantially affected their quality of life or led to discontinuation or non-adherence to medication [10].

The proportion of people with drug-resistant epilepsy has not changed substantially since the 1980s. However, the field of epilepsy has advanced over the last decade and is now entering the era of targeted and precision medicine. Increased understanding of the etiologies of epilepsy, including immune, genetic, and structural causes, has now made it possible, in some patients, to identify specific targets for therapies that go beyond anti-seizure drugs and allow treatment of the cause of epilepsy [10, 11].

Based on the above, the aim of the present study was to develop a predictive mathematical model for the development of antiepileptic medication resistance in women of reproductive age with epilepsy, using the logistic regression and likelihood calculus method, based on 5 years of follow-up of 159 patients.

Methods and materials

The given study is a bidirectional, cohort, descriptive-analytic study. The accumulation of primary material took place during 2016-2020 in the outpatient departments of the *Diomid Gherman* Institute of Neurology and Neurosurgery, the State Hospital of Republic of Moldova and the *Excellence* Private Medical Institution, based on bilateral collaboration contracts, signed with each institution, in compliance with bioethical clauses, confidentiality, and protection of personal data and informed consent of patients.

The study protocol was approved by the Research Ethics Committee of the *Nicolae Testemițanu* State University of Medicine and Pharmacy (minutes no. 55 of 03.06.2016).

The study subjects were women of reproductive age with epilepsy, where the first epileptic seizure started from birth to 49 years range.

Inclusion criteria were:

- women of reproductive age (15-49 years);
- signed informed consent;
- with no predetermined duration of illness before enrolment, regardless of seizure type;
- no comorbidities;
- onset of illness - from birth to the end of reproductive age (49 years).

Exclusion criteria were:

- refusal of informed consent;
- age of patients assessed for enrolment outside the age range 15-49 years;
- undocumented or unconfirmed epileptic seizures;
- patients with persistent epileptic encephalopathy.

After confirming eligibility and obtaining informed consent, prior to the initiation of the investigation, patients enrolled in the study were informed in detail about the purpose of the study, the requirements, benefits, and risks of the investigations and the treatment administered.

Thus, 159 complete records of patients of reproductive age with epilepsy, who met the inclusion criteria and had none of the exclusion criteria, were included in the final analysis.

All patients enrolled in the study were examined according to the National Clinical Protocol "Epilepsy in Adults" and institutional protocols[5].

The clinical examination included the recording of patient data - medical history with epidemiological and demographic data: age, residence, profession (working conditions), level of education (studies), medical history - personal and hereditary medical history (presence of epilepsy in first and second degree relatives), vicious habits, sexual history (menarche, menstrual cycle characteristics, obstetrical and gynecological history, sexual activity and menopause). Objective general clinical examination and neurological examination was performed. Patients were examined by standard clinical methods to assess general condition and neurological status. General and focal neurological symptoms, possible psychiatric, cognitive disorders were identified and entered into the standardized data recording form.

In order to clarify certain events, with the consent of the patients, their relatives and eyewitnesses of the seizures were interviewed. The technique of guided interview questions was applied.

Patients also completed a seizure diary (paper format), which described the symptomatic nature of the seizure, frequency and duration, aura and post seizure signs, time of onset and triggers. This data were then transcribed into the standardized data recording form. Disease- and patient-specific variables with epilepsy were collected *a priori*, based on the individual and clinical characteristics of each patient. Information about clinical events accompanied by loss of consciousness, amnesia, confusion was assessed based on family and witness information and no objective screening tool was applied to the subjects of the present study.

Before starting the collection of primary material, the number of patients required for enrolment in the study (sample size) was estimated, which would allow confirmation or rejection of the null hypothesis. Thus, the required number of patients was calculated using the free online software GPower 3.1 [12].

Since the main outcome parameters of the study are categorical, with non-Gaussian (non-normal) distribution, and the number of data series was 4 (according to the number of visits), the Kruskal Wallis Test was selected for calculation.

The following calculation steps have been carried out in the Gpower 3.1 software (1) Selected F tests from the Test family menu; (2) Selected ANOVA: fixed effects, omnibus, one way from Statistical test; (3) Selected A priori from power analysis.

Background information was entered: 95% confidence interval for significance of the results, minimum statistical power - 80%; difference of result f = 0.25; number of groups n = 3; number ratio between data series - 1:1.

Calculation results: non centrality parameter (describing the degree of difference between H1 and H0 values) $\lambda = 9.94$, critical F-value = 3.05, numerator of degrees of freedom df = 3 (because the calculation was performed based on 4 data series), denominator of degrees of freedom df = 156. Total number of patients required for research = 159.

Regarding the collected parameters, as criteria for their non-inclusion in the logistic regression analysis and the construction of the predictive model for the onset of antiepileptic drug resistance were established: (1) parameters with many individual data missing or having low variability over time; (2) parameters that are in close correlation with others, to avoid collinearity effect problems; (3) parameters that showed insufficient statistical significance ($p \geq 0.2$) in univariate analysis.

Finally, for the creation and testing of variants of the antiepileptic drug resistance prediction model, the following parameters were selected (with consideration of the conditions stated above): age at onset of illness (by age categories, at 10, 20 and 30 years), drop-out or poor adherence to prescribed antiepileptic treatment, presence of a brain lesion on brain MRI scan, prolonged confusion after an epileptic seizure (postictal sign), focal activity on electroencephalog-

raphy without seizure symptoms, anxiety; depression, seizure duration over 6 minutes, status epilepticus occurrence, seizure frequency (discrete quantitative variable).

Since the selected variables were found to be binary categorical, ordinal (with a small ordinal number), or discrete quantitative, the best-fitting predictive models were developed based on multivariate analysis (multiple logistic regression).

The calculation of the probability of occurrence of an event, in models based on logistic regression analysis was done according to formula (1):

$$P = \frac{e^{\beta 0 + \beta 1 X}}{1 + e^{\beta 0 + \beta 1 X}} \quad (1);$$

where,

β0: the mean value of the response variable (the interceptor) when X = 0;

β1: the average change in the response variable (constant) for a one unit increase in X;

X: value for the predictor variable (in this case, the clinical sign).

For each model version, the final number of remaining parameters were selected according to the results of the collinearity test, the variance inflation factor (VIF) and the Akaike informativeness criteria. All analysis was performed with the help of Graph Pad Prism software, version 9 trial. For practical convenience, the same results were also reflected by Odds ratios (OR).

Results

The general description of the patients enrolled in the study in terms of age, level of education, living environment, socio-familial status, etc., is presented in Table 1. These characteristics will define the profile of patients of reproductive age with epilepsy for whom the predictive model for the onset of antiepileptic drug resistance is constructed.

The results of logistic regression of the parameters that met the eligibility criteria (anxiety, depressed state, brain structural abnormality on MRI image, discontinuation of antiepileptic treatment, epileptic seizure lasting more than 6 minutes, confusional state after epileptic seizure, status epilepticus, and frequency of epileptic seizures) are presented in Table 2. Of note, the parameters in Table 2 do not show collinearity with each other because the variance inflation factor, VIF ≤2, R^2à0.

Of all the parameters mentioned in Table 2, only "annual frequency of epileptic seizures" is a discrete quantitative parameter. As the number of observations increases, it tends to have a normal (Gaussian) distribution and behaves as a continuous quantitative parameter. In this respect, it was examined whether a cut-off value could be identified, which would allow a more accurate prediction (with clinically acceptable sensitivity and specificity) of the onset of resistance to antiepileptic drug treatment. Respectively, figure 1 shows the ROC curve of antiepileptic drug-resistant epilepsy cases (from the study group) versus annual seizure frequency. Although it is clearly shown that increased frequency of epileptic seizures is one of the relevant clinical indicators (AUC = 0.71; 95CI: 0.65 - 0.76; p=0.0001) of the onset of resistance to antiepileptic treatment, an exact cut-off value in this respect is impossible to define (identify).

Table 1. The general description of the patients enrolled in the study.

Parameters	All patients (n = 159)
Age of onset of the disease, years	14.0±6.3 [2-34]
Age of first referral to a neurologist, years	24.0±7.2 [2-46]
Level of education	
• primary	4 (2.5%)
• secondary	100 (62.3%)
• high education	56 (35.2%)
Living environment	
• rural	84 (52.8%)
• urban	75 (47.2%)
Family status	
• single	109 (68.6%)
• married	45 (28.3%)
• divorced	5 (3.1%)
• widowed	0 (0.0%)
Social class	
• industrial workers	83 (52.3%)
• rural workers/farmers	61 (38,3%)
• intellectuals	15 (9.4%)
Vulnerabilities	
• unemployed*	12 (7.6%)
• degree of disability**	11 (6.9%)
Reproductive function	
• no. pregnancies	40 (25.2%)
• no. births	33 (20.8%)

Note: Age data are expressed as mean and standard deviation, with extreme values presented. * - patients with official unemployment status; ** - patients with disability status, granted by the National Disability Determination Council of the Republic of Moldova. Data are presented as mean and standard deviation [extremes] or as absolute (relative) values.

Table 2. Multiple logistic regression parameters for the onset over time of patients' antiepileptic drug resistance calculated according to the clinical indicators proven to be relevant.

Parameters	β (SE)	OR (95CI)	VIF	R^2	Mean Probability
Interceptor (β0)	0.78 (0.34)	2.19 (1.13 – 4.32)	-	-	69%
A (β1)	0.06 (0.24)	1.06 (0.67 – 1.70)	1.046	0.04	52%
B (β2)	0.75 (0.26)	2.11 (1.2 – 3.50)	1.066	0.06	68%
C (β3)	0.68 (0.26)	1.97 (1.20 – 3.30)	1.095	0.09	66%
D (β4)	-0.34 (0.25)	0.71 (0.43 – 1.17)	1.075	0.07	42%
E (β5)	0.24 (0.25)	1.28 (0.78 – 2.08)	1.127	0.11	56%
F (β6)	-0.43 (0.77)	0.65 (0.15 – 3.42)	1.039	0.04	39%
G (β7)	0.16 (0.25)	1.17 (0.71 – 1.91)	1.049	0.05	54%
H (β8)	-0.001 (0.001)	0.99 (0.99-1.00)	1.068	0.06	50%

"Nota:": A - anxiety; B - depression; C - brain structural abnormality on MRI image; D - drop-out of antiepileptic treatment; E - epileptic seizure lasting more than 6 minutes; F - status epilepticus; G - confusional state after epileptic seizure; - annual frequency of epileptic seizures; VIF - variance inflation factor; R2 - multiple correlation coefficient.

Figure 2 reflects the interrelationship between the likelihood of developing epilepsy resistant to antiepileptic treatments and the annual frequency of epileptic seizures, where the trend is evident. This finding leads to the conclusion that in a probabilistic model for predicting the development of antiepileptic drug resistance over time, seizure frequency must necessarily be part of the model.

From figure 2, for practical guidance - a number of 20 epileptic seizures per month, for 2 consecutive months (according to the following calculations: 250 seizures per year with 50% average probability of resistance installed / 52 weeks of the year = 5 epileptic seizures per week), against the background of correct treatment, as drug combination and appropriate dosage, will indicate a high probability of antiepileptic drug resistant epilepsy. It should be noted that in this case, other concurrent symptoms (depression, anxiety, post-seizure symptoms), which increase the probability of antiepileptic drug resistance, were not taken into account, but only the epileptic seizure itself as an on event (unspecified, however, in duration and intensity).

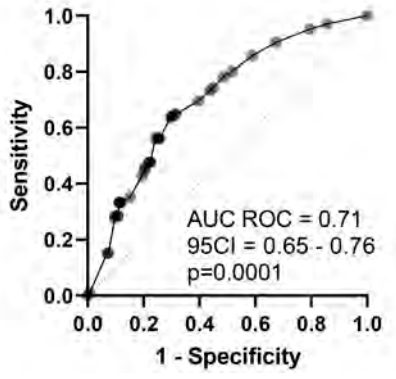

Fig. 1. ROC curve of cases of epilepsy resistant to antiepileptic treatment depending of annual number of epileptic seizures.

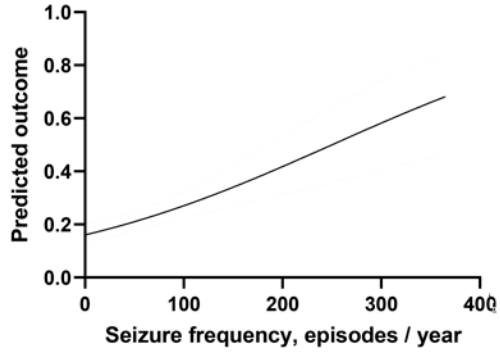

Fig. 2. Likelihood of resistance to antiepileptic drug treatment depending of the annual number of recorded epileptic seizures.

Table 3. A comparison of two multiple logistic regression models for the onset of resistance to EC treatment over time, calculated on the basis of clinical indicators with significant odds ratios.

Parameters	Model *rez_1*		Model *rez_2*	
	β (SE)	OR (95CI)	β (SE)	OR (95CI)
Interceptor (β0)	1.16 (0.97)	3.2 (0.5 – 24.2)	0.99 (0.86)	2.68 (0.52 – 16.37)
A (β1)	0.10 (0.25)	1.1 (0.7 – 1.8)	0.11 (0.25)	1.12 (0.69 – 1.82)
B (β2)	-0.21 (0.65)	0.8 (0.2 – 2.9)	0.22 (0.54)	1.24 (0.42 – 3.55)
C (β3)	-0.91 (0.79)	0.4 (0.1 – 1.9)	-1.14 (0.72)	0.31 (0.08 – 1.33)
D (β4)	-0.48 (0.67)	0.6 (0.2 – 2.3)	-0.35 (0.26)	0.70 (0.41 – 1.16)
E (β5)	0.20 (0.72)	1.2 (0.3 – 5.3)	0.23 (0.62)	1.26 (0.37 – 4.34)
F (β6)	0.45 (0.77)	1.6 (0.3 – 6.8)	0.47 (0.77)	1.60 (0.31 – 6.85)
G (β7)	0.23 (0.68)	1.3 (0.3 – 4.8)	0.01 (0.55)	1.01 (0.34 – 2.99)
H (β8)	-0.01 (0.002)	0.9 (0.8 – 1.0)	-0.01 (0.002)	0.98 (0.96 – 0.99)
B + C (β9)	0.74 (0.66)	2.1 (0.5 – 7.5)	0.74 (0.64)	2.10 (0.58 – 7.30)
B + D (β10)	0.77 (0.59)	2.2 (0.7 – 7.1)	-	-
B + E (β11)	0.12 (0.60)	1.1 (0.3 – 3.7)	0.17 (0.59)	1.19 (0.38 – 3.80)
B + G (β12)	0.13 (0.67)	1.1 (0.30 – 4.2)	0.23 (0.65)	1.27 (0.35 – 4.57)
C + D (β13)	-0.42 (0.58)	0.6 (0.2 – 2.0)	-	-
C + E (β14)	0.50 (0.57)	1.6 (0.5 – 4.9)	0.47 (0.56)	1.60 (0.53 – 4.81)
C + G (β15)	1.19 (0.67)	3.3 (0.8 – 12.4)	1.16 (0.65)	3.17 (0.89 – 11.55)
D + E (β16)	-0.06 (0.53)	0.9 (0.3 – 2.7)	-	-
D + G (β17)	-0.37 (0.57)	0.7 (0.2 – 2.1)	-	-
E + G (β18)	-0.55 (0.54)	0.6 (0.2 – 1.6)	-0.66 (0.53)	0.52 (0.18 – 1.43)
B + C + H (β19)	0.01 (0.005)	1.0 (1.0 – 1.1)	0.01 (0.005)	1.01 (1.00 – 1.03)
B + G + H (β20)	0.001 (0.004)	1.0 (0.9 – 1.0)	0.001 (0.004)	1.00 (0.99 – 1.01)
C + G + H (β21)	-0.006 (0.007)	0.9 (0.9 – 1.0)	-0.007 (0.007)	0.99 (0.98 – 1.01)
AICc (β0)	512.0		513.0	
AICc of the model	492.0		486.0	
AUC ROC	0.74 (0.69 – 0.79)		0.74 (0.68 – 0.79)	
PPN, %	62%		59%	
PPP, %	83%		83%	
Probability, %	96%		96%	

"Nota:" A - anxiety; B - depression; C - brain lesion on MRI examination; D - discontinuation of antiepileptic treatment; E - epileptic seizure lasting 6 minutes or more; F - epileptic status; G - confusional state after epileptic seizure; H - seizure frequency; AICc - Akaike informativeness criterion.

Based the parameters described in table 2, it is possible to develop several prediction models, based on multiple logistic regression, by summing the individual contribution of each parameter to the final characteristics of the model, with or without taking into account the influences produced by the interaction between variables.

Table 3 presents the characteristics of two probabilistic prototype models, named rez_1 and rez_2, which also take into account the contribution of interaction effects between 2 and 3 variables, in addition to the individual contribution of each model parameter to its summary predictive ability. Model rez_2 differs from model rez_1 in that the contributions of interaction effects between B-D (depression, drop-out of antiepileptic treatment), C-D (MRI brain lesion and drop-out of antiepileptic treatment), D-E (drop-out of antiepileptic treatment and seizure lasting more than 6 min) and D-G (drop-out of antiepileptic treatment and confusional state after seizure) were not included.

Both models (rez_1 and rez_2) possess absolutely similar performance characteristics (AUC ROC, PPN, PPP, likelihood) (Figure 3). At the same time, the Akaike informativeness criterion points to a low contribution to the final characteristics of the models of interactions between 2 and 3 variables, which means that they can be omitted from the calculations, with the deduction of a simpler predictive model.

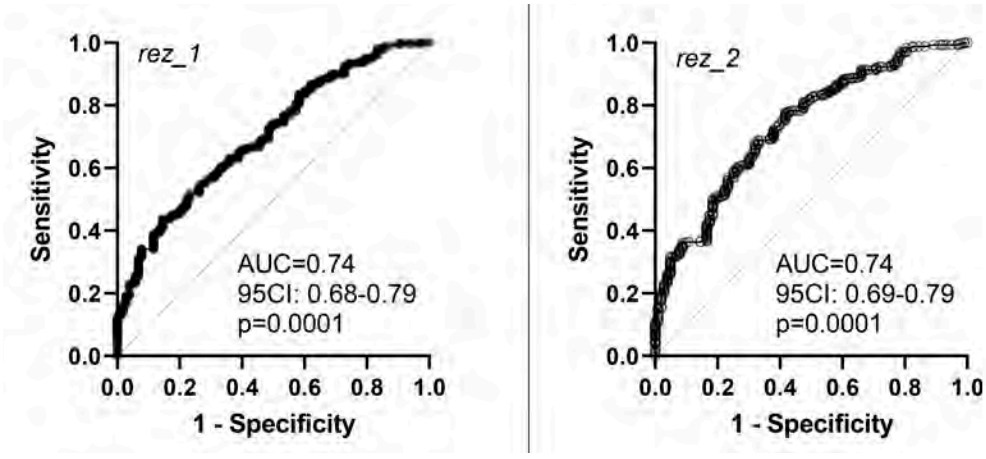

Fig. 3 Parameters of the ROC curves of the probabilistic models rez_1 and rez_2 for predicting antiepileptic drug resistance over time, based on the characteristics of the variables in Table 3.

After simplification, the AErez model is the "successor" of models rez_1 and rez_2, which was developed with consideration of the findings made based on the information in Table 3. A mandatory parameter in the model was the annual frequency of epileptic seizures. A frequency of 20 or more epileptic seizures per month was proposed, somewhat empirically, as a condition for including the variable in the model. Thus, the AErez model is presented in formula (2 and 3).

$$P(AErez) = \frac{e^{1.16+DEP^{-0.21}+RMN^{-0.92}+FREQ^{-0.01}}}{1+(e^{1.16+DEP^{-0.21}+RMN^{-0.92}+FREQ^{-0.01}})} \quad (2)$$

$$P(AErez) = \frac{3.19+0.81+0.40+0.99}{1+(5.39)} = 0.84 \quad (3)$$

So, according to the AErez model, patients with epilepsy who suffer from depression and have a brain lesion on MRI imaging, who have, for example, 20 or more epileptic seizures per week, have an 84% probability of developing a treatment-resistant form of epilepsy. In other words, patients who have all of the above characteristics will have at least (0.84 / 1-0.84) 5.3 times more frequent treatment-resistant epilepsy than patients who do not have these characteristics.

Discussions

A PubMed search on predictive models (of any kind) in epilepsy (any category), covering the period 1975-2022, returned 2821 results based on the keywords "predictive models", "outcome prediction", "epilepsy". An exponential increase in the number of publications selected by the search engine based on these keywords began to emerge after 2010 (86 publications per year), reaching 355 publications in 2022. A search by restriction criteria (articles *in extenso*, published between the years 2012-2022, in English, that directly address the topic of predictive models in epilepsy), led to a final list of 25 publications, which have been analyzed and systematized in this section.

In adults, the rate of resistance is about 4% per year. In the study group, the rate of resistance, expressed as the

total number of episodes observed during the 4 visits, was 107 episodes per 462 patient-visits (23.1%), which is an extremely high rate. In this context, the identification of clinical predictors, framed in a mathematical model, is particularly motivating and important.

Prediction models have long been used as clinical decision support (CDS) tools in medical practice. CDS is a complex term that encompasses a variety of tools designed to assist clinicians in their workflow and improve patient outcomes. Established examples include alert systems, computerized ECG interpretation, automated dose adjustment assistance for patients with renal failure, diagnostic tools, and models that can aid decision making (e.g., medication choice) and outcome prediction [13]. However, the data revolution in medicine and science is spurring increased interest in CDS and personalized prediction.

Unlike traditional approaches, which rely on the assumption of a data model, algorithms developed by machine learning (ML) models are retrieved directly from data [14].

Examples of ML models include decision trees (a decision tree is a type of supervised machine learning used to classify or make predictions based on how a previous set of questions was answered; the model is a form of supervised learning, meaning that the model is trained and tested on a data set containing the desired categorization), support vector machine (SVM), k-nearest neighbor algorithm, random forests study method, artificial neural networks, and K-means. Machine learning models are divided into supervised learning and unsupervised learning. Supervised learning involves "training" ML algorithms on datasets consisting of inputs (or features) and "labeling" outputs. Unsupervised learning does not use labeled data, but attempts to extract underlying patterns from a dataset [13]. To calculate prognosis, numerical values are often simplified as categorical variables, and the weight given to predictive factors is rounded to integers and only a limited subset of variables can be included in the prognostic index. ML is a branch of artificial intelligence, which is entering the clinical research field at an increasing pace. ML allows digital algorithms to learn from experience without being explicitly programmed to do so [15].

In the study by Lamberink H. (2017) [16], it was outlined that the strongest predictors included in nomograms for crisis recurrence were: duration of epilepsy, duration of seizure-free interval, age at seizure onset, history of febrile seizures, ten or more seizures before remission, absence of a self-limited epilepsy syndrome (such as, absence or Rolandic epilepsy, or Panayiotopoulos syndrome), intelligence quotient (IQ) below 70, and epileptiform abnormality on EEG before discontinuation of antiepileptic drugs. To predict long-term seizure outcome, the eight independent predictors selected were: duration of epilepsy, duration of seizure-free interval, number of antiepileptic drugs before discontinuation, female gender, family history of epilepsy in first- or second-degree relatives, ten or more seizures before remission, presence of focal seizures, and epileptiform abnormality on EEG before discontinuation of antiepileptic drugs. Validation, or assessing how well a prediction works on data other than that on which the model was built, is arguably the most important issue in prognostic modelling.

Comparing with accessible scientific evidence, it was found that a number of common parameters were found in the own models developed, reported by a number of authors - namely, (early) age, drop-out from antiepileptic treatments, EEG abnormalities, structural abnormalities on MRI. However, the investigated cohort (patients of reproductive age) has not been mentioned in predictive models in the literature. Also, for the first time, attention was paid to depression - a symptom frequently encountered in reproductive age patients with epilepsy, which contributes significantly to the increased likelihood of an adverse outcome scenario.

Finally, it is found that in predictive models, based on clinical indicators ("categorical data"), taking into account the contribution to accuracy of the interactions between 2 and/or 3 parameters, added to the contribution given by the sum of the results of the individual variables is not so relevant for clinical practice, brings unnecessary complexity and decreases the clinical practice adoption medical practitioners. It is therefore recommended to use the developed simplified models.

Future studies are needed to build a model that is applicable to a wider range of patients with epilepsy, which will provide an easier, faster and more reliable method to predict the risk of antiepileptic drug resistance.

Conclusions

The predictive model developed (AErez), based on 3 parameters (depressive state, annual frequency of seizures, presence of brain lesions on MRI) has a positive predictive value of 83%, negative of 62%, with an area under the ROC curve of 0.72 (95%CI = 0.56 to 0.88) and a probability of event occurrence of 96%. Depressed patients with documented structural lesions on MRI and a high frequency of epileptic seizures have a progressive, significant (5.3-24.0-fold) risk of developing resistance to antiepileptic drugs.

Competing interests

None declared.

Authors' contribution

VC conceived conceptualization, methodology, data collection, analysis and interpretation, writing - original draft preparation. MG conceived writing review and editing, supervision, funding acquisition, validation. The authors read and approved the final version of the manuscript.

Patient consent

Obtained.

Funding Statement

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Ethics approval

The study protocol was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (minutes no. 55 of 03.06.2016).

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RESEARCH ARTICLE

Humoral immune status in patients with parasitic arthritis

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ABSTRACT

Introduction. The description of clinical manifestations of parasitic infestation includes joint damage manifested by arthralgia and arthritis, however, until today; the form of joint damage in parasitic infections is not clinically defined. The multitude of parasites creates a heterogeneous joint involvement, and the intensifying of specific manifestations would be an important premise in clinical diagnosis.

Objective. Assessment of immunological changes in the context of parasitosis and their ratio depending on the clinical variant of parasitic arthritis.

Material and methods. A group of 161 patients with parasitic arthritis was selected, established in two stages of compliance according to specific and serological criteria. The average age was 47.0±2.1 years, 72 men / 89 women. The average duration of joint syndrome was 50.4±15.6 months. The first group (97 patients) had parasitic arthritis of echinococcosis infestation, the 2nd (31 patients) – parasitic arthritis of *Toxocara cannis* and the 3rd (33 patients) had parasitic arthritis of *Giardia lamblia* infestation.

Results and discussion. IgA was increased above normal in all patients with parasitic arthritis (4.25±0.001; $p < 0.001$). IgM showed significant changes, depended on infestation agent ($p < 0.05$). Patients with normal IgG levels predominated, except for patients from the group with echinococcosis parasitic arthritis ($p > 0.05$). The amount of IgE exceeds normal values in *Echinococcus* parasitic arthritis (38.40 ng/ml), *Toxocara canis* (34.16 ng/ml), *Giardia lamblia* (45.06 ng/ml).

Conclusions. This suggests that the key aspects of immunity against parasites are mainly determined by the size of the harmful organisms that the immune system needs to combat. Macroorganism includes unique defense mechanisms that can be effective against multicellular helminths: high production of IgA and IgE and activation of key effector cells – eosinophils.

Keywords: parasitic arthritis, immunoglobulins, *Echinococcus*, *Giardia Lamblia, Toxocara Canis*.

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Key messages

What is not yet known about the issue addressed in the submitted manuscript

Most authors attest that parasitic arthritis has a non-specific immunological response and until today, the most important reactions remain unclear.

The research hypothesis

Due to the fact of the difference in infesting agents, a different clinical manifestation of parasitic arthritis is assumed, with a varied expression of immuno-inflammatory syndrome.

The novelty added by the manuscript to the already published scientific literature

The presentation of variability in parasitic arthritis is based on the

fact of different immuno-inflammatory reactions generated by different parasitic agents. Thus, parasitic arthritis in *Echinococcus* is characterized by the most severe development, followed by *Giardia lamblia,* and then *Toxocara cannis.*

Introduction

The immunological nature of parasitosis has always been of major interest, and there is existing literature that documents elevated levels of immunoglobulins, particularly IgA. [1-3]. Thus, the authors stipulate that the increase in the blood level of IgA is evidentiary for the hypothesis of infectious triggers in the mucous tunics in parasitic arthritis [2, 4]. Cases of IgA-induced nephropathies in patients with parasitic arthritis have been described [3, 5, 6]. Similarly, it is known that IgG and IgM have a protective effect and are able to damage the body of helminths, to conjugate the enzymes secreted by them, to form precipitation around their membranes, disrupting the physiological processes of the parasite. Many authors note that helminths secrete a complex of carbohydrates, which stimulate the production of IgE antibodies. One of the functions of IgE is to stimulate the formation and migration of eosinophils towards parasites for the fight against them. In this case, it appears that parasitic invasions at the initial stage are always accompanied by an increase in immunoreactivity in two ways – activation of the general mechanism of IgE antibody production and synthesis of specific IgE antibodies [1, 3, 7]. Perhaps this is why parasitic invasions are accompanied by an increase or manifestation of the hypersensitivity of the host organism, and with successful treatment – a decrease in all systemic inflammatory manifestations, including musculoskeletal [3, 8].

The most active processes of immunogenesis of helminthiasis are IgE-mediated inflammation, proliferation of eosinophils, cytotoxic action, increase in the activity of effector cells, that is, a cascade of reactions, aimed at the death and elimination of parasites, but not the development of persistent immunity [5, 9]. An active manifestation in the fight against helminths is hyper-eosinophilia, hyper-IgE production, release of mast cells mediators, hypersecretion of mucus, secretion of interleukin characteristic of allergies and systemic inflammatory immuno-pathological processes.

In recent years, the main antiparasitic mechanism, the effector cells of which are eosinophils, and the humoral side expressed by immunoglobulins, have been well studied. But, till this day, remains a great conundrum the development and progression of parasitic arthritis, generated by persistent immunopathological reactions. Likewise, it is necessary to mention the heterogeneity of clinical and laboratory manifestations, which presents a great diagnostic difficulty. Late diagnoses, in turn, increase the rate of morbidity and invalidation.

Thus, the study of the immunopathology of parasitic arthritis will allow to broaden the range of clinical assessment and will contribute to the elaboration of international consensus in classification and diagnosis criteria.

The wide prevalence of helminthiases *Echinococcus*, *Giardia lamblia* and *Toxocara canis* in the Republic of Moldova, the duration of the life cycle of parasites in the host body, the variety of clinical manifestations caused by them, the severity of complications and consequences determine the extreme relevance of this problem, with the need to detect solutions. A particular interest is the study of the specific anthelmintic immune response, which provokes osteo-articular pathological changes, as well as the development of diagnostic criteria and prognosis indicators.

The purpose of the study: Assessment of immunological deviations in the context of parasitosis and their ratio depending on the clinical variant of parasitic arthritis.

Materials and methods

This study was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (No. 83, from 19.06.2018), according to the WMA Declaration of Helsinki. A group of 161 patients with parasitic arthritis was selected, established in two stages of compliance according to specific and serological criteria. The first stage included the correspondence of the diagnosis in accordance with the criteria of osteoarticular damage of the inflammatory type. The second stage of definition of diagnosis concerned the compliance in accordance with the positive results of the serological or parasitological diagnosis confirmed positive. The average age was 47.0±2.1 years, 72 men / 89 women. The average duration of joint syndrome was 50.4±15.6 months.

Patients were divided into 3 groups by the pathogen of infestation and the clinical variant of parasitic arthritis. The first group (97 patients) included parasitic arthritis on the background of echinococcosis infestation, the 2nd (31 patients) – of *Toxocara cannis* and the 3rd (33 patients) group included parasitic arthritis of *Giardia lamblia* infestation.

The patients from the studied groups were subjected to a detailed evaluation, carried out according to a program of complex paraclinical examination, with quantitative determination of IgA, IgM, IgG and IgE, with the assessment of specific anthelmintic cellular immunity: eosinophils, degranulation capacity etc. The data obtained was processed through statistical package StatSoft ver. 8.0.

Results

The assessment of the level of immunoglobulins in the blood revealed the increase of the average IgA indices above the normal physiological values (4.25±0.001; $p < 0.001$) in all patients with parasitic arthritis (Table 1).

The average indices of IgG level were within the reference range in all the subjects, but they were at the upper limit of normal value and showed no differences between the studied groups ($p > 0.05$). However, a slight tendency towards decreasing IgG concentration was observed in patients with *Giardia lamblia* parasitic arthritis compared to the other studied groups, but these differences, are not statistically significant ($p > 0.05$).

Table 1. Immunoglobulins level in peripheral blood in parasitic arthritis

Immunoglobulins	Parasitic arthritis *Echinococcus* n = 97	Parasitic arthritis *Toxocara canis* n = 31	Parasitic arthritis *Giardia lamblia* n = 33	Normal values
IgA level, g/l	4.5±0.17**	4.07±0.21**	4.39±0.12**	0.9-3.5
IgG level, g/l	1.25±0.3	1.23±0.4	1.19±0.7	0.8-2
IgM level, g/l	1.99±0.017	1.64±0.032	2.09±0.011*	0.6-2.1

Note: ** - p < 0.05; ** - p < 0.001.*

The average indices of IgM levels in the blood showed significant changes (p < 0.05) (see Table 1). As well as in the case of IgG, in parasitic arthritis, patients with physiological levels of immunoglobulins predominate (91%), except for a tendency of their elevation in the groups of patients with parasitic arthritis *Echinococcus* and *Giardia lamblia* compared to *Toxocara canis*. However, the differences were not statistically significant (p > 0.05).

Our study has shown that IgE is the only antibody that increases systematically and reaches the maximum values in the evolution of all 3 helminthiases. Its amount exceeds normal values by more than 10 times - with *Echinococcus* (38.40 ng/ml), 9 times - with *Toxocara canis* (34.16 ng/ml), 11 times - with *Giardia lamblia* (45.06 ng/ml) (see Table 2).

Table 2. IgE level in peripheral blood in parasitic arthritis

Immunoglobulins	Parasitic arthritis *Echinococcus* n = 97	Parasitic arthritis *Toxocara canis* n = 31	Parasitic arthritis *Giardia lamblia* n = 33	Normal values
Ig E (ng/ml)	38.40±0.16*	34.16±0.23*	45.06±0.12**	<4

Note: *p < 0.05; **p < 0.01

As far as eosinophils are concerned, the results of this study were generally consistent with the literature. Thus, absolute values of eosinophils in peripheral blood resulted in significant eosinophilia in all three groups of patients studied, but significantly higher in patients with echinococcal infestation, followed by *Giardia lamblia* and then *Toxocara canis* (see Table 3). A significant increase (relative to the norm) in the content of leukocytes of the eosinophilic series in the blood of the examined patients was observed (up to 45.43±8.71%, p = 0.001 - in patients with *Echinococcus*; in patients with *Giardia lamblia*, eosinophilia was determined in 60% of cases and was recorded at the level of 39.51±1.76%, p = 0.019) (see Table 3).

Table 3. Eosinophils number (%) and the capacity of degranulation (%, abs)

Eosinophil content, %	Parasitic arthritis *Echinococcus* n = 97	Parasitic arthritis *Toxocara canis* n = 31	Parasitic arthritis *Giardia lamblia* n = 33
Absolute indexes, $x10^9$/L (M±m)	4.55±0.97**	3.35±0.72*	4.0±0.56**
Relative indexes, % (M±m)	45.43±8.71***	33.5±0,3*	39.51±1.76**
Degranulation capacity, % abs	7.79±2.04%*** 0.21±0.01 G/l*	6.05±1.13%* 0.27±0.03 G/l*	6.37±0.83%* 0.07±0.00 G/l**

Note: ** - p < 0.05; ** - p < 0.01; *** - p < 0.001

Another factor that causes a significant increase in the bactericidal function of eosinophils may be an increase in their ability to degranulate and undergo cytolysis. This affirmation is confirmed by the results of our study, which revealed a significant increase (in relation to the norm) in the content of eosinophils with affected morphological properties (without antigenic stimulation: in patients with echinococcosis 7.79±2.04% (p < 0.001) and 0.210±0.010 G/l (p < 0.05), respectively, with giardiasis 6.37±0.83% (p < 0.05) and 0.070±0.000 G/l (p < 0.01) at a rate of 2.54±0.05% and 0.002±0.00 G/l) (see Table 3). When *Toxocara cannis* antigen was added to *in vitro* samples, the absolute and percentage number of eosinophils in peripheral blood with altered morphological properties was found to be greater than the mean values of these parameters (6.05±1.13% (p < 0.05) and 0.270±0.030 G/l (p < 0.05)). It should be noted that changes in the morphology of leukocytes of the eosinophilic series were mainly in the nature of cytolysis: swollen cells were 2 or more times larger than the size of neutrophils, granules were visualized next to the cells. Along with this, increased vacuolization of the nucleus and cytoplasm of eosinophilic granulocytes was recorded.

Discussions

At the present time, the following hypothesis is accepted „atopy has appeared as an evolutionary adaptation to the increased antigenic load, but in the absence of timely antigenic exposure, it does not protect, but leads to the development of allergic and autoimmune diseases" [2, 5, 9]

As known, the production of common serum immunoglobulins IgE and IgG is regulated by IL-4 [6, 8, 10]. These immunoglobulins, and especially IgE – are an important

physiological regulator of immunological homeostasis [2-4], a fact also proven by our study. As can be seen by our results, but also from the data of the specialized literature, in helminthiases the protective role of antibodies of the IgE class is the most important. Numerous data according to which the level of antibodies of the IgE class in helminth infection is significantly increased, suggest that IgE actively contributes to the protection of the host against parasites.

In helminthiases, there is often a 100-fold increase in IgE titers [3, 8, 11].In our study, we obtained similar results. Numerous studies show that IgE class antibodies activate mast cells and induce the release of mediators from them, which are able to act directly on the parasite by increasing vascular permeability and releasing eosinophilic chemotactic factor, which can lead to the accumulation of adjuvant antibodies (IgG and IgA) and cells that directly affect the parasite [1, 4].

It has been established that the characteristic features of antiparasitic immunity are primarily due to the size of pathogenic objects against which the immune system must act, a fact assumed by some scientists [5, 7]. Macroorganism includes unique defense mechanisms that can be effective against multicellular helminths: high IgE production and activation of eosinophilic – effector cells. It is known that an increased content of granulocytes is one of the first, and sometimes the only sign of the pathological process caused by helminthic invasion. According to the authors, in the acute phase, the content of eosinophils reaches 20-40%, sometimes up to 90% [6, 9]. At the same time, in the chronic stage, according to various authors, eosinophilia occurs in about half of the patients – from 44 to 59% [2, 4, 7].

Eosinophils involved in the fight against helminths along with mast cells, begin to secrete various cytokines. However, it is known that the "processes occurring along the line mastocyte – eosinophils also have a significant impact on the formation of allergic reactivity with a significant impact on the musculoskeletal system" [3, 8].

Eosinophils perform various functions in helminthiases and differ from other cells by the presence of granules that are intensely stained with acid dyes, especially eosin. One of the main functions of eosinophils is cytotoxicity [4, 10]. The products of the granules of these cells can participate both in oxygen-dependent lysis, when toxic oxygen metabolites are released, and independent of oxygen, which is mainly associated with the release of a large base protein, eosinophilic cationic protein and peroxidase [6, 11]. These mechanisms can act both in isolation and synergistically, which in this case ensures the maximum efficiency of the lysis [8, 9].

Conclusions

Therefore, it has been established that the characteristic features of antiparasitic immunity are primarily due to the size of pathogenic objects against which the immune system must act. Human organism includes unique defense mechanisms that can be effective against multicellular helminths: high production of IgA and IgE and activation of key – eosinophilic – effector cells.

The assessment of the level of immunoglobulins in the blood revealed the increase in the average indices of IgA in all patients with parasitic arthritis ($p < 0.001$).

The average indices of the IgG level were presented within the limits of the norm in all the investigated groups, but they were posted at the upper limit of normal and showed no differences between the studied groups ($p > 0.05$). However, a slight tendency towards a decrease in IgG concentration was observed in patients with *Giardia lamblia* parasitic arthritis compared to the other studied groups, but these differences are not statistically significant ($p > 0.05$).

The average indices of IgM levels in the blood showed significant changes ($p < 0.05$). In parasitic arthritis, patients with physiological levels of immunoglobulins predominate, except for a tendency to their elevation in the groups of patients with parasitic arthritis through *Echinococcus* and *Giardia lamblia* compared to *Toxocara canis*.

The most active processes of immunogenesis of helminthiasis are IgE-mediated inflammation, proliferation of eosinophils, cytotoxic action, increased activity of effector cells. It is such a cascade of reactions, aimed at the death and elimination of parasites, and not the development of persistent immunity. Our study have shown that, most visibly in the evolution of all 3 helminthiases, IgE systematically increases and reaches a maximum. Its quantity exceeds the normal range more than 10 times with *Echinococcus*, 9 times - with *Toxocara canis* and 11 times - with *Giardia lamblia*.

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

This study was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (Minutes No. 83, from 19.06.2018).

Abbreviations

ASDAS – Ankylosing Spondylitis Disease Activity Score; BASDAI – Bath Ankylosing Spondylitis Disease Activity Index; BASFI – Bath Ankylosing Spondylitis Functional Index; BASRI – Bath Ankylosing Spondylitis Radiology Index; CRP – C-reactive protein; DAREA – Disease Activity REactive Arthritis; FC – functional class; VAS – visual analogic scale.

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RESEARCH ARTICLE

Analysis of *SMN1*, *NAIP* and *GTF2H2* gene status in correlation with spinal muscular atrophy

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ABSTRACT

Introduction. Spinal Muscular Atrophy (SMA) is a genetic disorder caused by the loss of the survival motor neuron (SMN1) gene in over 95% of cases. Additionally, mutations in genes associated with the SMA chromosomal region can influence disease progression. Aim: To analyze the status of the NAIP and GTF2H2 genes in correlation with SMA.

Material and methods. The study included 105 patients suspected for SMA of which 50 with confirmed with SMA and 55 without causative deletions, and 107 healthy, unrelated individuals. The molecular genetics methods used were mPCR, PCR-RFLP and MLPA.

Results. From 105 patients, 50 were confirmed with SMA. In this group were identified in 8 patients (16%) with a homozygous deletion of exon 5 of the NAIP gene, 4 patients (8%) had a heterozygous status, and 2 (4%) had duplications. In the rest of the patients (55), in which deletions of SMN1 exon 7 were not identified, homozygous deletion of exon 5 of the NAIP gene was established in one patient (2%), 3 patients (5%) had duplications of exon 5 of the NAIP gene, and one patient had 5 copies of the NAIP gene. In the 107 healthy controls, one patient (1%) was identified with a deletion of exon 5 of the NAIP gene. None of the patients with combined deletions of SMN1 and NAIP had deletions in GTF2H2.

Conclusions. The frequency of deletions in the NAIP gene was found to be higher in the SMA patient group compared to the control group. Thus, a significant relationship was identified, the P value being <0.00001. The significance threshold was set at p<0.05. The genetic patterning of genes associated with SMA is an important aspect in the study of molecular pathophysiology and assessment of disease prognosis, especially in the approach to gene therapies.

Keywords: SMA, *NAIP, GTF2H2, SMN1*, deletions, frequency, molecular genetics**.**

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

The worldwide understanding of Spinal Muscular Atrophy (SMA) genetics remains incomplete, with ongoing research needed to uncover the precise molecular mechanisms, global variations in genetic patterns, and the full spectrum of genotype-phenotype correlations. Additionally, the therapeutic implications of genetic profiles and the long-term outcomes of individuals with SMA, especially in complex cases, are areas that require further investigation to enhance SMA diagnosis and treatment strategies.

The research hypothesis

The research endeavors to explore the relationship between the genetic status of the *NAIP* and *GTF2H2* genes, located in the SMA

chromosomal region, and the diversity of mutations associated with Spinal Muscular Atrophy (SMA).

The novelty added by manuscript to the already published scientific literature

It has been demonstrated that there is a significant correlation between the presence of deletions in the NAIP gene and the occurrence of Spinal Muscular Atrophy (SMA), suggesting that NAIP gene deletions may play a crucial role in SMA susceptibility and severity. Also this research aims to reflect the deletion profile of the patients from Moldavian population in relation to the prevalence of deletions in the genes associated with Spinal Muscular Atrophy (SMA).

Introduction

Spinal muscular atrophy (SMA) is an autosomal recessive disorder that causes muscular atrophy and hypotonia [1, 2]. In over 95% of cases, SMA is caused by homozygous deletion of exon 7 in the *SMN1* gene [3-5]. However, mutations in other genes in the SMA region can contribute to the disease, such as *SMN2*, *NAIP*, and *GTF2H2* genes [6-8].

The *NAIP* gene (OMIM: 600355) [9] is located near the SMN gene and is also duplicated in the 5ql3 region. However, the copy associated with deletions in SMA patients can be distinguished because only this copy contains exon 5 [10-12]. Therefore, in some studies, deletion of exon 5 is present in approximately 50% of patients with SMA type I and ~20% in patients with type II and type III [13]. Experiments demonstrating that the *NAIP* gene is responsible for expressing a protein that suppresses cellular apoptosis support the idea that the protein acts as a negative regulator of motor neuron apoptosis [14]. When this protein is deficient or absent, it contributes to the SMA phenotype [15-17]. Thus, a moderate correlation has been demonstrated between mutations in the *NAIP* gene and the pathophysiology of SMA, especially when considered together with the number of *SMN2* copies, particularly in SMA types I, II, and III [18].

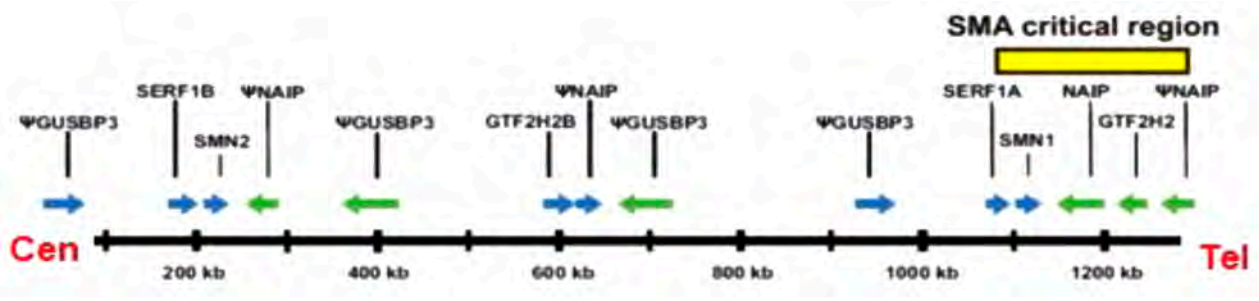

Fig. 1. Map of the genomic localisation of "SMA critical region" showing all genes and their orientation.
SMN1 and *SMN2* are in the same orientation and are approximately 848 kb away from each other. Near *SMN1* are *NAIP and GTF2H2* genes. Also are represented the pseudogenes that are indicated with a (Ψ) [19].
Note: *Cen – centromer; ΨGUSBP3 – Glucuronidase, Beta 3 Pseudogene; SERF1B – Small EDRK-Rich Factor 1B (Centromeric); SMN2 – Survival Of Motor Neuron 2, Centromeric; ΨNAIP – Neuronal Apoptosis Inhibitory Protein pseudogene; GTF2H2 – General Transcription Factor IIH Polypeptide 2; SERF1A – Small EDRK-Rich Factor 1A (Telomeric); SMN1 – Survival Of Motor Neuron 1, Telomeric; NAIP – Neuronal Apoptosis Inhibitory Protein; Tel – Telomeric; kb – kilobases.*

The gene *GTF2H2* (OMIM: 601748) a subunit of the basal transcription factor TFIIH, involved in the transcription process, DNA repair mechanisms and probably in other cellular processes was also characterized and located in the SMA region.

Thus, studies were reported on unrelated patients with SMA that showed that large deletions involving *SMN, NAIP, GTF2H2* gene loci are associated with the most severe SMA phenotype (SMA type I) [20, 21]. The identification of deletions in these genes can be performed by different molecular genetics methods such as PCR, qPCR or MLPA [13, 17, 21].

Objective

The aim of this study was to analyze the profile of modifier genes associated with SMA, such as *NAIP* and *GTF2H2*, in patients with SMA.

Material and methods

The study was conducted at the Institute of Mother and Child, Human Molecular Genetics Laboratory. A total of 105 patients suspected of having SMA were enrolled in the study, including 50 patients with a molecular-genetic diagnosis of SMA, 55 patients with hypotonia but without confirmed molecular-genetic SMA, and 107 unrelated healthy individuals from the Human Molecular Genetics Laboratory's database. All patients have signed the consent for participation (approved by Research Ethics Committee of *Nicolae Testemițanu State University of Medicine and Pharmacy*, Act No. 3, from February 16, 2021). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and multiplex ligation-dependent probe amplification (MLPA) (The innovation Act "*The method of diagnosing Spinal Muscular Atrophy by the MLPA technique*" No. 483 from 17.03.2022) were used to detect deletions of exons 7

and 8 of the *SMN1* gene for diagnostic purposes. Multiplex polymerase chain reaction (mPCR) was used for exon 5 of the *NAIP* gene and exon 4 of the *GTF2H2* gene. Primers for identifying mutations in exon 5 of *NAIP* (The innovation Act *„ Diagnostic method for the deletion of exon 5 of the NAIP gene based on the PCR genetic molecular technique*" No.517 from 14.03.2023) and exon 4 of *GTF2H2* through mPCR (The innovation Act "*Diagnostic method of deletion of exon 4 of the GTF2H2 gene based on the PCR genetic molecular technique*" No. 516 from 14.03.2023) were custom-designed, along with the parameterization of the amplification program. The innovative diagnostic methods used, as outlined in the acts mentioned above, contain the detailed protocol information, safeguarding that more specific data will not be made public to facilitate the filing of a patent application. Statistical analyzes were calculated via *Social Science Statistics* (Online) [22].

Results

In the pursuit of unraveling the intricate genetic landscape underlying Spinal Muscular Atrophy (SMA), our study meticulously examined the distribution and implications of deletions within the *SMN1, NAIP* and *GTF2H2* genes, elucidating potential links to SMA. The following results present a comprehensive analysis, detailing the frequency of deletions in the *NAIP, GTF2H2* and SMN1 genes across distinct patient cohorts, providing numerical insights crucial for advancing our understanding of SMA pathogenesis. The distribution of patient groups was predicated on clinical manifestations and the analysis of exon 7 status in the *SMN1* gene. After carrying out molecular genetics analyzes by PCR and MLPA technique, within the SMA group, 8 out of 50 patients (16%) exhibited a homozygous deletion of exon 5 of the *NAIP* gene, while 4 patients (8%) showed a heterozygous status, and 2 patients (4%) displayed duplications. In the cohort of 55 patients with hypotonia suspected of SMA but lacking deletions in *SMN1* exon 7, one patient (2%) had a homozygous deletion of exon 5 of the *NAIP* gene, 3 patients (5%) exhibited a duplication of exon 5 of the *NAIP* gene, and one patient showcased 5 copies of the *NAIP* gene. Among the 107 unrelated healthy controls, one patient (1%) manifested a deletion of exon 5 of the *NAIP* gene (Figure 2).

Fig.2 The deletion profile of the *NAIP* gene identified in the patients included in the study.
Representation of the number and type of mutations: Blue-homozygous, normal, Orange - deletions, Gray -Duplications, in each subgroup: I- SMA patients, II- Suspects for SMA, III- Control group; SMA - Spinal Muscular Atrophy.

Notably, the frequency of exon 5 deletions of the *NAIP* gene was calculated for the entire SMA patient group without categorization by SMA types.

Furthermore, molecular genetic analysis of exon 4 of the *GTF2H2* gene was exclusively performed for patients with combined deletions of *SMN1* and *NAIP*, revealing no deletions of exon 4 of the *GTF2H2* gene in any patient.

Statistical analysis, employing the Chi-squared test to explore the relationship between mutations in the *SMN1* gene and those in the *NAIP* gene, yielded compelling results. The Chi-squared statistic was X^2=24.97, with an associated p-value of < 0.00001, indicating a statistically significant association between deletions in the *SMN1* gene and deletions in the *NAIP* gene.

These findings, backed by numerical data and percentages, strongly support the hypothesis that genetic alterations in the *NAIP* gene are intricately linked with susceptibility to SMA. This underscores the paramount importance of delving into these genetic patterns for a nuanced understanding of SMA pathophysiology and a more accurate prognosis.

Discussions

SMA is an inherited disease that leads to progressive hypotonia and damage to the lower motor neurons in the ventral horn of the spinal cord [23]. According to previous studies, changes in genes located near the SMN gene locus have been correlated with the severity of SMA (Jiang et al., 2019) [24], and an important factor influencing the clinical severity of SMA is the duplication of exon 7/8 in the *SMN2* gene, which exerts a modifying effect on the disease. Additionally, prior research in various populations has linked severity to alterations in the *NAIP* and *GTF2H2* genes (Liu et al., 2016) [21]. Additionally, it has been demonstrated that *NAIP* plays a role in preventing motor neuron apoptosis and is homozygously deleted in approximately 50% of SMA type 1 cases [25], while the *GTF2H2* gene is important in transcription and DNA repair [8].

Other studies, regarding the gene expressions of *GTF2H2, NAIP*, and others related to SMA, have been reported in the literature. For example, a study conducted in the

Turkish population examined the expression levels of *SERF1A, GTF2H2, NCALD, ZPR1, TIA1, PFN2*, and *CORO1C* genes for the first time in SMA patients (Zhuri et al., 2022) showed statistically significant differences ($p = 0.037$, $p = 0.001$) between *SERF1A* and *NAIP* genes compared between control group and patients groups [26]. Another study conducted between 2018 and 2021 included 58 SMA patients and 40 healthy individuals as a control group also in Turkish population (Karasu et al., 2022) showed that the genes *NAIP* (p = 0.0095) and *GTF2H2* (p = 0.0049) exhibited a significant difference between healthy subjects and those with SMA [8].

In a study involving the Egyptian population, Hassan et al. in 2020 determined that *SMN2* and *NAIP* are the primary modifier genes, and alterations in their copy numbers can impact the severity of SMA. They found that homozygous deletion of exon 5 of *NAIP* was observed in 60% to 73% of SMA cases, depending on the SMA type [2].

In this study, our aim was to present the mutational profile of the *NAIP* and *GTF2H2* genes and examine the relationship between the *SMN1, NAIP*, and *GTF2H2* genes to determine the frequency of *NAIP* and *GTF2H2* deletions in patients with SMA. According to our findings, combined homozygous deletion in both the *SMN1* and *NAIP* genes was found in 16% of SMA patients. Therefore, a significant association between deletions in the *SMN1* gene and deletions in the *NAIP* gene was established. The chi-square statistic was $X^2 = 24.97$, and the p-value was <0.00001. These data once again underscore the presence of deletions in other genes in the immediate vicinity of the SMA-causing genes in the case of patients from the Republic of Moldova.

However, in our study, no relationship was demonstrated between the *GTF2H2* gene and patients with deletions in *SMN1* and *NAIP*. This phenomenon was described in a study by Arkblad et al. in 2009 [27], although the presence of mutations in the *GTF2H2* gene has been reported to be closely associated with a severe form of SMA (type I) by He et al. in 2013 [28].

Due to the fact that the frequency of deletions in genes associated with SMA was calculated for the entire group of SMA patients, without categorizing them by SMA types, the percentage of deletions is different compared to other populations. However, this still demonstrates that such genetic profiles are characteristic of this disease in the Moldovan population, especially given that the p-value (<0.00001) and the chi-square test statistic (X^2=24.97) showed a highly significant correlation between these two gene mutations. Regarding patients with duplication of exon 5 of *NAIP*, both in the SMA patient group and the hypotonia group, Tomoko Akutsu et al. in 2002 reported in their work that approximately 2 to 5 copies of intact or truncated *NAIP* gene have been identified in the general population, suggesting that duplications in the *NAIP* gene do not have clinical significance [29].

These findings suggest a complex link between *SMN1, NAIP* and *GTF2H2* genes in the pathogenesis of SMA and highlight the importance of molecular genetic studies for understanding and characterizing the disease in different populations. Our study contributes to the knowledge of the mutational profile of the *NAIP* and *GTF2H2* genes in the context of SMA in Moldova, while underlining the need for continued research to develop more effective therapies and to increase the understanding of this complex condition.

Some aspects that are not yet known or require investigation can be categorized as limitations but also ideas for further research:

- *Perspectives on Molecular Mechanisms*: Further investigations are warranted to elucidate the molecular pathways involved in establishing causality and risk factors.

- *Genetic Variability:* Examination of a broader spectrum of possible genetic factors implicated is essential.

- *Clinical Implications*: Subsequent research could shed light on how this genetic information can be practically applied in a clinical framework, with long-term monitoring of individuals with associated mutations.

Conclusions

The present study revealed a higher prevalence of *NAIP* gene deletions within the SMA patient group as compared to the control group, establishing a significant relationship with a *p*-value of $p < 0.00001$. This suggests that the likelihood of this relationship occurring by chance is exceedingly low. Consequently, these alterations merit consideration in the assessment of molecular pathophysiology and disease prognosis. This observation is particularly pertinent in the context of genetic therapies, as it signifies that the genetic profile characterized by modifications in genes within the SMA region is also representative of the population in the Republic of Moldova.

Competing interests

None declared.

Authors' contribution

IC conceived conceptualization, methodology, data collection, analysis and interpretation, writing - original draft preparation. VS conceived writing review and editing, supervision, funding acquisition, validation. The authors read and approved the final version of the manuscript.

Patient consent

Obtained.

Ethics approval

This study was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (Act No. 3, from February 16, 2021).

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RESEARCH ARTICLE

Platelet-rich plasma role in the local protection of the colon anastomosis

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ABSTRACT

Introduction. Intestinal anastomosis dehiscence has been and remains a critical problem in modern colorectal surgery, associated with significant morbidity and mortality. Currently, there are various studies focused on the development of a method to protect the intestinal anastomosis. The new method with the use of biological substances is the local application of platelet-rich plasma.

Material and methods. The experimental study included 42 rats, which were divided into two groups. Group 1 – application of unprotected colon anastomosis (n = 21); group 2 – local application of platelet-rich plasma on the colon anastomosis (n = 21). 37 patients were included in the clinical study, who underwent colon anastomosis. The patients were divided into 2 groups: group I (n = 16) had unprotected colon anastomosis and group II (n = 21) - protected anastomosis with platelet-rich plasma.

Results. It was experimentally proven that platelet-rich plasma does not aggravate the adhesion process and actually increases significantly the mechanical resistance of colon anastomosis. Microscopical examination demonstrated the acceleration of regenerative processes, in particular, angiogenesis and fibrillogenesis. The clinical study showed significant improvement of the postsurgical results – absence of colon anastomosis dehiscence in cases where local application of platelet-rich plasma was used.

Conclusions. A statistically significant increase in dehiscence pressure of the anastomosis at 3rd, 7th and 8th day after surgery was noticed in group 2 vs. group 1. Using platelet-rich plasma does not influence significantly the process of abdominal adhesion, leads to increased regeneration process in the anastomosis area, especially neogenesis and fibrillogenesis ($p < 0.5$). Using platelet-rich plasma significantly improves the post-surgical results.

Keywords: colon anastomosis dehiscence, platelet-rich plasma.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

Currently, the effects of platelet-rich plasma on the healing process of the colon anastomosis are not fully known.

The research hypothesis

Clinical and experimental evaluation of platelet-rich plasma effectiveness for local protection of the
colonic anastomosis: benefits and disadvantages.

The novelty added by manuscript to the already published scientific literature

A complex clinical and experimental evaluation of platelet-rich plasma effect on healing of colon anastomosis was performed. Postoperative results were studied, in particular the development

of complications, such as dehiscence of colon anastomosis or postoperative peritonitis. In medical literature, this subject is presented vaguely, while the scientific data is insufficient. Further studies are required.

Introduction

Intestinal anastomosis dehiscence is a surgical complication that remains an important issue in modern public health and has a major medical, social and economic impact. Anastomosis dehiscence can be considered one of the quality indices of medical care in surgical departments [1-3]. According to medical literature, the incidence of the development of colon anastomosis dehiscence ranges widely, from 3.3% to 25.1% [4, 5]. The occurrence of this complication is associated with a significant increase in postoperative morbidity and mortality [6], and, respectively, reflects the quality of the surgical service [7].

Currently, in order to protect the intestinal anastomosis and prevent the occurrence of anastomosis dehiscence, various studies are carried out based on studying the role of human blood elements and the use of various synthetic substances. A new proposed method is the use of platelet-rich plasma. This term was first proposed in 1998 by Marx [8]. Platelet-rich plasma ensures the penetration of platelets in excessive quantity, accelerating the wound healing process. The regenerative effect can be explained by modulating growth factors such as platelet-derived growth factor, insulin-like growth factor, transforming growth factors β1 and β2 [9]. The active secretion of these factors is initiated due to the blood coagulation process and begins within 10 minutes of coagulation. More than 95% of growth factors are synthesized during the first hour [10]. Clinical and experimental studies are necessary for the correct assessment of the effectiveness of the use of platelet-rich plasma in the local protection of the anastomotic area.

The study objective was to assess the efficacy of platelet-rich plasma in local protection of colon anastomosis.

Material and methods

Experimental part. Forty-two rats were divided into two groups: group I – unprotected colon anastomosis (n = 21); group II – protected colon anastomosis with local application of platelet-rich plasma (n = 21). Anesthesia was performed by intraperitoneal administration of ketamine hydrochloride solution (Kalypsol®, Gedeon Richter, Hungary). The experimental study was carried out in accordance with the „Directive 2010/63/EU of the European Parliament and of the Council" regarding the protection of animals used for scientific purposes [11]. The research project was examined at the meeting of the *Nicolae Testemițanu* State University of Medicine and Pharmacy Research Ethics Committee, which took place on September 15, 2014.

Colon anastomosis was performed according to the standardized method, which included the following steps: opening the abdominal cavity through mid-median laparotomy; transection of the transverse colon at a distance of 1 cm from the cecum with the application of end-to-end unprotected colon anastomosis with continuous suture, using Polypropylene monofilament thread 5/0 in rats from group I (Fig. 1). In rats from group II, platelet-rich plasma was applied on the line of anastomosis. Layered closure of the abdominal wall was performed.

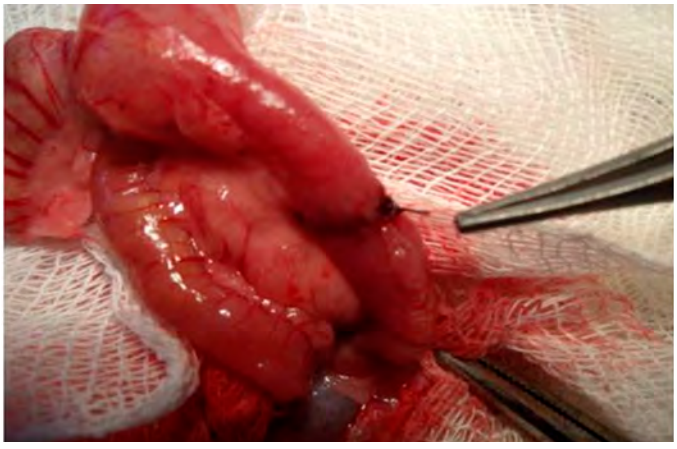

Fig. 1. Colon anastomosis, intraoperative photograph.

Animals were euthanized in CO_2 chamber. The autopsy of the rats was performed at 3rd, 7th and 14th postoperative day, 7 rats for each group. The anastomoses were examined macro- and microscopically to assess for abscesses, dehiscence, signs of peritonitis, adherences and to evaluate mechanical resistance. During autopsy, a 4 cm portion of the colon was taken, with the suture in the center and 2 cm on both sides of the anastomosis (fig. 2 a, b, c; fig. 3 a, b, c). The fragments were prepared for histological examination and to evaluate the anastomosis burst pressure.

Clinical part. 37 patients were included in the clinical study, who underwent colon anastomosis. The patients were divided into 2 groups: group I (n = 16) had unprotected colon anastomosis and group II (n = 21) - protected anastomosis with platelet-rich plasma. The anastomosis was applied in 2 steps: internal with suture polydioxanone 3/0 - 4/0 and external - polypropylene 3/0 (fig. 4 A, B).

The patients were carefully monitored in the postoperative period. Procalcitonin was used for the laboratory diagnosis of colon anastomosis dehiscence. Postoperatively, the changes in the level of serum procalcitonin on the 3rd, 5th and 7th day were studied. For the assessment of procalcitonin, an immunoenzymatic analysis kit (Вектор Б, Novosibirsk, Russia) was used performed on the automated immunological ELISA analyzer Uno (Human), Germany. The normal value of procalcitonin is < 0.1 ng/ml.

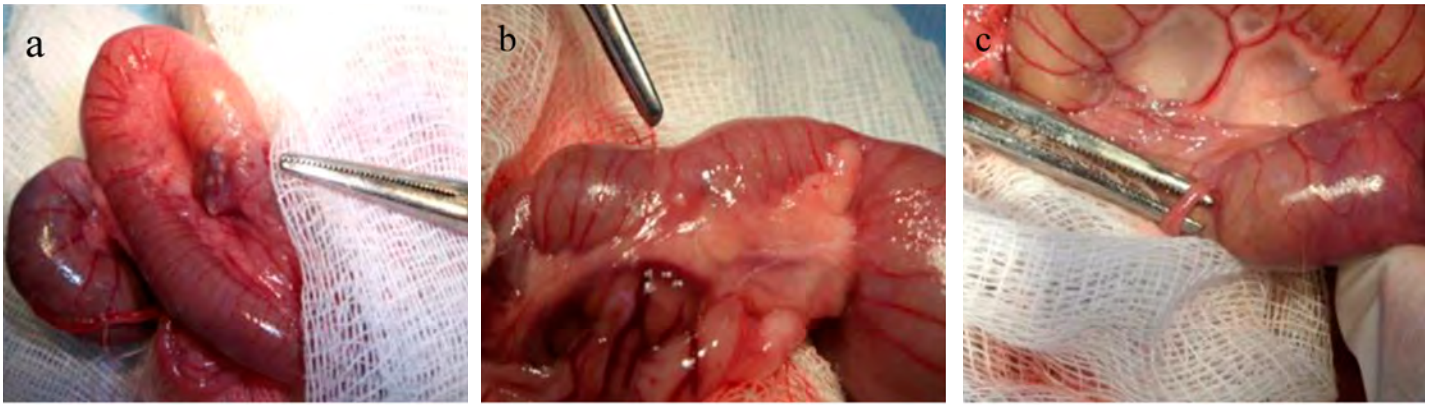

Fig. 2. Group I: a) 3rd day after anastomosis, b) 7th day after anastomosis, c) 14th day after anastomosis

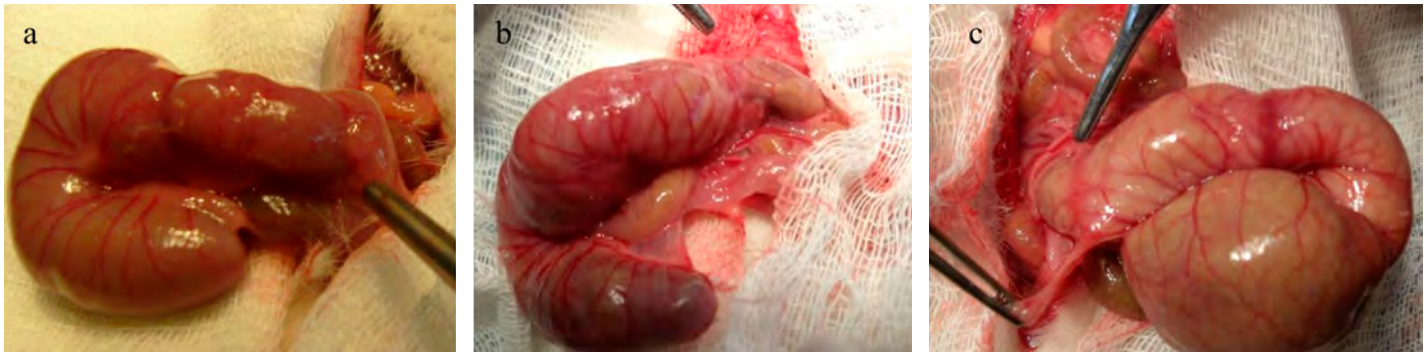

Fig. 3. Group V: a) 3rd day after anastomosis, b) 7th day after anastomosis, c) 14th day after anastomosis

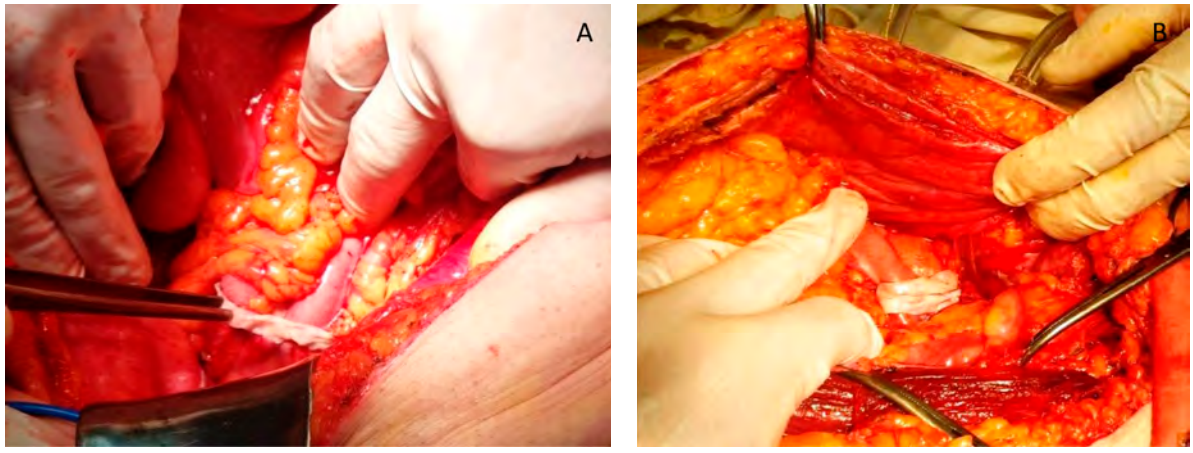

Fig. 4. End-to-end sigmorectal anastomosis protected by local application of platelet-rich plasma.

Results

Experimental study. Analyzing the degree of adhesion formation according to van der Hamm's score [12], we can conclude that there is a statistically insignificant increase in the degree of intra-abdominal adhesion formation from the 3rd to the 7th day, with a statistically insignificant decrease from 7th to 14th day.

According to the data obtained in the current study, a statistically insignificant increase in the degree of adhesion formation was demonstrated in group II vs. group I (NS).

The burst pressure of the anastomosis was also studied. According to the obtained data, there is a statistically significant increase in the burst pressure of the anastomosis from the 3rd to the 7th postoperative day and a statistically insignificant decrease of this parameter from the 7th to the 14th postoperative day (Fig. 5).

Analyzing the obtained data, a statistically significant increase ($p < 0.05$) of the anastomosis burst pressure was demonstrated on the 3rd, 7th and 14th postoperative day in group II vs group I.

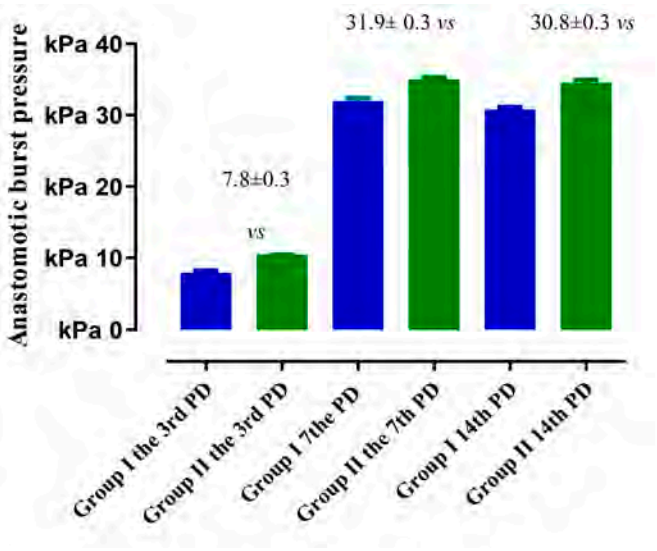

Fig. 5 Assessment of anastomosis burst pressure.

Note: *Group I – unprotected colon anastomosis (n = 21); Group II – protected colon anastomosis with local application of platelet-rich plasma (n = 21).*

Histological examination. Microscopic examination of the samples from group I revealed deformations and volume changes, reactive edema, adhesions and slowing of the regenerative processes. An important key in this group was the activation of the local bacterial microflora, which on the 3rd and 7th day, due to its proteolytic features, manifested itself through excessive activity, forming the bacterio-necrotic-purulent demarcation line. In the anastomoses with a predominance of bacterial flora, the necrolytic and inflammatory processes in some places were significantly more aggressive, sometimes with penetration into the depth of the anastomosis, thus contributing to the appearance of anastomotic leakages, abscesses, deformations of the anastomosis, as well as the appearance of diverticula (fig. 6). The formation of granulation tissue was manifested by increased proliferation of fibroblasts and the presence of collagen deposits (fig. 7). Persistence of dystrophy of the ganglioneuronal structures of the Auerbach plexus was frequently detected, at a distance of up to 2.5 cm from the anastomosis.

The microscopic examination of the samples from group II demonstrated that in most cases the anastomoses had a tubular appearance, with preserved permeability. The histological examinations in this group were demonstrated by the significant regenerative processes.

In group II, in the internal area of the anastomosis was noticed a decrease in the activity of thrombo-vascular and exudative processes, while in the external area these processes were absent, unlike group I (fig. 8). From the external examination, a mantle of newly formed tissue with an insignificant tissue-tuberous appearance was observed, prominent outside the anastomotic area with the activity of subtotal/total epithelization attested on the 14th day. In group II, it was attested a numerical increase of mast cells from

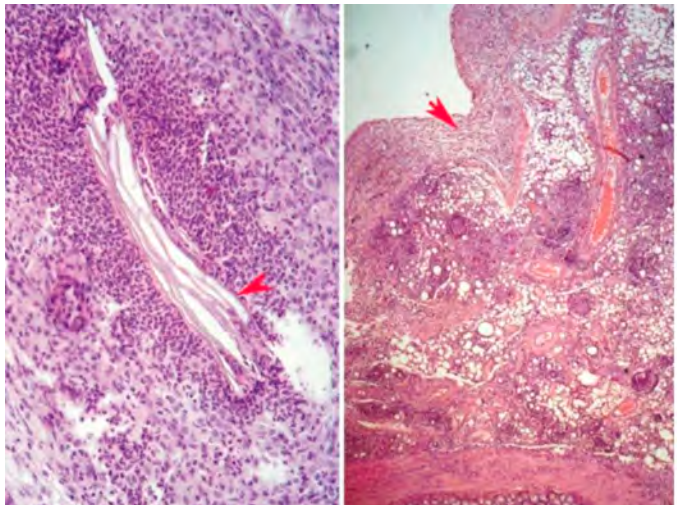

Fig. 6 Anastomotic leakage: fecaloid elements in the cellular mass (red arrow). *Coloration H&E.*

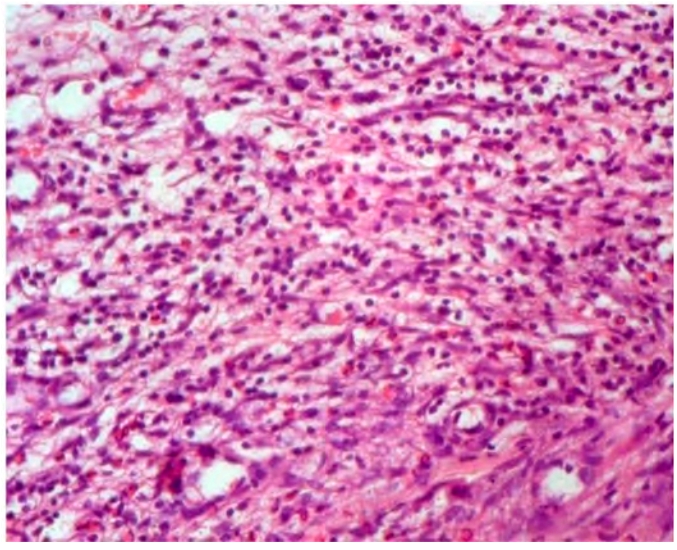

Fig. 7 Vascular granulation tissue with collagen arrangement in fibers x200. Coloration *H&E.*

2-3 to 8-9 at x20 high-power field, mainly in the external area of the anastomosis. In parallel, the emphasis of the proliferative-fibroblastic process was observed at the level of the cellular-adipose tissue, directed towards the anastomosis, with voluminous hyper granulated mast cells present or with the spread of granules in the extracellular matrix. Compared to the anastomoses in group I, the dystrophy of the nerve plexuses was insignificant, with the exception of 2 cases. It is necessary to mention that no anastomotic leakages were detected in this group. On the 14th day, the line of anastomosis in some places was completely diminished macroscopically (Fig. 9).

According to this study, the mast cells were actively involved in the initial stages of triggering the acute inflammatory process. The attested cellular morphological manifestations were characterized by hypergranulation, which reflects the activation of mast and degranulation cells, the

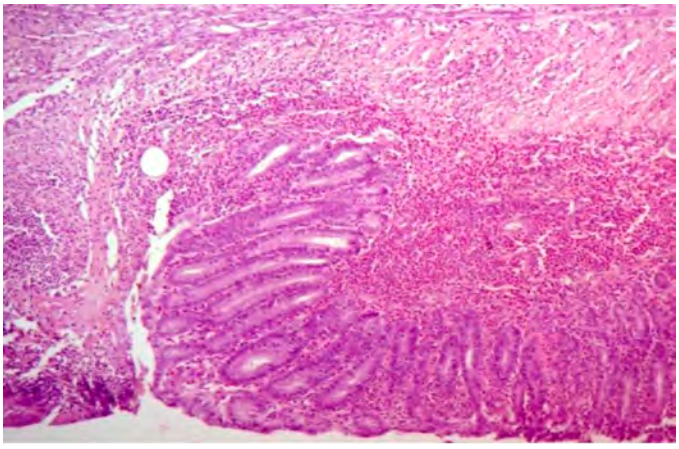

Fig. 8 Necrolytic process at the level of the fold and leukocyte reaction with the predominance of eosinophils in the adjacent areas x25. Coloration H&E.

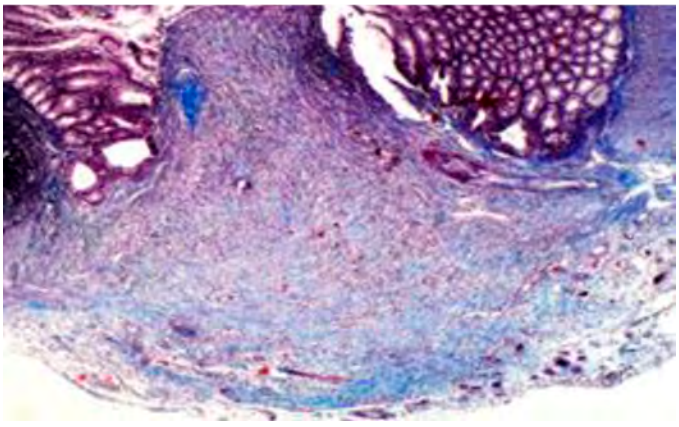

Fig. 9 Cicatricial remodeling of the anastomosis on the 14th day x25. Masson's staining.

phenomenon of release in the extracellular matrix of mediators and chemotactic substances [13, 14], which contribute to the initiation and migration of leukocytes towards the anastomosis areas. It is also necessary to mention that the degranulation phenomenon reflects the activation of the neovascularization process through the release of endothelial-vascular growth factors and platelet activation. Based on the experimental data, we can conclude that the application of platelet-rich plasma on the anastomosis line does not worsen the adhesion process and increases statistically significant the burst pressure of the colonic anastomosis. The microscopical examination showed the acceleration of regenerative processes, in particular, angiogenesis and fibrillogenesis, in group II vs. group I ($p < 0.05$).

Clinical study. Thirty-seven patients were included in the clinical study, who were hospitalized urgently or electively in the Institute of Emergency Medicine. The average age was 59.49 ± 2.16 (23-78) years. There were 21 males (56.75%), and 16 females (43.24%), the ratio M:F = 1.3:1. 16 patients were included in group I, of which 10 – males and 6 – females; in group II – 11 males and 10 females. The distribution of patients by sex and age is shown in table 1.

Table 1. Distribution of patients according to age

Age (years)	20-30	31-40	41-50	51-60	61-70	71-80	Total
Males	1 (%)	4 (%)	-	7 (%)	7 (%)	2 (%)	21 (%)
Females	-	-	1 (%)	2 (%)	9 (%)	4 (%)	16 (%)
Total	1 (%)	4 (%)	1 (%)	9 (%)	16 (%)	6 (%)	37 (%)

According to the etiology of the pathological process, the most predominant cause was neoplasia. Colon cancer was diagnosed in 17 cases (45.94%), other localized cancer - 2 (5.4%) cases, terminal colostomy – 7 (18.9%) cases, terminal ileostomy – 4 (10.8%) cases, colonic fistula – 2 (5.4%) cases, adhesion disease - 1 (2.7%) case, appendicular plastron - 1 (2.7%) case, appendicular mucocele - 1 (2.7%) case, sigmoid diverticulum - 1 (2.7%) case, colonic endometriosis - 1 (2.7%) case.

There were miscellaneous types and indications of surgeries. In 10 cases - the surgical procedures were performed urgently, while in 27 cases – planned. In group I, right hemicolectomy was performed - 7 cases, colostomy reversal - 4 cases, ileostomy reversal - 3 cases, ileum resection with ileocolic anastomosis - 1 case, ileosigmoid bypass - 1 case. In group II, right hemicolectomy was performed - 6 cases, segmental colectomy - 4 cases, left hemicolectomy - 4 cases, colostomy reversal - 4 cases, subtotal colectomy - 2 cases, previous rectal resection - 1 case.

Currently, there are numerous definitions and classifications of intestinal anastomosis dehiscence. This study used the classification of anastomotic dehiscence proposed by the International Rectal Cancer Study Group in 2013 [15]. In group I, grade B anastomosis dehiscence was diagnosed in 2 cases after planned right hemicolectomy, in one case after colostomy and one after ileostomy reversal. Grade C anastomotic dehiscence was detected in one case after emergency right hemicolectomy. This patient required repeated surgery – relaparotomy with anastomosis resection and ileostomy. In group II, there were no cases of anastomosis dehiscence. Thus, from the results of the clinical study data, the incidence of anastomotic dehiscence was in group I – 4 (25%) cases of grade B and 1 (6.25%) of grade C vs. group II – 0 cases ($p = 0.01$).

In the postoperative period, procalcitonin level was evaluated. Based on the obtained data, in uncomplicated cases with the development of anastomotic dehiscence, there is an increase in the serum level of procalcitonin on the 3rd postoperative day with a subsequent decrease on the 5th and 7th postoperative day. During the progressing of the colon anastomotic dehiscence a statistically significant increase in the procalcitonin serum level is determined, in particular - in grade C anastomotic dehiscence, on the 3rd, 5th and 7th postoperative day ($p < 0.0001$).

Discussions

Colorectal surgery represents an important field of contemporary surgery, due to the increasing incidence of surgical pathology of the colon. Despite the breakthroughs of modern medicine in general, and surgery in particular, dehiscence of intestinal anastomosis was and remains one of

the most dangerous postoperative complications [16], without significant improvements [5].

Currently there are different definitions of intestinal anastomosis dehiscence. The Surgical Infection Study Group in 1991 defined anastomosis dehiscence as the leakage of intestinal contents through the surgical connection between two cavity organs [17]. According to the results from Komen N. *et al.*, anastomosis dehiscence represents the leakage of intestinal content into the peritoneal cavity through the defect of anastomosis [18]. According to other authors, anastomosis dehiscence can be defined as the defect of the intestinal wall, which leads to the communication between the intra- and extraluminal compartment [19]. The International Study Group of Rectal Cancer defines dehiscence of the intestinal anastomosis as a communication between the intra- and extraluminal compartment through an anastomosis defect in the intestinal wall between the colon and the rectum or between the colon and anus. According to the data of this group, the abscess near the anastomosis, even without an obvious fistula, is to be interpreted as anastomotic dehiscence [15].

Early diagnosis and timely surgical intervention have a considerable influence on the final result. The diagnosis is primarily based on clinical data; however, epidural block [20], administration of analgesic drugs, including opioids, antibacterial therapy, and infusion therapy can reduce the clinical signs and symptoms. The increase in the concentration of serum inflammatory markers can be suggestive for anastomosis dehiscence, but the accuracy and specificity of the method is low, and the respective changes appear with a significant delay. The role of procalcitonin as a serological marker of intestinal anastomosis dehiscence is currently being researched. According to literature data, the increase in the serum concentration of procalcitonin was detected in all patients undergoing colorectal interventions, without complications, on the first postoperative day, with subsequent normalization on the 4th postoperative day in patients without complications and with a statistically significant increase on the 3rd to 5th postoperative day in patients with major anastomotic dehiscence [21]. Mokart *et al.* demonstrated the increased sensitivity and specificity of procalcitonin regarding the early diagnosis of septic complications after oncological surgery [22]. Thus, procalcitonin can be used as an early serological marker of major anastomotic dehiscence.

Protecting intestinal anastomosis remains an important problem in colorectal surgery. Various research is currently being carried out focusing on studying the natural factors that influence the regenerative processes. A new method in this field is the local application of platelet-rich plasma. Currently, platelet-rich plasma is used in various fields of contemporary medicine, such as: periodontology [23], maxillofacial surgery [24], dental implantology [25], orthopedics and sports medicine [26], chronic skin ulcers [27]. In the specialized literature, there are case reports about the use of platelet-rich plasma in colorectal surgery. Yol S. *et al.* (2008) experimentally proved that the application of platelet-rich plasma on colonic anastomosis suture is associated with an increase in the burst pressure of the anastomosis, by increasing the tissue hydroxyproline concentration [28]. Microscopical examinations of the anastomoses protected with platelet-rich plasma demonstrated the improvement of regenerative processes, in particular, neoangiogenesis and fibrillogenesis. Therefore, the local application of platelet-rich plasma on the anastomosis suture does not significantly worsen the abdominal adhesion process, statistically increases the burst pressure of the anastomosis and increases the regenerative processes.

Thus, the use of platelet-rich plasma has a beneficial influence on the healing process of the colon anastomosis and improves postoperative results.

Conclusions

- A statistically significant increase ($p < 0.05$) in anastomotic burst pressure was demonstrated on the 3rd, 7th and 14th postoperative day in group II vs. group I. Also, the use of platelet-rich plasma does not significantly influence the abdominal adhesion process.
- The microscopical examination demonstrated that the local use of platelet-rich plasma leads to the improvement of regeneration processes in the anastomosis area, in particular, neoangiogenesis and fibrillogenesis ($p < 0.05$).
- The use of platelet-rich plasma for local protection of colonic anastomosis improves postoperative outcomes.

Competing interests

None declared.

Patient consent

Obtained.

Ethical Statement

This study was carried out in accordance with the *European Convention for the Protection of Vertabrate Animals Used for Experimental and Other Scientific Purposes (*„Directive 2010/63/EU of the European Parliament and of the Council"*)* and approved by the Research Ethics Committee of *Nicolae Testemiţanu* State University of Medicine and Pharmacy, Minutes No. 5 from 15.09.2014.

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RESEARCH ARTICLE

The profile of villous chorion vascularization in primary placental insufficiency

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ABSTRACT

Introduction. Placentation and autochthonous vascularization during the early period of intrauterine development in primary placental insufficiency are important stages in the establishment of embryo-haemochorial circulation. Vascular and angiogenesis dysfunctions in the early period of gestation are essential in the development of changes in placental vascular status with severe repercussions on pregnancy outcome in the first trimester of gestation.

The purpose of the work. Evaluation of vascular density by the application of the anti-CD31 antibody in the chorio-villar germinal site in short-term dysregulated pregnancies.

Material and methods. 184 cases were divided into 2 groups: stagnant pregnancies (SP (L1 = 144 cases) and early spontaneous abortion (ESA) (L2 = 40 cases). Control material: pregnancies resolved at social indications/desire (A) (n = 18) distributed according to gestational term (3-5 weeks), (6-9 weeks), and (10-12weeks). The estimation of the histomorphological features was performed by applying the classical conventional method with hematoxylin-eosin (H.E.), and the immunohistochemical evaluation of the vascular density in the chorio-villar profile with the help of the endothelial marker anti-CD31. The statistical relationships between fetal conceptus assessment forms, gestational term, and age were analyzed.

Results. Distribution of pregnancies by profile: (SP) – 78.2% vs (ESA) – 21.8%, with vascular disturbances attested in 100% of cases by increasing the density of avascular chorionic villi in the case of stagnant pregnancies in 75% of cases. The application of immunoexpression with anti-CD31 at the level of the stroma of the chorionic villi determined PVI with a maximum mean in the control group (91.51±0.71) vs the ESA and SP groups (82.29±12.96 and 57.47±6.53, respectively). The index of vascularization with lumen (IV/L+) and vascularization index without lumen (IV/L-) were statistically significantly different (p = 0.011) for (SP). Applying the student t-test revealed statistically significant differences for PVI in most groups, except PSD vs SP at the age of 10-12 gestation of weeks (w.g.) At the same time, there are statistically significant differences between the means of vessels with lumen within PSD and SP (t31 = 5.500, p = 0.000005), and the means of vessels without lumen registered statistically significant differences in the PSD and SP groups (t12 = 3.862, p = 0.002).

Conclusions. Pregnancy evolution depends on the degree of vascularization, as well as on the quality of the vascular network. Disruption of placental vasculature is one of the basic links in the development of primary placental insufficiency during placentation.

Keywords: anti-CD31, fetal concept, stagnant pregnancy, early miscarriage, chorio-villar vascular dyschronism.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

The vascularization stage of the chorio-villous stroma in the placental period is an important link in the establishment of hemo-

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chorionic circulation, and its destabilization is a component part in the onset of primary placental insufficiency, assessed by dysfunction of uteroplacental or placento-fetal hemodynamics. Assessment of vascularization particularity and the evolving dynamics of the primary vascular network and its establishment are topics of discussion regarding their impact on addressing vascular disturbances in chorio-ovarian germinal status in early term dysregulated pregnancies.

The research hypothesis

The identification of blood vessels with CD-31 antibody, a marker of vascular endothelium, may be of clinical significance for patients whose pregnancies are complicated by miscarriage or developmental stagnation.

The novelty added by manuscript to the already published scientific literature

The degree by which chorionic villi are vascularized and the quality of the vascular network play a significant role in the development of primary placental insufficiency of vascular origin, which is confirmed by statistically significant results.

Introduction

The first trimester of gestation is the most complicated period, determining the normal course and outcome of pregnancy. This includes 3 critical periods: *implantation, organogenesis,* and *placentation.* The stage of implantation site formation in the endometrium and placentation results from the vascularization process induction of the chorionic plate and villi with the invasion of the trophoblast into the implantation "window" in the endometrial decidua, growth, and organization of the vascular network in the choriovillous stroma.

Early placentation disturbance contributes to morpho-functional changes in the gestational and germinal sites, followed by impaired maternal-embryo-fetal metabolic exchange during the establishment of hemochorionic blood circulation, manifested by primary placental insufficiency.

According to literature data, within placental insufficiency, the primary form is included [1], the cause of most early-term pregnancies such as a miscarriage or stagnant evolving pregnancies [2]. It is known that major reproductive disorders occur early in pregnancy [3], and deficiencies in vasculogenesis and angiogenesis in the chorionic villi are components of primary placental insufficiency, evolving through the dysfunction of uteroplacental or placento-fetal hemodynamics [4]. In this light, a number of studies have aimed to assess vascularization in the first trimester of gestation of pregnancies managed by medical abortion [5-8]. These studies have shed light on the developmental peculiarities of vascular circulation through angiogenesis, as well as vascularization disturbances in abortive disease [8], which are important steps in hemochorionic circulation stabilization [9]. Lisman *et al.* evaluated the peculiarities of the architectural vasculature in the normal and the hollow germinal sac of the hydatidiform mole using confocal scanning microscopy [6]. The authors have mentioned the impact of vascular-syncytial membranes formation and observed the reduction of endothelial cords with the maturation of pregnancy [6, 7].

Thus, from the 3rd week of intrauterine development, with the vascularization of the mesenchymal stroma (autochthonous vascularization), the period of placentation evolving during the first 12 w.g. begins. At the same time, there is an increase in the vessels of the allantois with the onset of embryo-hemochorionic circulation, determined by the presence of vascularization in the chorionic plate and villi [10]. In this context, the placenta is an important connecting organ in the maternal-placental-fetal system, which carries out the transport of nutrients and gas metabolism through a vascular circulation adjusted to the periods of development. This is an important component in achieving the growth and development of the human conceptus. An important morphological index in the assessment of placental status, reflecting the activity of metabolic processes, is the vascularization of chorionic villi [11]. Shchegolev *et al.* mention the importance of assessing the vascular component in the disturbance of vasculature in progressively stagnant pregnancies [11]. Placental vascularization can be assessed by evaluating angioendothelial components expressing the CD31 marker [5, 12].

CD31 represents the platelet endothelial cell adhesion molecule (PECAM-1), a highly glycosylated protein used in immunohistochemistry for endothelial cell determination, assessing the degree of angiogenesis. PECAM-1 is involved in angiogenesis through cell-cell adhesion with the formation of vascular tubules [13].

In this respect, the knowledge of the vascularization features and the evolving dynamics of the primary vascular network are important in addressing the impact of vascular

perturbations in the chorio-villous germline status in pregnancies failure.

The aim of the current study was to assess vascular density by using anti-CD31 antibody in the chorio-villous germinal site in small term deregulated pregnancies.

Material and methods

The study was conducted at the Perinatal Center Level III, Institute of Mother and Child, during 2020. Tissue samples were obtained by uterine aspirate of 184 patients with term (3-12 w.g.) pregnancies clinically and morpho-pathologically diagnosed as evolving stagnant pregnancies. They were included in the study group, with evolving stagnant pregnancies (L1 = 144 cases), and those that evolved into early miscarriage (L2 = 40 cases) served as the study material. Patient ages ranged from 18-46 years (30.03±6.43) for group I and 17-42 years (30.85±6.36) for group II. All patients underwent ultrasonographic investigation (USG) to determine the gestational term after the first day of the last menstrual bleeding, as well as clinical diagnosis of early miscarriage (EM) or evolving stagnant pregnancy (SP). Tissue from the germinal sac of pregnancies terminated for social indications was used as a control group. This tissue was from 18 patients aged 22-40 years (30.5±5.6) and divided into two subgroups: a) 6-9 w.g. (n = 9 cases) and b) 10-12 w.g. (n = 9 cases).

The clinical data were obtained from patients' medical records based on their informed consent. The current research is part of a larger study of early-term dysregulated pregnancies within the state program "Morphological approach by conventional, histo- and immunohistochemical methods of the features of the pathological profile of early placentogenesis in early-term dysregulated pregnancies", number 20.80009.8007.17 P1P2 0750. Cases were selected in accordance with the inclusion and exclusion criteria:

- *Inclusion criteria*: pregnancies terminated with gestational term from 3 to 12 w.g. (clinically confirmed, by USG and resolved in IMC); pregnancies with pathological evolution: stagnant, early miscarriage; pregnancies with abortion at social indications; quality and volume of the aspirate: chorionic villi and decidual plates in sufficient volume to form the standard paraffin block (1.0x1.0x0.5cm); monofetal pregnancies; lack of an age threshold.
- *Exclusion criteria*: severe somatic pathology; multiple pregnancies; pregnancies terminated on medical indication; lack of clinical-anamnestic data in clinical examination records; lack of gestational term specification and USG confirmation of pregnancy status.

The technical procedure included histoprocessing of tissue samples, application of the usual histological method (haematoxylin-eosin), immunohistochemical method (anti-CD31) with evaluation of histopathological features and immunoexpression, and statistical processing.

The primary processing. Conceptus tissue was harvested at short term in obstetric units with rapid fixation in a 10% formalin solution, pH 7.2-7.4, to reduce the risk of early lysis of tissue material and bacterial flora overgrowth. The fixation period in a 10% buffered formalin solution lasted 24 hours. For paraffin embedding, the DP500/CIT2002 (Bio-Optica, Italy) system has been used. For histochemical and histological processing of samples, the "TISSUE-TEK, VIP 6AI" (Sakura, Japan) histoprocessor has been utilized. The 3.5-µm thick sections were obtained using the HM325 microtome (Thermoscientific) (USA) and placed on positively charged slides (APTACA, Italy) to be dyed.

Histological method. The sections were stained by the conventional classical hematoxylin-eosin (H.E.) method using Mayer hematoxylin (HEMM-36/21, BIOGNOST, Slovenia) and 1% eosin Y (EOY10-35/21, BIOGNOST, Slovenia). The H.E. sections were automatically stained with the AUS-240 autostainer, (Bio-Optica, Italy) and automatically mounted (TISSUE-TEK, ClasTM, Sakura, Japan). Suitable sections (with sufficient tissue material) were selected for immunohistochemical staining.

Immunohistochemical method. Immunohistochemical assays were performed using manually adapted operational procedures for anti-CD31 antibody (clone JC70A) with the application of the EnVisionTMFLEX detection system, high pH (K8000) [14]. The conventional immunohistochemical method was applied (Table 1). Deparaffinization was performed in two toluene baths (code UN1294, Sigma-Oldrich), the first bath for 60 min at 59°C in a thermostat, followed by the second bath for 5 min at room temperature, a mixed bath of toluene and 96% alcohol for 5 min, then 2 baths of 96% alcohol with re-hydration in 2 taps of 10 min each in distilled water. For the epitope demarcation purpose, the sections planned for anti-CD31 antibody disclosure were exposed to dissolved Target Flex solution (1ml Target: 49 ml distilled water) at high pH, during 20 minutes at 95ºC-96ºC with a total pretreatment and posttreatment time of 60 minutes. Incubation with anti-CD31 was carried out for 20 minutes at room temperature. After incubation with the primary antibody, neutralization of endogenous peroxidase with peroxidase block was performed for 5 minutes, followed by the application of the secondary antibody (HRP) for 20 minutes and DAB (3,3'-diaminobenzidine) applied as a chromogenic substrate for 5 minutes. Nuclei were counterstained with Mayer hematoxylin (HEMM-36/21, BIOGNOST, Slovenia). CD31-positive reaction was manifested in endotheliocytes with brown staining of the cell membrane. Then, the histological slide panel was subjected to the dehydration and clarifying procedure by two absolute drops of alcohol, one mixed drop of alcohol and toluene and three drops of toluene, each exposure lasting 5 minutes. The final procedure consisted of mounting the slides with BMC-100 solution. In the manual immunohistochemical staining procedure, Sequenza TM Immunostaining Center was applied using Thermo Shandon Coverplate.

Immunohistochemical testing with anti-CD31 was performed on a numerically smaller batch selected after evaluation (n = 80) and divided into: L1 = 43 cases; L2 = 12 cases; L3 = 17 cases. The difference in cases was due to the loss of histological sections during the testing procedure, paraffin block completion, or lack of primary material.

Table 1. Antibody used: source, dilution, demasking system, detection system, incubation time.

Antibody/ clone	Source/incubation time/dilution	Retrieval system/ time	Detection/time
CD31/ JC70A	20 min, ready- to-use	Solution Target Flex, high pH /Water bath at temperature of 95°C-96°C/20 min	EnVision™FLEX, high pH

Microscopic evaluation. Expression of CD31 (endothelial cell adhesion receptor) protein was detected at the membrane level, expressed by the presence of brownish color in the evaluated tissue. In all sections, blood vessels were quantified by the hot-spot method. Initially, the highest density of chorionic villi areas was identified at ×100 magnification. Subsequently, chorionic villi with vessels (VC v+) and those without blood vessels (VC v-) were counted in 3 visual fields in the areas with the highest chorio-villous density at ×100 magnification, calculating the placental vascularization index (PVI, %). This presented the ratio of the number of vascularized chorionic villi vs the total number of chorionic villi ×100. The following score was assigned:

- negative – in the case of PVI ranging from 0-25%;
- +1 – in the case of PVI ranging from 26-50%;
- +2 – in the case of PVI ranging from 51-75%;
- +3 – in the case of PVI ranging from 76-100%.

Immunoexpression intensity was assessed as 0 (absent); + (positive).

Next, in 3 visual fields within the highest chorio-villous density areas, at x400 magnification, each cell, group of cells, cell cords, immunopositive vessels vs chorio-villous stroma area (mcm^2) were counted. Based on the presence/absence of lumen, they were classified as vessels with lumen (V/L+) and without lumen (V/L-). After that, chorio-vascularization indices (IV/L+ and IV/L-) were calculated according to the following formulas: (V/L+)/(V/T) x100 and (V/L-)/(V/T) x100, where (V/Total) - total number of cells, cell cords, immunopositive vessels which expresses vascular density. The above-mentioned structures were counted in each of the 3 study groups (EM, ASI, SP), grouped according to gestational term into the following groups: 3-5 w.g., 6-9 w.g. and 10-12 w.g. Microscopic evaluation was performed using the Axio Imager A2 microscope (Carl Zeiss, Germany) equipped with the AXIOCam MRc5 recording camera.

Data analysis. The results of the study were stored in an Excel 2007 database (Microsoft Office 2007) and were analyzed using SPSS software (SPSS Statistics 23.0, IBM, Chicago, IL, USA). The median, arithmetic average, and standard deviation were determined. The difference between groups of variables was tested by applying the t-student test. Results were considered statistically significant at $p \leq 0.05$.

Results

To assess the histomorphological vascularization features of the chorionic villi in the study groups, hematoxylin and eosin staining was initially applied. The majority of cases, 78.2% (n = 144), were presented by stagnant uterine pregnancies. Uterine pregnancies evolving into miscarriages accounted for 21.8% (n = 40). Vascularization disturbances were attested in the chorio-villous compartment and were manifested by vascular dyschronism: the presence of vascular and avascular chorionic villi in 100% of cases in all study groups, with a significant increase in the density of avascular chorionic villi in stagnant pregnancies (75%), Fig. 1.

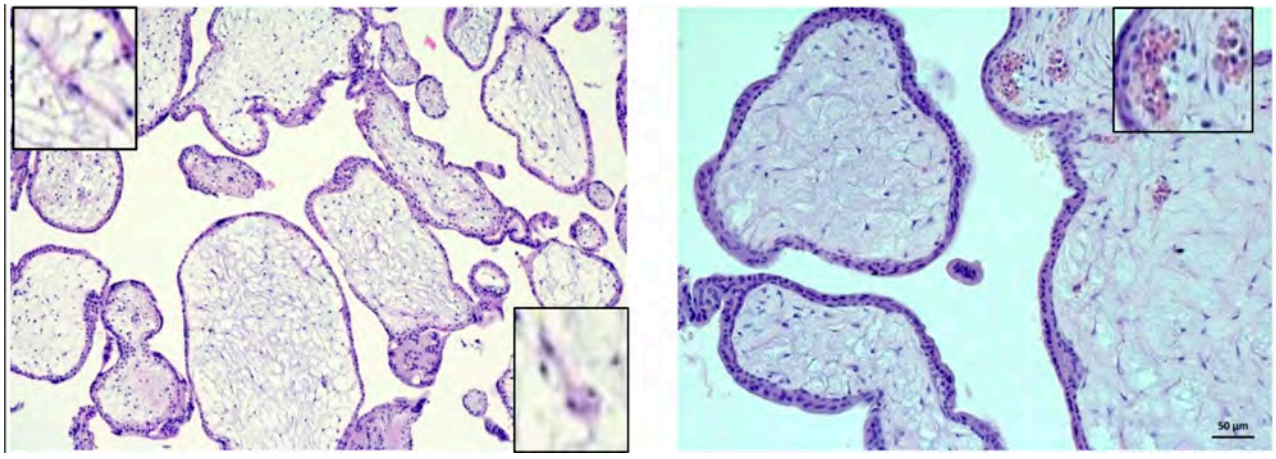

Fig. 1 Evolving stagnant pregnancies at 8-9 w.g.

(a) Avascular mesenchymal chorionic villi with angiogenic cords, with and without lumen, peripheral site; x100, 400; (b) Avascular chorionic villi with vascularization dyschronism (uneven distribution of the primary immature capillary network) with intravascular nucleated and anucleated erythrocytes, marginal (subepithelial) location. Staining hematoxylin-eosin, x200, 400.

To determine the percentage value of the placental vascularization index (PVI, %), the vascular density in the stroma of chorionic villi was assessed with the application of antibody with high specificity toward anti-CD31 endothelial cells. According to membrane immunostaining in the stroma of chorionic villi the following results were obtained, the

numerical values and distribution of which are elucidated in Table 1.

According to the obtained results, anti-CD31 immunoexpression in the chorionic villus stroma induced PVI with a maximum mean in the control group (91.51±0.71) vs the EM and SP groups (82.29±12.96 and 57.47±6.53, respectively) (Table 2).

Table 2. Descriptive statistical analysis of placental vascular density: mean values and standard error of mean (±).

Vascular profile	Lot SA/D (M±SD), w.g.			Lot EM (M±SD), w.g.			Lot SP (M±SD), w.g.		
	3-5	6-9	10-12	3-5	6-9	10-12	3-5	6-9	10-12
VCtotal	-	209.75±44.06	190.11 ±18.82	177.00 ±151.32	94.29 ±42.24	75.33 ±17.61	52.00 ±32.75	90.12 ± 40.98	77.64 ±22.15
VCv+	-	192.88±43.33	172.78 ±25.72	169.00 ±140.01	68.29 ±28.39	59.0 ±24.06	29,00 ±23.2	47.12 ±32.20	43,36 ±29.91
VCv-	-	18.38 ±14.41	17.33 ±21.29	8.00 ±0.31	26,00 ±18,18	16.33 ±10.79	23.00 ±32.64	43.08 ±32.69	34.27 ±28.48
PVI%	-	92.01±6.7	91.01 ±10.53	97.18 ±3.98	73.39 ±12.97	76.31 ±17.22	64.27 ±35.43	51.25 ±26.96	56.89 ±32.31
PVI% total	-	91.51±0.71		82.29±12.6			57.47±6.53		

Note: *M - average; SD - standard deviation; w.g. - weeks of gestation; ASI - abortion at social indications; EM - early miscarriage; SP - stagnant pregnancies; VCtotal - chorio-villous density; VCv+ - chorionic villus with vessel; VCv- - chorionic villus without vessel; PVI - placental vascularity index.*

In the group of pregnancies resolved at a social indication/desire, there was an indirect correlation between the total number of chorionic villi and gestational term (rs = -0.511, p = 0.036). Gestational term similarly correlated with the total number of vessels (rs = 0.702, p = 0.002) as well as with the number of vessels with lumen (rs = 0.746, p = 0.001).

In the miscarriage group, the total number of vessels was directly dependent on the gestational term (rs = 0.746, p = 0.001). In the group of stagnant pregnancies, no statistical correlations were determined for any of the analyzed parameters.

Examining overall cases, we identified a range of statistically significant correlations between gestational term, study group, total number of chorionic villi, villi with vessels, villi without vessels, total number of vessels, vessels with lumen, and vessels without lumen. These correlations are shown in Table 3.

Table 3. Statistically significant correlations between study group, gestational term and various indices representing the vascular density.

		TG	Lot	CV total	CVv^+	CVv^-	Surface	V/Total	V/L^+	V/L^-
TG	rs		-.245*	.209	.187	-.057	.237	.345	.237	.235
	p		.038	.077	.115	.633	.046	.003	.047	.049
Lot	rs	-.245*		-.620	-.740	.313	.390	-.480	-.554	-.175
	p	.038		<.01	<.01	.007	.0007	.00002	<.01	.14372

Note: *rs - Spearman's correlation coefficient; GT - gestational term; CVtotal - chorio-villous density; CVv+ - chorio-villous villi with vessel; CVv- - chorio-villous villi without vessel; V/Total - chorio-villous vascular density; V/L+ vessel with lumen; V/L- vessel without lumen; * - statistically significant results.*

Further, the placental vascular density as a whole was analyzed, with a score given for PVI in the groups studied. The assignment of cases according to the score distribution is shown in Table 4.

In relation to gestation term, IVP in the control group was dominant in the subgroup (6-9) with a distribution score of +3 in 100%, and in the subgroup (10-12), a maximum score was attested in 88.9% vs one case (11.2%) with a score of +2. Overall, the distribution score in the group of pregnancies resolved at social indications/desire shows a high placental vascularity index (+3) in 94.1%.

The EM group was characterized by an equalization of the distribution score per total between subgroups, with +2 and +3 each accounting for 50%, and the subgroup (3-5) rated +3 in 16.7%. However, the maximum IVP distribution score was set for subgroup (6-9) at 58.3%. The SEP group was the most diverse in relation to the IVP distribution

Table 4. Distribution of IVP anti-CD31 immunoreactivity (score).

Unit group/ subgroup/GT n = 72		Vessel/Stroma/score			
		0	+1	+2	+3
SP (n = 43)	3-5	2	-	1	4
	6-9	5	7	7	6
	10-12	3	3	-	5
EM (n = 12)	3-5	-	-	-	2
	6-9	-	-	4	3
	10-12	-	-	2	1
ASI (n = 17)	6-9	-	-	-	8
	10-12	-	-	1	8

Note: *0 = (0-25%); + = (26-50%); ++ = (51-75%); +++ = (76-100%). Immunoexpression intensity was assessed as: - (absent); + (present); GT - gestational term; SP – stagnant pregnancies; EM - early miscarriage; ASI -abortion at social indication.*

score. Thus, overall, the +3 score predominated (34.9%), the 0 and +1 scores each (23.25%), and the +2 score was at (18.6%), with the highest diversity in the corresponding subgroup (6-9 w.g.) with an average distribution value between the (0-1) vs (+2+3) score, respectively 12/13 cases.

Subsequently, the median was determined, which served as a reference value to elucidate the degree of vascularization of the chorionic villi (*low* for values lower than the median and *high* for values higher than the median) (Table 5).

Table 5. Descriptive statistical analysis of placental and chorio-villous vascular density, S/T and degree of vascularity: mean values and standard error of mean ±

Unit group/subgroup/TG n = 72		Vessel/Stroma/score			
		PVI (%)	S/T, μm²	V/Total mean values, m	Degree of vascularization
SP (n = 43)	3-5	64.27±35.43	36336.63±22207.69	5±1.88 m = 4.6	high
	6-9	51.25±26.96	40448.05±37666.43	5.23±2.73 m = 4.33	high
	10-12	56.89±32.31	53025.42±49273.26	6.89±5.51 m = 4.86	high
EM (n = 12)	3-5	97.18±3.98	12881.76±1021.90	7.23±0.44 m = 7.23	high
	6-9	73.39±12.97	13069.66±6215.77	7.26±3.11 m = 8.47	low
	10-12	76.31±17.22	30450.56±5780.98	16.28±1.93 m = 15.33	high
ASI (n = 17)	6-9	92.01±6.7	19935.37±4490.42	6.84±1.32 m = 6.825	high
	10-12	91.01±10.53	26784.26±19753.27	14.13±10.24 m = 9.58	high

Note: *m – median; SP - stagnant pregnancies; EM - early miscarriage; ASI - abortion at social indication; PVI - placental vascularity index; S/T - chorio-villous stromal area; V/Total - chorio-villous vascular density.*

In Table 5, we can see that practically in all cases, there was a pronounced degree of vascularization. Therefore, the next step was to assess the quality of vascularization by determining the ratio of vessels with lumen (functional) to those without lumen (non-functional). The vascularization index with lumen (VI/L+) and the vascularization index without lumen (VI/L-) were thus determined, and the data obtained are shown in Table 6.

Table 6. Descriptive statistical analysis of placental and choriovascular vascular density, VI/L+ and VI/L-: mean values and standard error of the mean ±.

Unit group/subgroup/TG n = 72		Vessel/Stroma/score			
		IPV (%)	V/Total (%)	VI/L⁺ (%)	VI/L⁻ (%)
SP (n = 43)	3-5	64.27±35.43	5±1.88	23.2	86.6
	6-9	51.25±26.96	5.23±2.73	23.5	76.4
	10-12	56.89±32.31	6.89±5.51	32.2	67.7
EM (n = 12)	3-5	97.18±3.98	7.23±0.44	7.6	92.3
	6-9	73.39±12.97	7.26±3.11	44.9	54.8
	10-12	76.31±17.22	16.28±1.93	21.3	74.8
ASI (n = 17)	6-9	92.01±6.7	6.84±1.32	53.6	46.9
	10-12	91.01±10.53	14.13±10.24	52.3	50.2

Note: *TG - term of gestation; PVI - placental vascularity index; V/Total - chorio-villous vascular density; VI/L+ - vascular index with lumen, VI/L- - vascular index without lumen; SP - stagnant pregnancies; EM - early miscarriage; ASI - abortion at social indication.*

In Table 6, we can see that the largest discrepancy between luminal and non-luminal vessels is found in the batch of stagnant pregnancies. Statistical analysis confirmed this observation. Thus, in this group, the difference between the density of vessels with lumen and those without lumen was statistically true (p = 0.011).

The hypothesis that each batch, depending on gestational term, was associated with statistically significant different mean values of PVI and blood vessels with lumen or without lumen was subsequently examined. The hypothesis was further investigated by applying the t-student test. The results of the intergroup differences in average means are reflected in Table 7.

As shown in Table 7, statistically significant differences were established for PVI in most groups except for spontaneous abortion vs stagnant pregnancies at 10-12 w.g. Concurrently, there were statistically significant differences between the means of vessels with lumen in SA/D and SP (t31 = 5.500, p = 0.000005). The means of vessels without lumen showed statistically significant differences in spontaneous abortion and stagnant pregnancies groups (t12 = 3.862, p = 0.002).

Table 7. The difference in average values of IVP and lumen vessels, t-student test.

Weeks of gestation, w.g.	Lot	Results for PVI	Results for vessels with lumen	Results for vessels without lumen
3-5	EM vs SS	$t_{6.467}$ = 2.405* p = 0.05	t_{7} = -0.563 p = 0.591	t_{7} = 2.289 p = 0.056
	AS/D vs AS	$t_{8.720}$ = 3.421* p = 0.008	$t_{7.182}$ = 0.457 p = 0.661	t_{13} = -1.296 p = 0.218
6-9	AS/D vs SS	$t_{30.287}$ = 6.92* p<0.01	t_{31} = 5.500* p = 0.000005	t_{31} = -0.952 p = 0.348
	EM vs SS	$t_{21.454}$ = 3.03* p = 0.006	$t_{6.905}$ = 2.266 p = 0.058	t_{30} = -0.02 p = 0.984
	AS/D vs AS	t_{10} = 1.812 p = 0.1	t_{10} = 0.91 p = 0.384	t_{10} = -2.067 p = 0.066
10-12	AS/D vs SS	$t_{12.502}$ = -3.294* p = 0.006	$t_{10.688}$ = -2.069 p = 0.064	t_{18} = 1.679 p = 0.11
	EM vs SPP	t_{12} = -0.983 p = 0.345	t_{12} = 0.588 p = 0.567	t_{12} = 3.862* p = 0.002

Note: *w.g. - weeks of gestation; ASI - abortion at social indication; EM - early miscarriage; SP - stagnant pregnancies; PVI - placental vascularization index; *- statistically significant results.*

Discussion

The assessment of fetal conceptus (FC) pathology in the early term remains a major challenge for obstetricians, sonographers, geneticists, and, not least, pediatric morphologists, as an integral part of clinical-morphological diagnostic management. According to the literature, as well as our own data, along with the increase in the number of abortions for social/medical indications, the incidence of stagnant pregnancies in evolution or those evolving into early miscarriages is reported to be quite high, ranging from 10% to 51% [15, 16].

Among the causes of miscarriages or stagnation of pregnancies are maternal-fetal factors and various multifactorial pathogenetic mechanisms that induce disorders of conceptus morphogenesis at the blastocyst and gastrulation stage, because of which various structural and phenotypic abnormalities develop [17]. Furthermore, for the formation of a long-term stable connection of the embryo/fetus placental feeding, it is necessary to initiate autochthonous vascularization within the chorio-villous mesenchymal stroma and to install the chorio-allantoic circulation in the formation of the embryo-hemochorionic circulation with the occurrence of the chorionic plate and chorionic villi vascularization [18].

In this regard, the placenta is an important connecting organ within the mother-placenta-fetus system, carrying out the transport of nutrients, conducting gaseous metabolism through a vascular circulation adjusted to the periods of development, serving as an important link in achieving growth and development of the human conceptus. An essential morphological index in placenta condition assessment, revealing the activity of metabolic processes, is the vascularization of chorionic villi [11]. Similarly, to our results, Shchegolev *et al.* mentioned the importance of assessing the vascular component in the disruption of blood supply in progressively stagnant pregnancies with a harmful effect on pregnancy [11].

The placental vasculature can be evaluated by assessing angio-endothelial components expressing the CD31 marker [5, 12], where CD31 represents platelet endothelial cell adhesion molecule (PECAM-1), a highly glycosylated protein used in immunohistochemistry to determine endothelial cells, assessing the degree of angiogenesis. Similarly, PECAM-1 is involved in angiogenesis through cell-cell adhesion with vascular tube formation [13].

In the current study, a pronounced disruption of villous chorion vasculature was established in evolving stagnant pregnancies with pronounced vascularity in most cases. Upon analysis of CD31 marker expression in endothelial cells in relation to the study groups, a discrepancy was determined at the vascularization stage with the involvement of vasculogenesis and angiogenesis with the dominance of vessels without lumen in the (SP) group, which in this case represents vascular retardation. According to Pereteatko L.P. *et al.*, the comparative analysis of pregnancies in abortive disease and chronic endometritis denotes the delayed impact of both chorionic villi and vasculo-angiogenesis [3]. At the same time, disruption of growth factor content together with the accentuation of inflammatory factors' effect in the given case contributes to the initiation of vascularization, the latter having a particular priority in the establishment of embryo-placental circulation [9, 19].

According to published data, vasculogenesis and angiogenesis during intrauterine development are important steps in establishing the embryo-feto-placental circulation and by the 3rd week, virtually all chorionic villi are secularized [20]. Another important milestone is the initiation of vasculogenesis in the yolk sac wall and embryo tissue, then in the mesenchyme of the chorionic plate and stroma of the secondary chorionic villi, with the fusion and formation of an integral embryo-hemochorionic blood circulation beginning around the 7th week. Any dysregulation of the formative stage of this system contributes to intrauterine death [21].

Conclusions

1. The degree of chorionic villi vascularization is different depending both on the mode of resolved pregnancy and gestational term, with statistically significant differences in PVI.
2. The evolutionary success of pregnancy is dependent not only on the degree of vascularization, but also on the quality of the vascular network, which can undergo changes through retardation or immature compensation (vessels with lumen and/or without lumen).
3. Disruption of placental vasculature is one of the basic links in the development of primary placental insufficiency during placentation.

Competing interests

None declared.

Authors' contribution

VD designed the study, conducted the laboratory work, interpreted the data, and drafted the first manuscript; VF collected the material, interpreted the data; LS collected the material, interpreted the data; EC performed the laboratory work, interpreted the data; EF collected the material, interpreted the data; VF conducted the laboratory work, collected the material, interpreted the data; LS revised the manuscript. All authors reviewed and approved the final version of the manuscript.

Ethical Statement and patient consent

No approval was required for this study.

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RESEARCH ARTICLE

Clinical and morphological forms of chronic pancreatitis: features of development, diagnosis and treatment

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ABSTRACT

Introduction. Chronic pancreatitis (CP) is a common disease with a complex pathogenesis, characterized by difficulties in its diagnosis and treatment.

Purpose of the study. To determine the main pathogenetic links of clinical and morphological forms of CP, markers of disease progression, to develop a diagnostic algorithm and principles of treatment of patients.

Material and methods. 210 patients with CP were examined, who were divided into 4 groups: I - obstructive, II - calcifying, III - fibrous-parenchymal, IV – CP, complicated by pseudocyst. Instrumental, functional, morphological, biochemical, immunological, microbiological methods were used. To study the main morphological and biological changes in the pancreas during the development of CP and to study the effectiveness of the proposed treatment, we conducted experimental studies on 45 laboratory white Wistar male rats weighing 180-230 g.

Results. Imbalance of the immune system, oxidative stress, toxic-metabolic disorders, and diseases of the biliary system are important in the development of various forms of CP. However, there are differences in the severity of these changes. The most pronounced activity of fibrotic processes in the pancreas is typical for patients with a long course of the disease and in the presence of complications (pseudocyst). The most unfavorable course and prognosis are seen in the calcifying form of CP. The markers of CP progression are the value of the calcification coefficient of 0.5-1.0, the translocation of DNase I from the cytoplasm to the nucleus of the acinar cell, the activation of collagen formation, the increase in the level of fibrosis activators (TGF-β1, TNF-a), and the intensification of lipid peroxidation processes. An early marker of apoptosis is the translocation of DNase I from the cytoplasm to the nucleus of an acinar cell.

Conclusions. The developed diagnostic algorithm allows assessing the pathophysiological features of functional and organic disorders of the pancreas, predicting the course of the disease and choosing the optimal treatment. The proposed treatment of patients with CP effectively reduces the severity of pain and oxidative stress, normalizes the cytokine profile, improves the general condition and quality of life of patients.

Keywords: chronic pancreatitis, clinical and morphological forms, pathogenesis, diagnosis, treatment, algorithm.

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Key messages

What is not yet known about the issue addressed in the submitted manuscript

Today there are still many unresolved issues of the pathogenesis and diagnosis of chronic pancreatitis (CP) and there is no specific treatment for CP. For effective treatment of chronic pancreatitis, it is necessary to influence the main pathogenesis in order to inhibit the development of fibrous and other structural changes in the pancreas.

The research hypothesis

The development and course of clinical and morphological forms

of chronic pancreatitis are determined by the ratio of regulatory factors of the immune system, apoptosis, and oxidative stress. The treatment, aimed at normalizing the cytokine profile and limiting oxidative stress, will improve the effectiveness of therapy and improve the quality of life of patients.

The novelty added by the manuscript to the already published scientific literature

The markers of chronic pancreatitis progression and calcification coefficient to predict the formation of pancreatic calcifications were developed; differences in the mechanisms of apoptosis in the exo- and endocrine parts of the pancreas were determined and an early marker of apoptosis was identified; an algorithm for diagnosing clinical and morphological forms of chronic pancreatitis and methods for treating patients have been developed.

Introduction

Chronic pancreatitis (CP) is a widespread disease that affects patients of working age, which makes this problem not only medical, but also medical and social [1, 2]. This pathology attracts the attention of many researchers, but today there are still many unresolved issues of the pathogenesis, diagnosis and treatment of CP [3, 4].

Until now, there is no specific treatment for CP, and the goal of conservative influence is the treatment of exacerbation of the disease, chronic pain syndrome, exocrine / endocrine insufficiency of the pancreas, correction of metabolic disorders and complications [2].

For effective treatment of chronic pancreatitis, it is necessary to influence the main pathogenesis in order to inhibit the development of fibrous and other structural changes in the pancreas.

Purpose of the study: To determine the main pathogenetic links of clinical and morphological forms of CP, markers of disease progression, to develop a diagnostic algorithm and principles of treatment of patients.

Materials and methods

Prospectively, we recruited 210 patients with CP who underwent a comprehensive examination Control groups without pancreatic diseases (n = 120) and a comparison group (n = 25) were established to determine the effectiveness of the proposed treatment. Patients in all groups were comparable in terms of gender and age. To determine the effectiveness of treatment, the main indicators were studied immediately after treatment and at 6-12 months. Among the examined patients, there were 169 men and 41 women, with patients ages ranging from 26 to 72 years, and an average age of (47.3±0.7) years. The ratio of women to men was 1:4.1. According to the 1998 Marseille-Rome classification, patients were divided into 4 groups: group I consisted of 26 patients (12.4%) with obstructive CP, group II - 56 patients (26.7%) with calcification, group III - 78 patients (34.1%) with the fibro-parenchymal form, and group IV - 50 patients (23.8%) with CP complicated by pseudocyst.

Methods of research included clinical, instrumental (esophagogastroduodenoscopy (EGD), endoscopic retrograde cholangiopancreatography (ERCP), radiography, enhanced computed tomography (CT), ultrasound), and functional methods (gastric sounding to study its secretory activity and duodenal sounding with the determination of enzymes and bicarbonates) [2]. To study the role of immune factors and oxidative stress in the progression of fibrosis and stone formation in the pancreas, the following methods were used: biochemical analysis of blood serum and the contents of the pancreatic duct to determine enzymatic activity of the pancreas (amylase, lipase, phospholipase A) [4]. Levels of glycated hemoglobin (HbA1c) and hyaluronic acid (HA) were determined [4], as well as indicators of lipid peroxidation (LP) and antioxidant protection (AP) [5, 6], hexosamines (Ha) content [4], protein-bound hydroxyproline (PBH) [7], and the content of medium-weight molecules (AWM) [8].

Levels of interleukins TNF-α, TGF-β1, REG-1α, lactoferrin, and fecal elastase-1 were determined in the blood by enzyme immunoassay. The immune status and nonspecific resistance of the organism were assessed [9, 10]. Morphological methods were used to study biopsies of the pancreas obtained from 60 patients (aspiration biopsy under ultrasound control and during planned operations on the pancreas). Staining was conducted with hematoxylin and eosin and according to Mallory-Slinchenko, and pancreatic histostructure was assessed according to Stolte (1987). Immunohistochemical typing of apoptotic nucleases and morphometry were performed [11, 12]. Microbiological methods were used to determine the microbial contamination of the stomach and pancreatic duct [13].

To study the main morphological and biological changes in the pancreas during the development of CP, we conducted experimental studies on 24 laboratory white Wistar male rats weighing 180–230 g. We used our experimental model of CP, which developed as a result of blocking NO synthase [14]. Rats (n = 12) were intraperitoneally administered NG-nitro-L-arginine (L-NNA), Sigma-Aldrich (USA), at a dose of 40 mg/kg for 6 (n = 6) and 12 (n = 6) days. The control group (n = 12) consisted of rats intraperitoneally administered with 0.9% NaCl solution. Rats were removed from the experiment on days 6 and 12.

To study the effect of the drug „Glutargin" („Health", Kyiv, Ukraine) on the state of the LP-AP system and collagen metabolism, an experimental study was conducted on 21 laboratory white Wistar male rats weighing 180-230 g. Group I rats (n = 7) were injected with L-NNA at a dose of 40 mg/kg for 12 days, and the rats in group II (n = 7) were injected with L-NNA at the same dose and glutargin at 20 mg/kg (intraperitoneally, 20 min. before the introduction of L-NNA) for 12 days. The control group (n = 7) consisted of rats that

received 0.9% NaCl solution intraperitoneally. Rats were removed from the experiment on the 45th day.

The withdrawal of animals from the experiment was carried out by introducing a lethal dose of ketamine hydrochloride. After the animals were withdrawn from the experiment, blood was taken to determine the concentration of PBH, malonic dialdehyde (MDA), glucose, nitrites/nitrates, and the activity of α-amylase, lipase, and trypsin in the blood serum. A histological study of the tissue of the pancreas of rats was also conducted.

The studies were carried out in compliance with the main provisions of the Declaration of Helsinki of the World Medical Association (1964-2013) and the European Convention for the Protection of Vertebrate Animals used for Research and other Scientific Purposes [15].

Descriptive and inductive statistics were used to analyze the obtained results. In the case of quantitative data and under the condition of their normal distribution, the mean and standard error of the mean were used. To determine the significance of differences, Student's t-test was used. In the absence of a normal distribution, the median, minimum, maximum, upper, and lower quartiles were used, and the significance of differences was determined by the Mann-Whitney U test. To describe qualitative data, the frequency of feature detection (%) was used. In this case, the χ-test was used to determine the significance of differences between groups. Differences were considered significant at $p < 0.05$. Correlation and factor analysis were performed. All calculations were carried out in SPSS 9.0 for Windows (or Statistica 6) [16, 17]. The work was performed at the Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine.

The study has been approved on September 10, 2008, by the „Research ethics committee of Institute of Gastroenterology of National Academy of Medical Sciences of Ukraine" (minutes No. 5). Informed consent was obtained from each subject at the beginning of the study.

Results

In the experiment involving the use of L-NNA in the pancreatic tissue of rats, morphological changes characteristic of inflammation with atrophy and fibrosis of the pancreatic parenchyma were observed, along with an activation of the exocrine function of the gland. The introduction of glutargin contributed to a decrease in the level of lipid peroxidation products, an improvement in the state of the antioxidant defense system, and the normalization of collagen metabolism (PBH).

In all patients, risk factors for the development of clinical and morphological forms of chronic pancreatitis and quality of life (QOL) were studied. An assessment of the possibilities of methods for diagnosing clinical and morphological forms of CP was carried out, and morphological features of the pancreas were determined. The role of immune factors and oxidative stress in the progression of clinical and morphological forms of CP was examined, and the diagnostic capabilities of markers of inflammation, fibrosis, stone formation, and apoptosis in clinical and morphological forms of CP were studied. A treatment and diagnostic algorithm for CP has been developed, taking into account the factors of disease progression.

As a result of the work, it was found that the activity of fibrotic processes (TGF-β1) is higher in patients with a long history of the disease (groups II and III, (29.06±0.55) ng/ml and (27.62±0.56) ng/ml, respectively, compared with I (23.50±0.64) ng/ml and IV (21.16±0.67) ng/ml, ($p<0.001$), against the background of a decrease in the level of apoptosis receptor protein – CD95 (II (15.08±0.83)% and III (15.78±0.43)% compared with control (17.24±0.57)%, ($p<0.001$). The level of pro-inflammatory cytokines (TNF-ά) is increased in all patients, but significantly higher in patients with impaired pancreatic secretion outflow - group I (197.33±2.21) pg/ml, and the presence of pancreatic pseudocyst - group IV (194.44±2.62) pg/ml compared with II (174.34±12.16) pg/ml, ($p<0.001$) and III (178.78±1.88) pg/ml, ($p<0.05$), and compared with the control (22.0±0.81) pg/ml, ($p<0.001$).

For a detailed characterization of immune disorders, the coefficient of diagnostic value was taken into account, allowing, given the average values of the parameters in the group and their dispersions, to select indicators that are significantly different from the norm. Consequently, a formula for immune system disorders (FID) was obtained, which includes the 3 most informative indicators (Table 1). According to FID, the most pronounced changes were found in patients of group III in both cellular and humoral immunity. In patients with chronic pancreatitis complicated by a pseudocyst, dysfunction of cellular immunity is characteristic, while in patients with calcifying chronic pancreatitis – humoral dysfunction is notable.

Table 1. Formula for immune system disorders in examined patients.

Groups of patients	FID	
	cellular immunity	humoral immunity
I group	$CD3_{2}^{-}$, $CD4_{2}^{-}CD8_{1}^{+}$	CIC_{2}^{+}, IgG_{2}^{+}, IgA_{1}^{+}
II group	$CD4_{2}^{-}$, $CD3_{2}^{-}$, $CD8_{1}^{+}$	CIC_{2}^{+}, IgG_{3}^{+}, IgA_{2}^{+}
III group	$CD4_{2}^{-}$, $CD3_{2}^{-}$, $CD4/CD8_{2}^{-}$	$CD19_{3}^{+}$, CIC_{3}^{+}, IgG_{2}^{+}
IV group	$CD4_{2}^{-}$, $CD3_{2}^{-}$, $CD4/CD8_{2}^{-}$	$CD19_{3}^{+}$, CIC_{2}^{+}, IgG_{1}^{+}

Note: the results were analyzed using Oneway ANOVA; FID – formula for immune system disorders; CD – cluster of differentiation (CD3 – T lymphocytes, CD4 – T helper cells, CD8 – cytotoxic T cells, CD19 – B lymphocytes); CIC – circulating immune complexes; Ig – immunoglobulin; (+) – hyperfunction; (-) – immune deficiency; 1 (2, 3) – the degree of immune disorders.

In patients with various clinical and morphological forms of CP, unidirectional changes in the level of stone formation markers were established. There was a significant increase in the level of lithostatin (REG-1α) by 8.7 times, reaching (1498.71±63.80) pg/ml compared to the control group's (185.0±23.0) pg/ml ($p<0.001$). Additionally, the level of lactoferrin increased by 18.7 times, reaching (12227.57±542.29) ng/ml compared to the control group's (653.57±11.89) ng/ml ($p<0.001$).

An intergroup analysis showed that in patients of groups I and II, the level of lithostatin ((1183.22±70.69)

pg/ml and (983.86±8.83) pg/ml, respectively) was significantly lower compared to patients in groups III and IV ((1779.91±109.86) pg/ml and (2256.94±57.79) pg/ml, respectively) (p<0.001). The increase in the level of lactoferrin was significantly higher in patients of groups I and II ((17669.44±841.39) ng/ml and (17461.892±261.18) ng/ml, respectively) compared with patients in groups III and IV ((7305.72±40.74) ng/ml and (7218.88±51.45) ng/ml, respectively) (p<0.001). Thus, the level of a specific protein of „pancreatic stones" - pancreatic stone protein (PSP, or lithostatin), which suppresses the growth of calcium carbonate crystals, is significantly lower in patients of groups I and II (obstructive and calcifying CP). In contrast, the level of lactoferrin, which is the basis of „pancreatic stones", is significantly higher in patients of these groups compared with patients of groups III and IV. The coefficients of the values of these indicators relative to the control and their ratio were calculated (the control values of the studied indicators were taken as 1.0) and it was found that the probability of stone formation is high - at a value of 0.5-1.0 of the calcification coefficient (REG-1α / lactoferrin), low - at 1.5 and above.

The possibilities of modern methods for diagnosing clinical and morphological forms of CP were determined, and the highest sensitivity in diagnosing structural changes in the pancreas was found with the CT method (group I - 98.6%, II - 95.1%, III - 89.1%, IV - 98.2%). Complete agreement among CT, ultrasound, and ERCP diagnoses occurred in 64.7% of cases, partial mismatch - in 28.5%, and complete discrepancy - in 6.8%. Ultrasound demonstrated the highest sensitivity and specificity in cases of calcific CP (93.8% and 85.6%) and CP complicated by pseudocyst (92.3% and 88.5%). For diagnosing other forms of CP, it is necessary to additionally use CT and, if necessary, ERCP. In the assessment of fibrotic changes in the pancreas in CP, a significant correspondence was found between the ultrasound parameters and the data from morphological studies of the pancreas, including the areas of fibrosis in the parenchyma of the gland (r = 0.91; p = 0.03) and the density of the parenchyma (r = 0.94; p = 0.05).

As a result of the study of the histostructure of the pancreas, it was found that the morphological picture of fibrotic changes in the pancreas does not depend on the form of the disease and is characterized by a mild (I) degree of fibrosis in 6.6% of patients, moderate (II) in 20.0%, severe (III) in 16.7%, and complete fibrosis (IV) in 56.7%. Immunohistochemical studies have revealed various mechanisms of apoptosis in the exocrine and endocrine parts of the pancreas. The proapoptotic protease DNase I is expressed in the cytoplasm of acinar cells, and endonuclease-G is expressed in some of the islet cells and in the epithelium of the ducts. It has been established that an early marker of apoptosis is the translocation of DNase I from the cytoplasm to the nucleus of the acinar cell.

The vast majority of patients with CP are characterized by endotoxemia (AWM level is increased in 67.8% to (706.67±26.84) mg/l compared with the control (445.60±18.20) mg/l, p<0.001), activation of fibrotic processes (PBH increased in 81.6% to (175.76±3.59) μmol/l compared with the control (136.04±4.3) μmol/l, p<0.001), HA level increased in 75.0% to (1.71±0.24) g/l compared with the control (0.76±0.12) g/l, p<0.02), and the level of Ha was increased in 50,9% to (6.81±0.16) mmol/l compared with the control (5.04±0.19) mmol/l (p<0.001)), and no significant intergroup differences in these indicators were found.

Activation of lipid peroxidation processes was detected in 90.0% of cases (MDA up to (3.32±0.09) nmol/ml versus (2.07±0.13) nmol/ml in control, p<0.05), isolated double bonds (IDB) up to (1.42±0.04) relative units/ml versus (0.99±0.04) relative units/ml in control, p<0,05), diene conjugates (DC) up to (0.87±0.03) relative units/ml versus (0.67±0.03) relative units/ml in control, (p<0.05). Moreover, in 97.4% of patients with CP, there was an imbalance between the accumulation of primary lipid peroxidation products and a significantly accelerated decay of the products of their transformation into dialdehyde products (MDA). That is, in patients with CP, there is an intensification of lipid peroxidation processes, which contributes to the formation of secondary LP products that inactivate cationic pumps, channels, and ionic conductivities, membrane proteins, and enzymes.

On the basis of the data obtained, markers of CP progression were determined, including: the value of the calcification coefficient (REG 1α/lactoferrin) between 0.5-1.0, translocation of DNase I from the cytoplasm to the nucleus of the acinar cell, activation of collagen formation (with a decrease below 0.5), an increase in the level of fibrosis activators (TGF-β1, TNF-a), and intensification of LP processes (MDA).

As a result of factorial and correlation analysis of the obtained data, and in order to develop a diagnostic algorithm, the main pathogenetic links of various forms of CP were formulated (Figs. 1-4). The analysis allowed us to explore the relationships between variables and interpret the „nature" of the factors that make the main contribution to explaining the variability of the sample variables.

In patients of group I (obstructive pancreatitis), four factors explain about 59% of the total variance (Fig. 1). The first factor (13.4% of sample variability) correlated mainly with indicators that reflect disorders of the biliary system (presence of cholelithiasis (Chl) r = 0.72; p = 0.001), LP-AP processes (ceruloplasmin/malondialdehyde (Cp/MDA) r = 0.72; p = 0.05), endogenous intoxication (AWM r = 0.66; p = 0.05) and activation of humoral immunity (IgG r = 0, 78; p = 0.001). Therefore, with cholelithiasis (Chl), bile reflux into the pancreatic duct is determined, which, along with an imbalance in the LP-AP system, leads to inflammation and damage to the pancreatic tissue. The volume of duodeno-pancreatic reflux affects the degree of oxidative damage. Prolonged exposure to refluxate leads to fibrosis of the pancreas. There is „a vicious circle", that is, the mutual influence of immune factors, LP indicators, disorders of the biliary tract, endogenous intoxication in the development and course of CP.

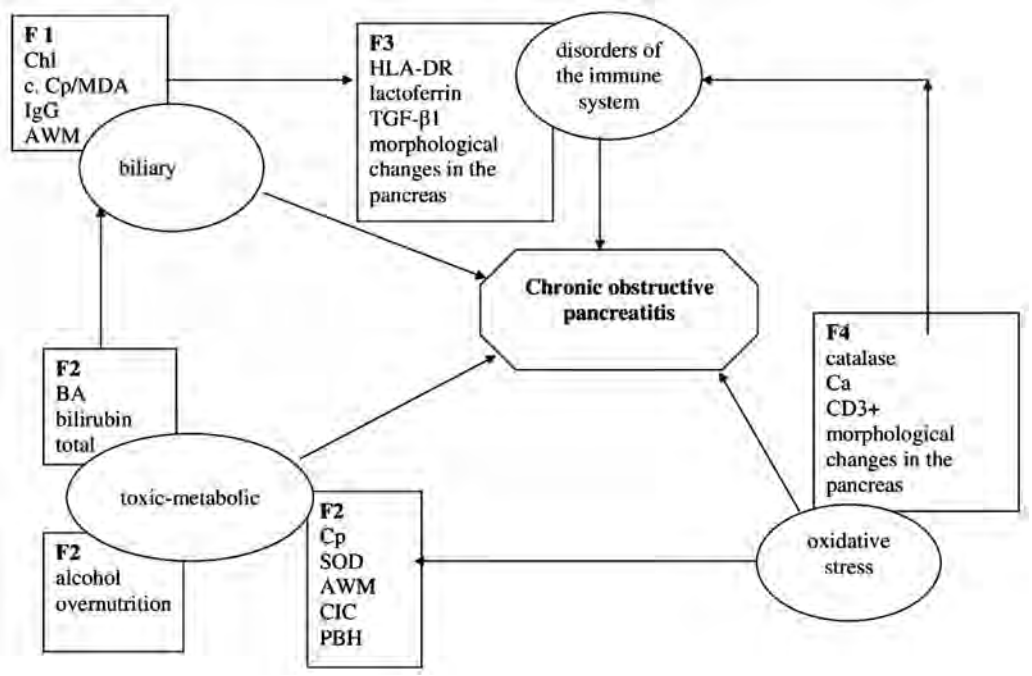

Fig. 1 Pathogenesis of chronic obstructive pancreatitis.

Note: Factor analysis was conducted using the STATISTICA system. F1 – factor 1 (disorders of the biliary system); F2 – factor 2 (toxic-metabolic); F3 – factor 3 (disorders of the immune system); F4 – factor 4 (oxidative stress); Chl – cholelithiasis; c. Cp/MDA – coefficient ceruloplasmin/malondialdehyde; IgG – immunoglobulin G; AWM – medium-weight molecules; BA – bile acids; Cp – ceruloplasmin; SOD – superoxide dismutase; CIC – circulating immune complexes; PBH – protein-bound hydroxyproline; HLA-DR – activated lymphocytes; TGF-β1 – transforming growth factor β1; Ca – calcium; CD3+ – T-lymphocytes.

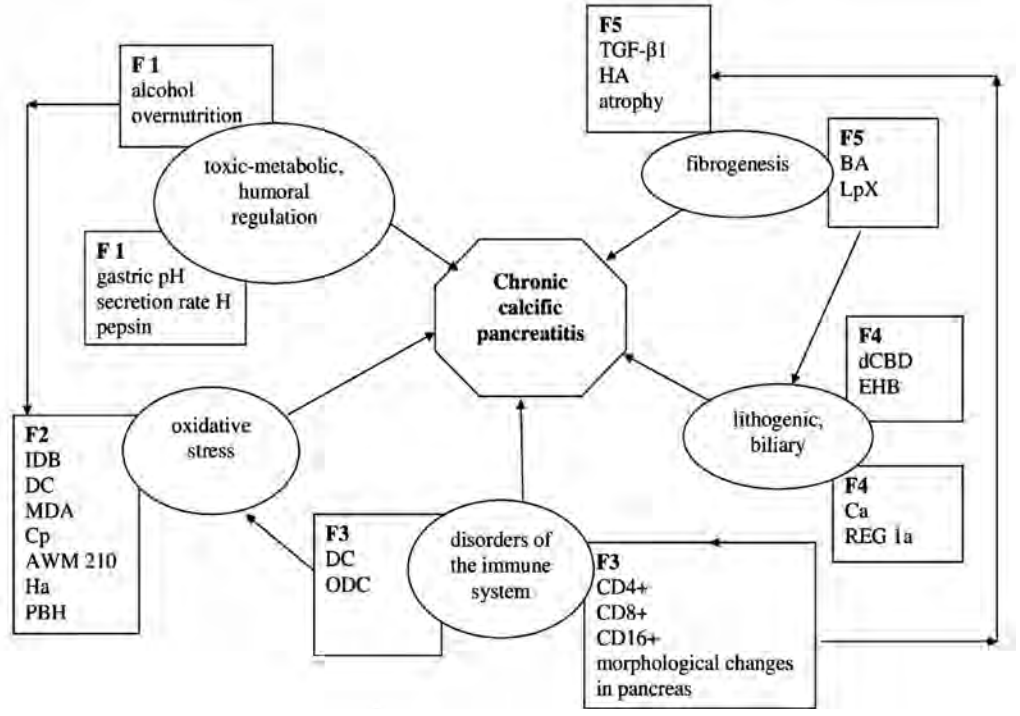

Fig. 2 Pathogenesis of chronic calcific pancreatitis.

Note: Factor analysis was performed in the STATISTICA system. F1 – factor 1 (toxic-metabolic and humoral regulation); F2 – factor 2 (oxidative stress); F3 – factor 3 (disorders of the immune system); F4 – factor 4 (lithogenic and disorders of the biliary system); F5 – factor 5 (fibrogenesis); IDB – isolated double bonds; DC – diene conjugates; MDA – malonic dialdehyde; Cp – ceruloplasmin; AWM – medium-weight molecules; Ha – hexosamines; PBH – protein-bound hydroxyproline; TGF-β1 – transforming growth factor β1; HA – hyaluronic acid; BA – bile acids; LpX – lipoprotein X; dCBD – diameter of the common bile duct; EHB – extrahepatic bile ducts; Ca – calcium; REG 1a – lithostatin; CD4+ – T helper cells; CD8+ – cytotoxic T cells; CD16+ – natural killer cells; ODC – oxadiene conjugates.

The second most important factor (16.0% of sample variability) is toxic-metabolic, correlated with indicators that reflect the toxic effect of overnutrition (r = 0.69; p = 0.001), harmful substances and toxins (alcohol r = 0.82; p = 0.05, CIC r = 0.77; p = 0.05, AWM r = 0.73; p = 0.05, bile acids (BA) r = 0.78; p = 0.01, bilirubin r = 0.76; p = 0.001), and shows their direct effect on acinar cells, cell metabolism, which leads to fatty degeneration, cell necrosis and the development of fibrosis (PBH r = 0.81; p = 0.05). Periductal fibrosis develops and, accordingly, dilatation of the ducts. Fibrosis of the pancreatic tissue with scarring in the periductal areas leads to obstruction of the ducts. Complete and prolonged obstruction leads to atrophy and fibrosis. Structural changes in the pancreas, which were detected by ERCP, CT, or ultrasound, correlate with the level of endogenous intoxication (AWM r = 0.78; p = 0.01), metabolic changes (LpX r = 0.76; p = 0.03, Ca r = 0.73; p = 0.028), and AP disorders (superoxide dismutase (SOD) r = 0.77; p = 0.04).

The third factor (15.0% sample variability) reflects disorders of the immune system; it is correlated with the level

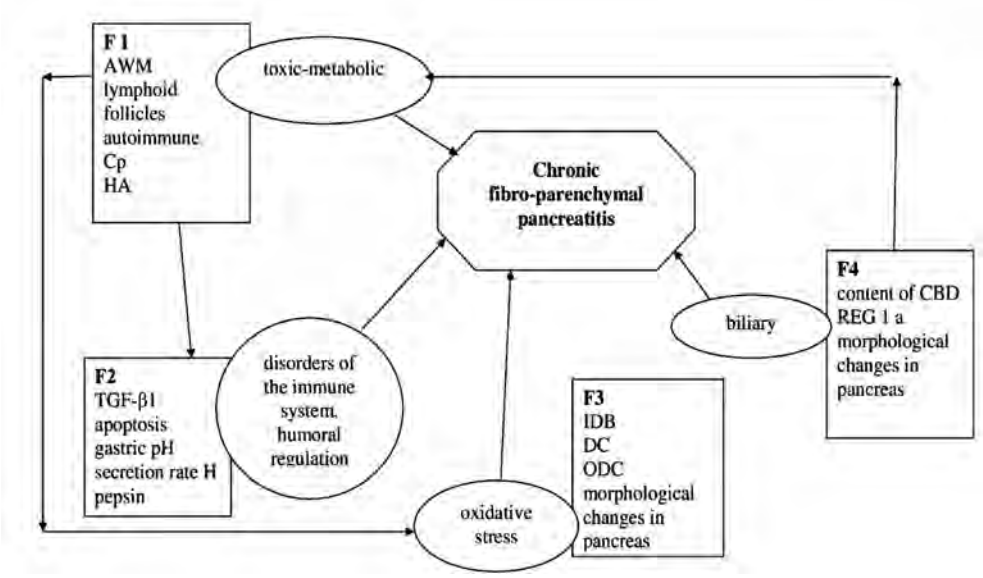

Fig. 3 Pathogenesis of fibro-parenchymal chronic pancreatitis.

Note: Factor analysis was performed in the STATISTICA system. F1 – factor 1 (toxic-metabolic); F2 – factor 2 (disorders of the immune system and humoral regulation); F3 – factor 3 (oxidative stress); F4 – factor 4 (disorders of the biliary system); AWM – medium-weight molecules; Cp – ceruloplasmin; HA – hexosamines; TGF-β1 – transforming growth factor β1; IDB – isolated double bonds; DC – diene conjugates; ODC – oxadiene conjugates; REG 1 a – lithostatin; CBD – common bile duct.

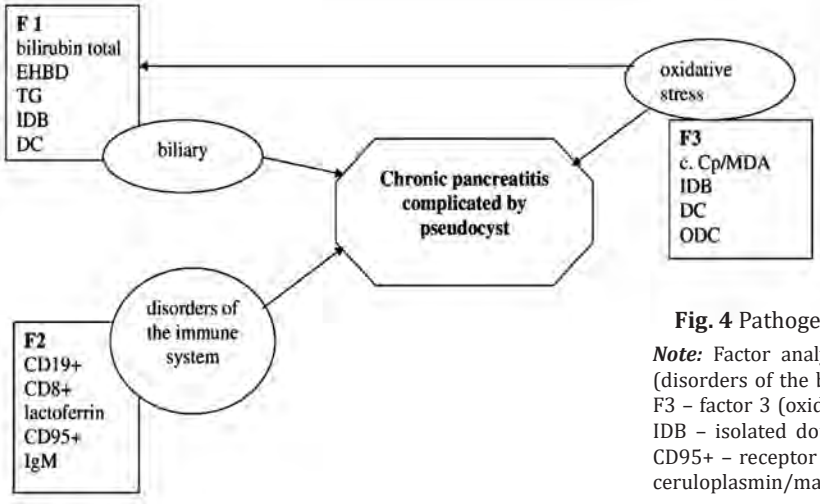

Fig. 4 Pathogenesis of chronic pancreatitis complicated by pseudocyst.

Note: Factor analysis was performed in the STATISTICA system; F1 – factor 1 (disorders of the biliary system); F2 – factor 2 (disorders of the immune system); F3 – factor 3 (oxidative stress); EHBD – extrahepatic bile ducts; TG – triglycerides; IDB – isolated double bonds; CD19+ – B lymphocytes; CD8+ – cytotoxic T cells; CD95+ – receptor of apoptosis; IgM – immunoglobulin M; c. Cp/MDA – coefficient ceruloplasmin/malondialdehyde; DC – diene conjugates; ODC – oxadiene conjugates.

of activated lymphocytes (HLA-DR r = 0.68; p = 0.001), lactoferrin (r = 0.86; p = 0.05), profibrotic cytokines (TGF-β1 r = 0.81; p = 0.001), which, in turn, leads to the activation of stellate cells with their subsequent production of an extracellular matrix with a predominance of collagen. This leads to the replacement of the functional parenchyma of the gland with fibrous tissue (fibrosis index r = 0.79; p = 0.03).

The fourth factor (14.3% of sample variability) reflects oxidative stress and correlates with the level of catalase (r = 0.70; p = 0.05), lipid peroxidation products (MDA r = 0.79; p = 0.003), (Ca r = 0.73; p = 0.028), the level of lymphocytes (CD3 r = 0.88; p = 0.001). Various xenobiotics during their metabolism cause oxidative stress in the pancreatic tissue, which leads to cell and organ damage (fibrosis index r = 0.56; p = 0.007).

In the development of clinical and morphological forms of CP, the same factors are important: toxic-metabolic, biliary, immune, oxidative stress, but there are differences. In patients of group II (calcifying pancreatitis), the largest number of factors was found - 5 (Fig. 2), which confirms the data obtained on the basis of a comprehensive clinical, laboratory, and instrumental study that the calcific form of CP is the most unfavorable for the course and prognosis. Five factors explain about 58.4% of the total variance.

The first toxic-metabolic factor (11.1% of sample variability) was additionally correlated with indicators of humoral mechanisms of regulation of pancreas activity (pH r = 0.81; p = 0.001; H+ secretion rate r = 0.73; p = 0.05; pepsin r = 0.87; p = 0.001). In combination with the second factor (oxidative stress, 12.0% of sample variability, correlated with Cp indicators r = 0.79; p = 0.05, Ha r = 0.75; p = 0.001, IDB r = 0.68; p = 0.05, DC r = 0.69; p = 0.003), third (disorders of the immune system, 13.6% sample variability, correlated with CD19 r = 0.74; p = 0.001, CD4 r = 0.74; p = 0.001, CD16 r = 0.65; p = 0.001), and disorders of the biliary system (fourth factor, 8.9% of the sample variability, correlated with the expansion of the choledochus r = 0.68; p = 0.001, choledochal calculi r = 0.63; p = 0.05); these changes lead to the activation of stone formation (REG-1α r = 0.78; p = 0.05; Ca r = 0.83; p = 0.001). Against the background of

an increase in the phenomena of cholestasis and metabolic disorders, the processes of fibrosis of the pancreas intensify (fifth factor, 9.1% of sample variability, correlated with BA values r = 0.80; p = 0.001, LpX r = 0.85; p = 0 .01, TGF-β1 r = 0.75, p = 0.01, HA r = 0.71, p = 0.001).

In patients of group III (fibro-parenchymal form of CP), similarly to the first group, 4 factors were identified that explain 50.8% of the variance (Fig. 3): the toxic-metabolic factor is determined in the first place (14.5% of the sample variability, correlated with AWM r = 0.77, p = 0.05, Cp r = 0.70, p = 0.05, HA r = 0.62, p = 0.05, lymphoid follicles r = 0.67, p = 0.01), on the second - disorders of the immune system and humoral regulation of the activity of the pancreas (13.9% of sample variability, correlated with TGF-β1 r = 0.65; p = 0.001, apoptosis r = 0.83; p = 0.05, gastric pH r = 0.88, p = 0.05, H+ secretion rate r = 0.78, p = 0.01, pepsin r = 0.88, p = 0.05) induced by oxidative stress (the third factor is 11.9% of the sample variability, correlated with IDB indicators r = 0.65, p = 0.05, DC r = 0.86, p = 0.05, oxadiene conjugates (ODC) r = 0.65, p = 0.05). All these changes, along with disorders of the biliary system (fourth factor - 10.5% of the sample variability, correlated with indicators of the content of the choledochus r = 0.83; p = 0.05, REG-1 α r = 0.67; p = 0.05, fibrosis index r = 0.74; p = 0.05), lead to damage to the pancreas. The high probability of stone formation is evidenced by REG-1α, which is a component of the fourth factor.

The smallest number of factors was found in patients of group IV - 3 factors (Fig. 4): biliary (13.3% of sample variability, correlated with bilirubin index r = 0.75; p = 0.05, dilatation of extrahepatic bile ducts r = 0.77; p = 0.05, triglycerides (TG) r = 0.76; p = 0.05, IDB r = 0.82; p = 0.05), disorders of the immune system (13.2% sample variability, correlated with CD19 r = 0.74, p = 0.05, CD8 r = 0.76, p = 0.05, lactoferrin r = 0.70, p = 0.05, CD95 r = 0.65, p = 0.05, IgM r = 0.66; p = 0.05), oxidative stress (10.7% of sample variability, correlated with Cp/MDA r = 0.63; p = 0.05, IDB r = 0.83; p = 0.001, DC r = 0.95, p = 0.001, ODC r = 0.81, p = 0.05). These three factors account for 37.1% of the variance. This coincides with the data on a short history of the disease in patients of this group and the development of pseudocysts after acute pancreatitis.

Based on the conducted studies and factor analysis data, the diagnostic algorithm for patients with CP was improved (Fig. 5).

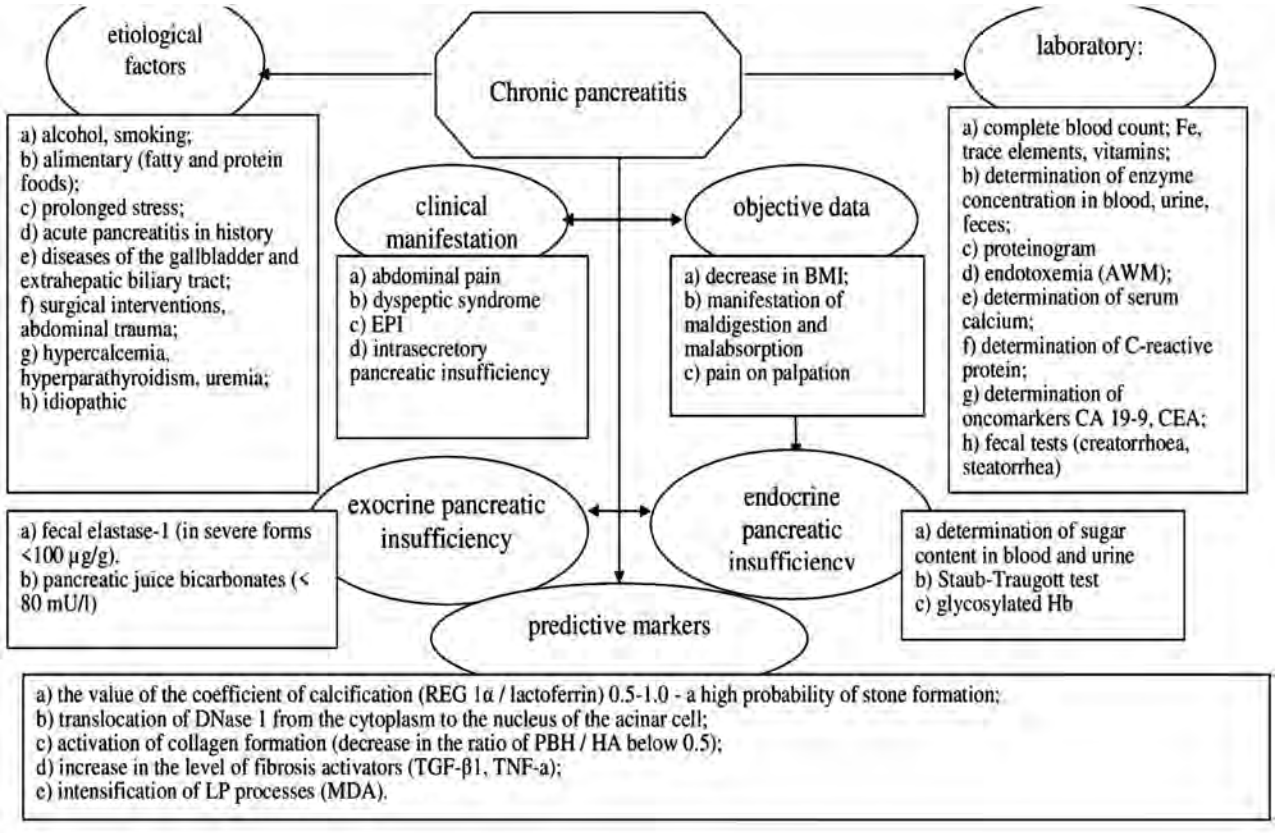

Fig. 5 Algorithm for diagnosing clinical and morphological forms of chronic pancreatitis.

Note: EPI – exocrine pancreatic insufficiency; BMI – body mass index; Fe – iron; CA 19-9 – carbohydrate antigen 19-9; CEA – carcinoembryonic antigen; AWM – medium-weight molecules; REG 1α – lithostatin; DNase 1 – deoxyribonuclease I; PBH/HA – protein-bound hydroxyproline/hyaluronic acid; TGF-β1 – transforming growth factor β1; TNF-a – tumor necrotic factor alpha; LP – lipid peroxidation; MDA – malonic dialdehyde.

The developed diagnostic algorithm, with the maximum information content of the data obtained, made it possible to evaluate the pathophysiological features of functional and organic disorders of the pancreas in patients with CP, choose a pathogenetically justified method of treatment, and reduce the length of stay in the hospital.

A group of patients with persistent and intermittent pain syndrome due to obstruction at various levels of the

pancreatic ductal system was identified, with the development of complications that could not be eliminated by conservative methods and were subject to surgical treatment. With obstructive CP, the treatment of patients is aimed at eliminating the cause of the obstruction, which is achieved only by surgical methods. Patients with CP complicated by pseudocyst formation also need treatment in the surgical department. They underwent gastro/duodenocystostomy or percutaneous puncture of the pseudocyst at the first stage of treatment, and then surgery was performed. Surgical treatment of patients with CP was aimed at improving the outflow of pancreatic juice from the main pancreatic duct or resection of the affected area of the gland.

The tactics of conservative treatment for patients with CP was the use of basic complex therapy to relieve exacerbation of the disease, chronic pain syndrome, exocrine/endocrine insufficiency of the pancreas, and correction of metabolic disorders and complications. Analgesics, non-steroidal anti-inflammatory drugs, enzyme preparations, antacids, and proton pump blockers were used.

In addition to the basic therapy, treatment is proposed aimed at stopping the pain syndrome, normalizing immune system disorders, eliminating oxidative stress, and inhibiting fibrotic processes. To stop the pain syndrome, a vortex pulsed magnetic field (VPMF) was applied with an effect on the projection of the pancreas and biologically active points for 5-15 minutes in a course of 10-15 sessions [18]. Glutargin (a salt of arginine and glutamic acid) was used to normalize the state of the LP-AP system, glutathione, and ultimately as an antifibrotic agent [19]. For immunocorrection and increasing the adaptive potential of the body, reducing the level of pro-inflammatory cytokines and profibrotic cytokines, patients with CP were prescribed autocytokine therapy in addition to the main treatment [20, 21].

As a result of complex treatment, good results were obtained in 73.5% of patients. The severity of pain, as measured by the visual analogue scale (VAS), decreased. There was a significant decrease in the levels of lactoferrin, REG 1α, TGF-1β, and normalization of the Tx/Tc ratios. There was also a significant decrease in the marker of collagen formation (PBH) and lipid peroxidation products (MDA).

Discussion

The development of CP is considered the result of exposure to various pathological stimuli. There is no single etiology that inevitably leads to CP, and it is considered a complex disease with several concomitant factors [3, 22, 23]. The diagnosis of CP requires the integration of clinical, laboratory, and imaging features [24, 25].

In our study, we demonstrated that the development and course of clinical and morphological forms of CP are determined by the ratio of regulatory factors of the immune system, apoptosis, and oxidative stress. The obtained interrelations of immune factors, oxidative stress, and structural changes in the pancreas made it possible to develop markers for the progression of CP: the value of the calcification coefficient (REG 1α / lactoferrin) 0.5-1.0; translocation of DNase I from the cytoplasm to the nucleus of the acinar cell; activation of collagen formation (decrease in the ratio of PBH / HA below 0.5); the growth of the level of fibrosis activators (TGF-β1, TNF-a), intensification of LP processes (MDA). In our study, it was shown that an early marker of apoptosis is the translocation of DNase I from the cytoplasm to the nucleus of an acinar cell.

Today, the treatment of patients with CP remains one of the most difficult areas of pancreatology [25, 26]. A search is being made for treatment methods that can influence the main links in the pathogenesis of the disease to inhibit the development of fibrosis and other structural changes in the pancreas [27].

In the ACG guideline on CP (2020), it is noted that the most frequent symptom of CP and a factor in patients' low quality of life is abdominal pain, which is often refractory to the treatment used [26]. There is a constant search for treatment methods that can reduce the severity of the pain syndrome and slow down the progression of CP.

One of the mechanisms of the pain syndrome is damage to nerve endings [28, 29]. In the hope of treating refractory pain in CP, various experimental treatment methods, such as spinal cord nerve stimulation, transmagnetic brain stimulation, or direct radiation therapy to the pancreas, have been performed, and they have shown their effectiveness in treating pain [26, 30].

We used VPMF to treat pain and showed that in patients with CP and refractory pain, VPMF has an analgesic effect, improves the patient's general condition and quality of life [18].

In the development and progression of CP, an important pathogenetic chain is the uncontrollable strengthening of LP processes. In the case of prolonged action, oxidative stress entails the destruction and death of cells, which further leads to the progression of fibrous changes, exocrine and intrasecretory pancreatic insufficiency [3, 31, 32]. Therefore, antioxidants are used to correct LP [33, 34].

In our work, we used glutargin in the complex treatment of patients with CP. The composition of glutargin includes L-arginine and glutamic acid; it is able to activate the synthesis of enzymes of antioxidase protection (glutathione, glutathione peroxidase); therefore, it is an antioxidant. In addition, L-arginine enhances the activating effect of glutamic acid, which, in turn, together with metabolites, changes the activity of phosphorylase A2 and promotes the removal of toxic products of peroxidized fatty acid residues of phospholipids [19].

Cytokines play an important role in the pathogenesis of CP, as in any inflammatory process. During pancreatic injury, atrophic acinar cells activate several key participants in inflammation, such as macrophages and granulocytes, which release a number of pro-inflammatory cytokines. Proinflammatory cytokines additionally activate pancreatic stellate cells, contributing to the development of CP [35-37].

Cytokines have promising potential as therapeutic agents. However, the clinical use of cytokines is limited since they have high pleiotropy, and their systemic administration can lead to severe side effects [38].

We used autologous cytokines (autocytokines) in addition to the main treatment of patients with CP. Autocytokines act according to the network principle and their immunocorrective effect is directed to the cells involved in inflammation, regeneration, and development of the immune response [20]. The main principles of the method are the use of a composition of cytokines secreted by mononuclear cells of the patient's peripheral blood, it allows to avoid the development of toxic and allergic side effects [20].

Conclusions

The obtained results contribute to a further understanding of the complex pathogenesis of pancreatitis, improving the diagnosis and treatment of patients with CP. The use of disease progression markers has enhanced the diagnosis of clinical and morphological forms of pancreatitis and the stratification of treatment. The proposed treatment for patients with chronic pancreatitis contributed to a decrease in the severity of pain, normalization of the cytokine profile, and limitation of oxidative stress, improving the general condition and quality of life of patients.

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

The study was approved on 10 September 2008 (minutes No. 5) by the Research ethics committee of the Institute of Gastroenterology of the National Academy of Medical Sciences of Ukraine.

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REVIEW ARTICLE

Algorithm of diagnosis and treatment of Gilles de la Tourette syndrome and tic disorder, adapted for the Republic of Moldova: a review

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ABSTRACT

Introduction. In the last years the high prevalence of the tic disorder and Gilles de la Tourette syndrome (GTS) was confirmed. The available therapies can temporarily suppress the tics, but not eliminate them definitively, that implies a strict individual assessment of the risks and benefits for every patient. The recent scientific studies confirmed the efficiency of some diagnostic and therapeutic options, and infirmed the other ones. Thus, a qualitative analysis and adjustment of the recommendations to the actual conditions and possibilities of the Republic of Moldova (RM) is required.

Material and methods. All the guidelines for the diagnosis and treatment of GTS and tic disorders were found in the *PubMed* database. The articles containing the keywords *„Tourette diagnosis", „Tourette treatment", „tic diagnosis"* or *„tic treatment"* and published after 2021 were selected. The availability in RM of the medications recommended by the international guidelines was verified on the site of the Medicines and Medical Devices Agency of RM. The final text includes the qualitative analysis and synthesis of the recommendations, adapted to the conditions and possibilities of RM.

Results. The diagnosis of tics needs only clinical observation, and usually other investigations are not indicated. It is extremely important to identify patients whose tics do not need treatment, but just monitoring and informing about their nature. There are three main therapeutic methods directed against tics: behavioural therapy, pharmacotherapy, and, in some particular cases, neurosurgical approach (deep brain stimulation). The first two methods are available in RM. The efficiency of other tics treatment had been not confirmed yet. A particular attention is paid to the management of the specific comorbidities of GTS and tics.

Conclusions. The diagnosis of tics is based on the recognition of their clinical manifestations and precise anamnesis. Only some patients with tics need treatment but all of them should be informed about the tics' nature, causes and evolution. The behavioural therapy could be effective in some patients, while in others would be more convenient the pharmacotherapy or the combination of both. The neurosurgical treatment (deep brain stimulation) is reserved to severe tics, that are resistant to other therapies.

Keywords: tics, Gilles de la Tourette syndrome, diagnosis, treatment.

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Key messages

What is not yet known about the issue addressed in the submitted manuscript

Tics and Gilles de la Tourette syndrome are common in the population but are frequently underdiagnosed and not treated appropriately. At the moment, there is no official guidelines for the diagnostic and treatment of tics adapted to the conditions of the Republic of Moldova (RM). Present work addresses this gap and suggests some diagnostic and treatment options available in RM,

based on the international guidelines**.**

The research hypothesis

This work aims to prove that many modern diagnostic and treatment options for tics are available in RM.

The novelty added by manuscript the already published scientific literature

This article is a unique review on the tic-related diagnostic and therapeutical options adapted for RM, and can improve practical skills of the adult and pediatric physicians.

Introduction

Tics are sudden, rapid, recurrent, arrhythmic movements and vocalizations in variable frequency, type, and intensity, which usually appear in bouts and are performed without any specific purpose [1]. Tics can be motor and vocal (phonic).

Gilles de la Tourette syndrome (GTS) is a combination of the multiple motor tics with at least one vocal, starting before the age of 18 and persisting more than 12 months [1]. The intensity of tics in GTS can widely vary from mild tics in some patients to the violent tics or even combination of tics of different intensities in other patients.

The prevalence of GTS, although previously underestimated, is relatively high. GTS is present in almost 0.3-1% of the population. GTS is found in about 1-2% of children and 0.3-0.5% of adults [1-4]. The prevalence of only chronic motor and of only vocal tics is of about 3-4% in the general population (adults and children) [5]. The transient tics are found in approximatively 20% of the pediatric population [6]. The tics are more common in children compared to the adults and more frequently affect boys than girls (ratio boys/girls of about 3-4:1) [1].

The exact prevalence of tics in the Republic of Moldova is not known yet, but the results of the screening study in children of the preschool age show that 2.05% of them had tics [7].

Tics represent a neurodevelopmental disorder with a neurologic background [1]. Although there are a lot of cases of primary tics without comorbidities, tics are often coexistent with some psychiatrically disorders as *attention-deficit/hyperactivity disorder* (ADHD) and *obsessive-compulsive disorder* (OCD) [1].

The right diagnosis of tics is the key element of their adequate management. The therapeutically approach to the tics should be adapted to their intensity, degree of the impact of the quality of life of the patient and to the presence or absence of the comorbidities. In some cases, not tics but comorbidities should be the major goal of the treatment.

The etiopathogenetic basis of tics includes changes in the basal ganglia and their connections (cortico-striato-thalamo-cortical circuits) during the development and maturation of the brain [8]. Tics are due to the dysfunction at the level of different neurotransmitters: dopamine, gamma-aminobutyric acid, glutamate, serotonin etc.

There is no treatment yet that would definitely eliminate tics forever. At the same time, the tics fluctuate spontaneously: they can increase or decrease or even disappear for some time, without any explicit cause. The majority of patients become tic-free at the age of 18-20. Therefore, if the tics are mild and do not affect significantly the quality of the patient's life, they need no treatment. The tics are temporarily suppressed by the drugs that act on the receptors of the neurotransmitters implied in tics' generation. However, these medications have some considerable side effects, and the decision to treat should be taken in each case individually.

Tics can be voluntarily suppressed for a short time. This property was used to develop particular behavioral strategies that facilitate this temporary suppression. GTS is not caused by psychoemotional reactions, but the already existent tics can be increased by stress, tiredness, and excitement [9]. Their intensity could be decreased by relaxation techniques.

Diagnosis and treatment of tics have been the subject of the in-depth research in the last years. The data of multiple studies have already confirmed some hypotheses and contradicted other ones. New drugs with fewer side effects were developed. However, the lack of some treatment options in RM significantly limits the possibilities of physicians and patients with tics. Therefore, an algorithm of diagnosis and treatment adjusted to the autochthonous conditions is required.

Material and methods

Initially the search by the key words *„Tourette guidelines"* and *„tic disorder guidelines*" was performed through the publications until October 2023 in the online database PubMed (*US National Library of Medicine, National Institute of Health*) [10]. After the examination of the titles, the author selected papers representing official guidelines for the diagnosis and treatment of the tic disorder and GTS.

Afterwards was performed the search by the key words *„Tourette diagnosis"*, *„Tourette treatment"*, *„tic diagnosis"* or *„tic treatment"* in the PubMed database through the publications between 2021 – October 2023. The option *„humans"* was active in the search settings to exclude preclinical studies. The inferior timeline limit was adjusted to the time of publication of the 2nd variant of the European clinical guidelines for Tourette syndrome and other tic disorders [1, 11-13], a critical evaluation of the valorous sources published until 2021. These guidelines were created by a large international group of specialists in GTS and consist of four parts.

To specify some notions and facts, additional sources of information were consulted. The diagnostic and therapeutic recommendations were assessed from the point of view of their relevance and accessibility in the Republic of Moldova (RM). The availability of the drugs in RM was checked on the site of the Medicines Agency and Medical Devices of RM [14].

The final text includes a synthesis of recommendations, with an algorithm of diagnosis and treatment of tics adapted for RM.

Results

Processing of the information. In the PubMed database were identified Canadian, German and two (1st and 2nd versions) European guidelines for GTS and tics. The 2nd variant of the European guidelines consists of four parts published apart, one of which includes the presentation and diagnostic approach to the GTS [1], and the remaining three parts describe the treatment of tics (behavioral, pharmacotherapeutic and neurosurgical approach) [11-13].

The Canadian guidelines for GTS, published in 2012, consist of two parts: the non-pharmacological treatment and the pharmacological treatment of tics [15-16]. The German guideline, appeared in 2012 is outdated and no more recommended by the German Neurological Society and is not available anymore on its official website. It shortly describes the clinical and diagnostical particularities of tics, as well as the therapeutic non-pharmacological and pharmacological opportunities, existent at the moment of publication. There is also a practice guideline recommendation summary from international authors, published in 2019, that summarized treatment recommendations for tics and Tourette disorders [17].

Of the articles published between the 2021-2015, 764 corresponded to the search criteria. After the analysis of the titles, 436 articles were qualified as probably most relevant for the topic of the present work. The text of 397 of them could be integrally accessed. Of them, the final bibliography of the article includes only the most relevant sources in the number of 95. In some cases (e.g., to define notions referring GTS, to precise statistical data, therapeutic methods or characteristics of some medications) additional literature (including publications before 2021) or the internet-sites were consulted.

Discussion

Diagnosis of tics. Tics represent the clinical manifestation of the neurodevelopmental disorder and belong to the spectrum of disorders including transient tics, chronic tics, GTS, ADHD and OCD. The phenotype and intensity of tics may significantly vary from barely observable and rare toviolent and frequent.

The main diagnostic option for tics is the clinical observation. The typical presentation does not need any supplementary investigation. The diagnosis of tics is based on the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition, Text Revision [18], that specifies:

- *Gilles de la Tourette syndrome:* (1) Both multiple motor and at least one vocal tics have been present during illness, although not necessarily concurrently; (2) The tics may wax and wane in frequency but have persisted for more than 1 year since the first tic onset; (3) The onset is before the age of 18; (4) The disturbance is not attributable to the effects of a substance (e.g., cocaine) or another medical condition (e.g., Huntington's disease, post viral encephalitis).
- *Persistent (Chronic) Motor or Vocal Tic Disorder:* (1) Single or multiple motor or vocal tics have been present during illness, but not both motor and vocal; (2) The tics may wax and wane in frequency but have persisted for more than 1 year since the first tic onset; (3) The onset is before the age of 18; (4) The disturbance is not attributable to the effects of a substance (e.g., cocaine) or another medical condition (e.g., Huntington's disease, postviral encephalitis); (5) Criteria have never been met for GTS.
- *Provisional Tic Disorder:* (1) Single or multiple motor and/or vocal tics; (2) The tics have been present for less than 1 year since the first tic onset; (3) The onset is before the age of 18; (4) The disturbance is not attributable to the physiological effects of a substance (e.g., cocaine) or another medical condition (e.g., Huntington's disease, post viral encephalitis); (5) Criteria have never been met for GTS or persistent (chronic) motor or vocal tic disorder.
- *Unspecified tic disorder:* tics that do not meet the full criteria for a tic disorder or for any of the disorders in the neurodevelopmental disorders (e.g., the ones that appeared after the age of 18, the ones persisting less than 4 weeks etc.).
- *Other specified tics:* refers to the tics that do not meet the criteria for a tic disorder or any specific neurodevelopmental disorder.

The tics in DSM-5-TR are included in the section „Neurodevelopmental Disorders", category „Motor Disorders".

There are also criteria of diagnosis defined by the International Classification of Diseases, 11th edition (ICD-11) [19] that are similar to those of DSM-5-TR.

The diagnosis of tics is based on:

1. Anamnesis: The anamnesis is indispensable for the tics' diagnosis, because it allows to establish their specific particularities. It is important to collect not only the anamnesis referring to the tics, but also to their common comorbidities (ADHD, OCD, impulse-control disorder) and to ask about the clinical signs of a neurological disorder with secondary tics. The antenatal and family history in children are indispensable for the differential diagnosis.

Therefore, during the discussion, the patient should be asked about [1, 20, 21]:

- Tics' particularities (which tics the patient has, where are they localized, how frequent and how strong are they);
- Onset age (usually tics begin between the 5th and 8th years of life, mean age 5 years, although lower ages of onset reported in up to 40% of cases [1]), circumstances of the first manifestation;
- Evolution of tics – diurnal fluctuations, decrease or absence during sleep, long-time fluctuations with possible vanishing for hours, days or even months, changes in the tics' pattern, increase between 8-12 years of age, decrease after 18 year of age;
- Short-time voluntary suppression of tics (not always possible in little children) and particular unpleasant feeling of an internal tension during the suppression;
- Family history: although the transmission is non-mendelian, and tics are due to the combination of the genetic and ambient factors, the relatives of the patients can also present tics, ADHD or OCD.

- Physical consequences of tics (pain, lesion etc.) and psychosocial impact (on the social relations, learning, work, sleep);
- Evaluation of the psychosocial intrafamilial situation, financial state, interpersonal conflicts caused by tics;
- Presence of the comorbidities: OCD (obsessions, rituals), ADHD (attention disorder, hyperactivity), depression, anxiety, impulse-control disorder;
- Other pathological signs (including the neurological ones), other diseases in the anamnesis;
- Treatment anamnesis, doses, and efficiency of the medication.

The diagnosis of GTS can be supported by the so-called Diagnostic Confidence Index [22].

2. Physical examination: The physical examination contributes to the right diagnosis. The general examination of the patient (symptoms of other somatic disorders or of a dysmorphism indicating a genetic syndrome) and an exhaustive neurological examination (detection of some secondary causes of tics) should be performed. The tics' phenotype, their suggestibility (they increase when somebody talks to the patient about them) and the voluntary suppressibility for a short time are assessed. During the medical consultation, patients can consciously or unconsciously suppress their tics. In such a case, parents or relatives of the patient are asked to film the tics at home.

3. Paraclinical examination: Today there is no paraclinical examination indispensable for the diagnosis of typically manifested primary tics [1, 20]. The paraclinical exams are useful only if tics are atypical, e.g., first-time appearance of tics after the age of 18, presence of the symptoms suggesting another disease, antecedent of a cranial trauma, etc.

If the symptoms suggest a metabolic (e.g., Wilson disease) or an autoimmune (e.g., Sydenham chorea) condition, a blood analysis is performed (blood count, creatin kinase and ceruloplasmin level, glycemia), the antistreptolysin O titer is assessed, and the presence of the antiphospholipid or antineuronal antibodies is tested [20, 23]. If the additional symptoms suggest a metabolic, inflammatory or neurodegenerative disease, the urinalysis or cerebrospinal fluid analysis are indicated.

If the clinical presentation is more suggestive for an epileptic condition (convulsive or myoclonic seizures, absences etc.), electroencephalography should be performed. For the diagnosis of typical tics, without any additional suspect symptoms, the electroencephalography is useless [1, 23]. In some rare cases, electromyography could help to differentiate tics and myoclonus, although these two entities usually have different clinical appearance.

The magnetic resonance imaging scan is ordered in case of suspicion of traumatic, infectious, metabolic or neurodegenerative brain lesion. There are no specific structural changes on the magnetic resonance imaging scan of a patient with primary tics [23].

Genetic analysis is performed only for the scientific purpose. No consistent genetic changes common for a large population of patients with tics have been detected yet [1, 23-26].

The neuropsychological testing could discover comorbid ADHD, OCD or depression. If there is a suspicion of a coexistent autistic spectrum disorder, the patient should be evaluated by a specialist in this domain. The uncomplicated tic disorder and GTS do not induce any mental or physical abnormality. If there is a mental and/or physical handicap, another explanation of its origin should be searched [23].

As it has been already mentioned, the paraclinical examinations are useful only in some particular cases, when the clinical manifestation of the tics is atypical or there are other pathological signs. The unjustified and excessive use of different paraclinical methods is non-contributive for the diagnosis and treatment of tic disorder and GTS.

Differential diagnosis of the primary tics. The differential diagnosis of tics is a complex process that requires a sufficient knowledge of the clinical manifestation of neurological diseases similar to tics. In the majority of cases, the anamnesis and clinical examination can be sufficient for the right diagnosis of tics. It is important to take into account the individual variability and intensity of tics in GTS.

Primary tics and GTS should be differentiated from [1, 5, 26-31]:

1. *Primary tics, associated with the major psychiatric comorbidities,* such as autistic spectrum disorders, mental retardation, major ADHD, major OCD, etc. If comorbidities have a severe evolution while tics are mild, the treatment should be oriented against the symptoms most affecting patient's quality of life. As an example, in a patient with major ADHD and extreme mild tics (e.g., blinking) only ADHD should be treated, even if the stimulant medications could somehow increase the tics.
2. *Secondary tics, that could be:*
   - Caused by drugs and other substances, e.g., amphetamines or other central nervous system stimulants, serotonin reuptake inhibitors, cocaine, levodopa, carbamazepine, phenytoin, phenobarbital, lamotrigine, caffeine and other dopamine receptor blockers (tourettism and tardive tics);
   - Caused by hereditary diseases, e.g., neuroacanthocytosis, tuberous sclerosis, Wilson's disease, neuroferritinopathy, Lesch-Nyhan syndrome (purine metabolism disorder), phenylketonuria etc. Tics are not the only manifestation of these diseases, and usually there are other pathologic manifestations;
   - Caused by infectious or autoimmune diseases, e.g., Sydenham's chorea, pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS), antiphospholipid syndrome, viral encephalitis, neurosyphilis, and Lyme's disease. Other infectious, inflammatory and central nervous system dysfunction signs are usually present;
   - Tics associated with chromosomal syndromes, e.g., fragile X syndrome, Down's syndrome, Kleinfelter's syndrome, XYY karyotype, X trisomy, partial trisomy 16, Beckwith-Wiedemann syndrome, etc. A somatic dysmorphism and other specific symptoms are usually observed;

- Tics caused by brain traumatisms or even by peripheral traumatism (e.g., tics after shoulder injury);
- Other causes of tics: vascular stroke with lesion in the fronto-subcortical circuit, hypoxic-ischemic encephalopathy, etc.

3. *Other movement disorders similar with tics*: stereotypies, habits, mannerisms, rituals, chorea, ballism, myoclonus, dystonia, tremor, seizures (especially the myoclonic ones), akathisia, hyperekplexia, synkinesis, compulsions, restless legs syndrome, psychiatric disorders with pathological movements, psychogenic movement disorders.

Tics are not psychogenic and should be differentiated from the pathological movements in somatoform disorders that should be treated differently. It could be difficult to differentiate tics from compulsions in OCD, especially if they are comorbid. In table 1 are presented some differential criteria of tics and compulsions.

Table 1. Differences and similitudes between tics and compulsions in OCD*

Tics	Compulsions in OCD
Differences	
Purposeless	Goal-oriented actions, with a purpose (e.g.: *„If I would not flap my hands three times, I will not be lucky today*").
Accompanied by urge to tic	Accompanied by obsessive thoughts
Usually are not associated with anxiety	Usually are associated with anxiety
Semivoluntary or involuntary	Voluntary
Typical onset age of 6-8	Typical onset age of more than 8
Fluctuant natural evolution	No significant fluctuations in evolution
Similitudes	
Suppressible for a short time	Suppressible for a short time
Decrease if the attention is focused on other things	Decrease if the attention is focused on other things
Increased by emotions	Increased by emotions

Note: * The table is adapted from the European clinical guidelines for Tourette syndrome and other tic disorders-version 2.0. Part I: assessment [1].

The diagnostic algorithm of tics is described in figure 1.

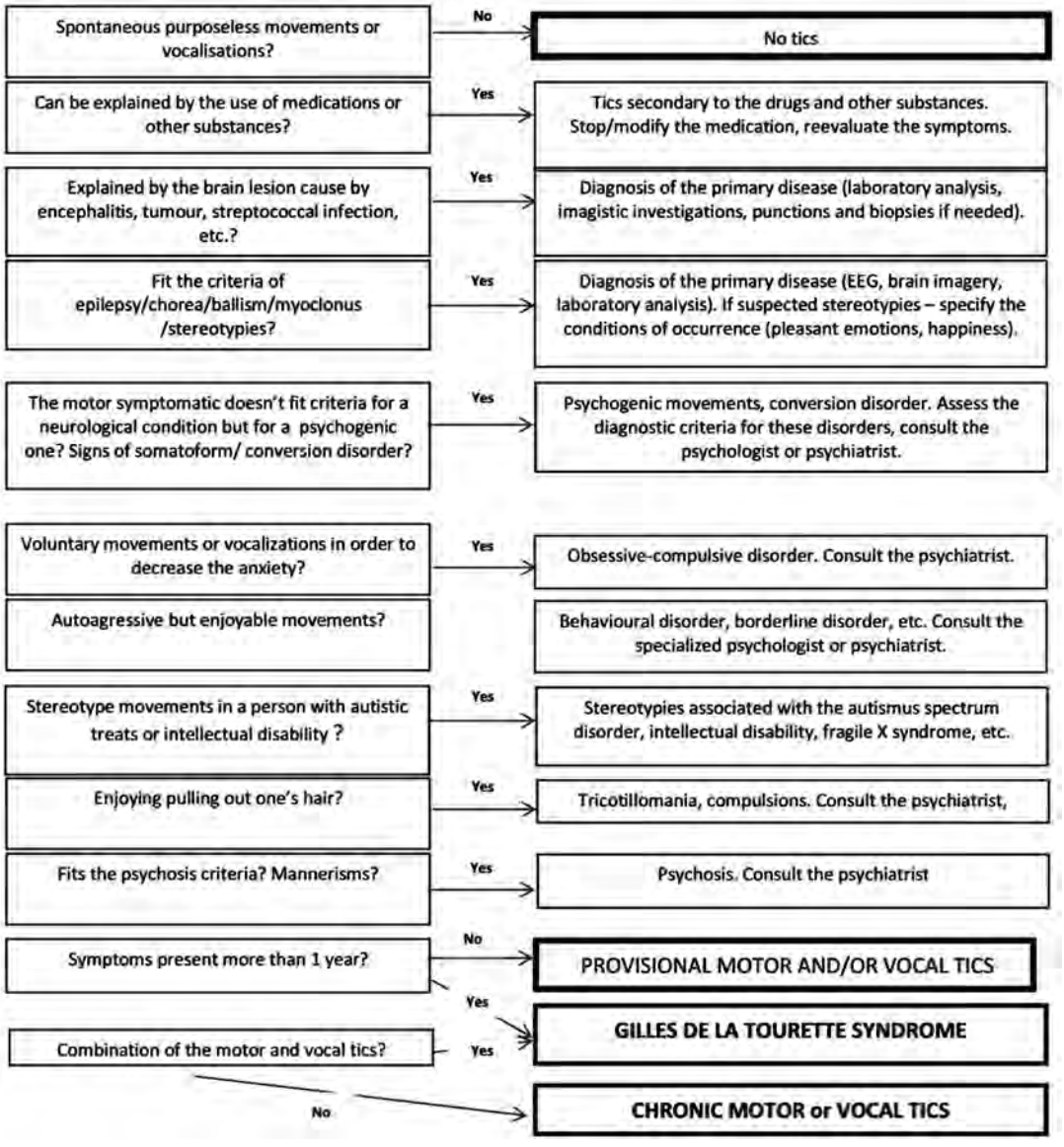

Fig. 1. Decisional algorithm for the differential diagnosis of tics (adapted from the European clinical guidelines for Tourette syndrome and other tic disorders-version 2.0. Part I: assessment [1]).

Treatment of tics. *Selection of patients that need treatment.* Only some patients with tics seek medical advice and only a part of them really need to be treated. The existent GTS medication does not provide total cure but just temporarily suppresses tics during the treatment. These drugs also have important adverse effects.

The modern scientific studies proved that tics have neural origin and represent a variant of the normal cerebral maturation. About 20% of all children have transient tics that will disappear spontaneously [6]. Other children have mild chronic not disturbing tics that do not affect their social life. The evolution of tic disorder and GTS is fluctuating and there could be weeks and even months free of tics. At the age of 18-20, the tics decrease or vanish in 90% of patients, although it is not possible to individually predict their evolution [1, 26].

Therefore, a decisive moment of the therapeutic management of tics is the individual decision to treat or not. Tics with a recent onset usually disappear in some weeks or months and do not need any treatment. In the majority of cases, the chronic tics will spontaneously disappear after some years, too. However, tics should be treated in the following conditions [1, 26, 27, 32]:

- Tics are violent and cause muscular or neuropathic pain or even skin lesions (e.g., biting tics), muscular lesions, articular luxation, bone fractures.
- Tics cause social problems, for example isolation and stigmatizing. As the parents frequently tend to overestimate the impact of tics, as well as adolescent patients, it is important to evaluate the real influence of tics and comorbidities on social life of the patient.
- Tics induce major negative psychoemotional reactions as reactive depression, phobias, anxiety, low self-esteem, etc. In this case, psychological support and treatment should be also offered.
- Tics interfere with the functional abilities of the patient. For example, the academic performance could decrease if a patient is all the time busy with tics' suppression. Sometimes the motor or vocal tics could affect efficient communication with other people.

Management of patients needing no treatment. The only desire of the parents to treat their child with tics should not serve as indication to start the treatment if tics are mild and do not affect the psychoemotional, physical state and the social integration of the child. In such case, parents should be informed about the side effects of the tics' medication that exceed the benefits for this patient. In the same way adult patients with mild tics should be informed. These people should be supervised and psychoeducated, and a psychological support should be offered if needed. In other words, for mild non-disturbing tics, the so-called „watch and wait" approach is preferred [1, 26, 27].

The psychoeducation includes [1, 17, 26, 27, 33-35]:

- Information of the patient or parents in a clear and simple way about the clinical and etiopathogenetic particularities of tics. It is important to specify, that tics are not a malign disease, not a rare disorder, and represent a variant of a normal development;
- Information about the fluctuant (waxing and waning) evolution and age-related particularities of the tics. The factors that could positively or negatively influence the tics' intensity and frequency should be specified. The high probability that tics will disappear after the age of 18 should be accentuated;
- Description of the typical comorbidities of the tics;
- Explanation of the importance to inform the preschool or school staff (for children with tics), the coworkers, and relatives. An adequate and clear information about the tics' nature frequently ameliorates the social relationships.

Some people with mild tics could have severe comorbidities (major ADHD or OCD, impulse control disorder, depression, etc.), that need a specific treatment. As tics fluctuate in time, there could be periods when they increase and need a short-term treatment until the next „remission" when tics calm again.

Management of patients needing treatment: non-pharmacologic non-surgical methods. The already described psychoeducation is a key element in the management of any patient with tics. Recent studies proved that paying attention to tics contribute to their exacerbation, while they decrease when ignored. Therefore, some experts consider that the person with tics and his or her ambiance should try to ignore the tics, and that the voluntary suppression of tics decreases the probability that they will disappear [36].

At the same time there are many behavioral methods based on voluntary suppression of tics. They proved to be efficient in some patients. The disadvantage of behavioral treatment is the need for a permanent concentration on tics' suppression, what is difficult for a person with an active everyday life. Additionally, the majority of patients feel a specific unpleasant sensation during the tics' suppression, similar to a pressure, internal tension or itching – the so-called *urge*. Sometimes this feeling is unsupportable. There are also some persons with tics who have no premonitory sensation and, thus, are not eligible for behavioral treatment. Patients with few mild tics could especially benefit from such kind of therapies.

The two most capable behavioral methods are [11, 26, 37-43]:

- The *Habit Reversal Therapy* – the patient is trained to observe the moment when a tic appears and to activate the antagonist muscles in order to stop the tic with a competitive movement;
- The *Exposure and Response Prevention* – the patient is trained to suppress the tic, trying to habituate to the unpleasant sensations.

The behavioral therapy must be performed by a psychotherapist specially trained for this. Psychoanalysis or other methods used for the treatment of psychogenic movement disorders and conversion disorders are not recommended for GTS and tic disorders, because tics have a neural origin. At the same time, a psychological support could be used to cope with depression, stress, low self-esteem, and intrafamilial conflicts caused by tics.

Several novel treatment delivery formats of psychotherapy in tics are currently being evaluated, of which videocon-

ference has the most evidence to date [11, 26, 39, 40, 44].

The anti-stress relaxation methods decrease the excitability of the neural structures connected with the neuronal source of tics. These methods have a benefic effect against the intensity and frequency of tics. Sport activities and, especially, swimming, have a positive effect on tics. Stress, anxiety, insufficient sleep, stimulant's consumption (e.g., strong coffee and tea) increase tics [1, 11, 26].

Management of patients needing treatment: pharmacotherapy. The etiopathogenetic mechanism of tics is not yet known. Therefore, GTS and tic disorders are treated with the drugs acting on different neurotransmitter systems (e.g., dopaminergic, serotoninergic, noradrenergic, glutamatergic, GABA-ergic, cholinergic, opioid systems). During the evaluation of the efficiency of the drug, therapy should consider the natural fluctuant evolution of the tics. If the period of the spontaneous remission or, vice versa, of intensifying of tics by chance coincide with the use of medication, it could lead to false conclusions about its efficiency. As there is a large phenotypic variability, no medication has proven effective for all patients with GTS and chronic tic disorder [12].

The treatment should be started with low doses that will be gradually increased until the desired effect is obtained. Total elimination of tics is not always aimed, nor possible. The necessary doses of medications are usually much lower than the doses of the same drugs for other diseases. After some months of an efficient treatment the doses could be progressively decreased even to the point of discontinuation of the drug. It is important to take into account the fact that existent medications do not cure, but only suppress the tics while administrated [12, 15, 45-50].

For the last 40 years in tics' treatment are used the *post-synaptic dopaminergic D2 receptors' blockers* that are efficient in about 70% of cases [50]. This group of medication includes *neuroleptics*, typical ones (haloperidol, pimozide) and atypical (aripiprazole, risperidone, olanzapine, quetiapine, ziprasidone). Although neuroleptics are the most used and very efficient anti-tics drugs, they can cause severe side effects (extrapyramidal symptoms, hyperprolactinemia, abnormality of the cardiac repolarization) [12, 45, 50-52]. At the same time, without any clear explanation, neuroleptic-induced tardive dyskinesia is rare in GTS patients [53]. Atypical neuroleptics are preferentially recommended for the treatment of tic disorder and GTS, because they have less side effects than the typical ones [15, 23, 12, 44-50].

Aripiprazole, olanzapine, quetiapine, and risperidone are atypical neuroleptics accessible in the Republic of Moldova [14]. Of them, aripiprazole and risperidone seem to be the most efficient. The way of administration and the dosage of these drugs are presented in table 2.

The atypical neuroleptic aripiprazole is largely used in the worldwide practice, and has a good effect on tics in patients older than 6 years, with less side effects than other neuroleptics. Aripiprazole should be started with very low doses (1.25-2.5 mg/day) and increased by 1.25-2.5 mg every week or by 5 mg every 2 weeks, until the desired effect (lower intensity and frequency of tics) is observed. The usual maintenance doses are 3-5 mg/day; in case of severe tics, they could be progressively increased up to 10-15 mg/day. Aripiprazole has a good effect on tics, which could be seen in 70% of cases [12, 15, 45, 54-58].

Table 2. Medication recommended in international guidelines for the Gilles de la Tourette syndrome and tic disorder*

Drug	Indication	Initial dose (mg/day)	Therapeutical dose (mg/day)	Frequent side effects	Paraclinical examinations at the start of the treatment and its maintenance
Alpha-adrenergic agonists					
Clonidine	ADHD†/GTS‡	0.05 or 0.1 patch	0.1-0.4 (divided in 1-4 doses) patch – 0.1-0.3	Orthostatic hypotension, sedation, somnolence, xerostomia, headache	BP‖, ECG¶
Guanfacine		0.5-1.0	1.0-4.0 (in 1-2 doses)		
Atypical neuroleptics					
Aripiprazole	GTS	2.5	2.5-3.0	Sedation, akathisia, ES**, headache, increased appetite (less than other neuroleptics), orthostatic hypotension	Blood count, BP, ECG, weight, transaminases, blood glucose
Olanzapine	GTS/OCD§	2.5-5.0	2.5-20.0 (once a day)	Sedation, increase in appetite, akathisia	
Quetiapine	GTS	25-50	100-600 (in 2 doses)	Sedation, increase in appetite, agitation, orthostatic hypotension, rarely – ES	Blood count, BP, ECG, weight, electrolytes, transaminases, prolactin, lipidic profile, blood glucose
Risperidone	GTS/OCD	0.25	0.25-6.0 (in 2 doses)	Sedation, increase in appetite, orthostatic hypotension, depression and dysphoria, rarely – ES	
Benzamides					
Sulpiride	GTS/OCD	50-100 (2 mg/kg)	2-10 mg/kg (in 2-3 doses)	Sedation, dyssomnia, increased weight, hyperprolactinemia with amenorrhea and galactorrhea	Blood count, ECG, weight, transaminases, prolactin, electrolytes
Tiapride	GTS	50-100 (2 mg/kg)	2-10 mg/kg (in 2-3 doses)		

Note: *Adapted from the Clinical European Guidelines for Tourette syndrome and tic disorders, part II [45] and the European clinical guidelines for Tourette syndrome and other tic disorders-version 2.0, part III [12]. With **bold** are marked the drugs approved in the Republic of Moldova. Typical neuroleptics are not included, because nowadays there are other medications with lesser or fewer side-effects.

Abbreviations: † – attention deficit hyperactivity disorder; ‡ – Gilles de la Tourette syndrome; § – obsessive-compulsive disorder; ‖ – blood pressure; ¶– electrocardiography; ** – extrapyramidal symptomatic.

Less side effects, but also a lesser efficacy have the *alpha-adrenergic agonists*: clonidine and guanfacine. Only clonidine is available in RM [14]. There are no large studies investigating the role of clonidine in tics' treatment, but the small trials concluded that it has a better effect on tics in patients with a comorbid ADHD and has a positive influence on the ADHD symptomatic [45, 58-60]. Clonidine seems to play a role in the sensorimotor gaiting [61]. This drug is used in doses of 0.0025-0.0055 mg/kg/day in tablets or as a patch 0.1-0.3 mg/day (the patch is not available in RM). Usually, it starts with 0.05 mg/day before the sleep. The dose is gradually increased. The maximal maintenance dose is 0.3-0.4 mg/day divided in 2-3 doses [12, 15, 45]. Side effects of clonidine are presented in table 2.

Benzamides (tiapride, sulpiride, amisulpride) represent another group of drugs used for tics' therapy, acting on the D2 dopaminergic receptors of the ventral striatum and limbic system, as well as on the 5HT3 and 5HT4 serotonin receptors [12, 15, 45, 62-64]. For the amisulpride large studies are missing. Sulpiride has a proved efficiency against tics of 59% [63]. This drug is accessible in RM. The particularities of the sulpiride administration are presented in table 2.

Benzodiazepines, GABA-ergic modulators, have a long story of use in GTS. Although they are efficient in tics' treatment (more efficient than clonidine, the positive result is present in about 71% of cases [59]), their use is limited by the development of dependence in some weeks and, particularly, tolerance (progressively higher doses with an increasing frequency of administration are needed) [64]. Benzodiazepines also have other major side effects, e.g., sedation, somnolence, short-memory disturbance, concentration disorder, confusion, ataxia, paradoxical insomnia, and anxiety). The withdrawal can lead to important reactions: anxiety, insomnia, phobias, psychotic reactions, tremor, headaches, dyskinesia, etc., therefore, the discontinuation of the treatment should be gradual. One of benzodiazepines used for tics' therapy is clonazepam (initial doses of 0.125-0.5 mg/day and maintenance doses up to 6 mg/day). It could be sometimes used as short-term acute treatment (days-weeks) in case of violent tics [65].

Vesicular monoamine transporter type 2 (VMAT2) inhibitors (*tetrabenazine*, *deutetrabenazine*) deplete the presynaptic stock of dopamine and serotonin, and block the postsynaptic dopamine receptors [66-67]. VMAT2 are not available in RM. *Tetrabenazine* is largely used worldwide for tics' therapy, being the first intention drug in the United States of America [12, 15, 68-71]. The benefic action of tetrabenazine on tics was proved in many studies, oscillating between 33% and 94% [69]. *Deutetrabenazine* is well tolerated according to the trial data [72]. Some studies suggest that it can improve tics in GTS [73], while other trials failed to show its benefits [71]. Because of its pharmacokinetic properties, deutetrabenazine can be given twice daily, thus improving compliance [74].

Ecopipam, a first in class, selective dopamine 1 receptor antagonist, reducedw tics in some trials to a greater extent than placebo, without observable evidence of common antipsychotic-associated side effects [75, 76].

A small double blind, randomized, controlled crossover trial of *cannabis* in adults with GTS showed an improvement of tics with the Δ^9-tetrahydrocannabinol (THC) 10% compared with placebo [77]. Cannabinoids and cannabis are not available as medicine in RM.

Injections of botulinum toxin could be a local therapeutic option against tics, although the quality for evidence is very low [78-80]. Their action is explained by a focal denervation with an influence on the sensory component of the tics-related brain circuits [78]. The efficacy of the botulinum toxin is variable. It could be used for the treatment of simple motor tics, especially at the level of the face and neck, the dosage being of 2500-3000 U [15]. The side effects are usually local and temporary, and include hypophonia, ptosis, temporary loss of muscular force etc.

Although a randomized controlled trial confirmed a high efficiency of *valproic acid* in GTS [81], there are no large-scale studies confirming it, and the side effects after the long-term therapy with this drug (teratogenicity, endocrine hormonal side effects, and hepatotoxicity) limit its use especially in women of childbearing age and in children [82]. Thus, valproate is not recommended anymore for the tics' treatment.

The coexistence of comorbidities implies an adjuvant treatment of ADHD (e.g., clonidine, methylphenidate, atomoxetine) [83, 84] or OCD (e.g., sertraline, fluoxetine, fluvoxamine) [85, 86] in usual doses.

The European guidelines for GTS and other tic disorders recommend to initiate pharmacotherapy with dopamine blocking agents, preferably aripiprazole. Other agents that can be considered as first-line therapy include tiapride, risperidone, and especially in case of co-existing attention deficit hyperactivity disorder (ADHD), clonidine, and guanfacine [12].

Management of patients needing treatment: surgical methods. GTS is considered refractory to treatment if there is no response to 3 different drugs, including neuroleptics of both types – typical and atypical (but not „typical or atypical"), adequately dosed and administered for a sufficiently long period of time, without any significant amelioration of tics or with development of severe side effects imposing the discontinuation of the treatment [87-89]. If possible, at least 12 sessions of behavioral therapy should also be tried. At the same time, there is no generally established definition available for "treatment refractoriness" in GTS, and exact number of "treatment-refractory" patients is unknown [13]. If nothing helps, another therapeutic option could be *deep brain stimulation* (DBS) [13, 16]. Due to the possible major complications [90-94], this method is limited to severe tics. The selection of the candidates for DBS is made on rigorous criteria [13, 16, 47, 90-98]. DBS' targets are represented by different regions of striatum (e.g., *globus pallidus internus*) and thalamus. At the moment the neurosurgical treatment of GTS is not available in RM.

Other methods of tics' treatment. Acupuncture, meditation, massage, phytotherapy, and physiotherapeutic methods

give contradictory results, and the mechanism of their action on tics is not clear yet [45, 12]. Relaxing (listening to the music [99], resting) or directing attention towards other things (e.g., computer games), one could have less tics than usually, and this kind of activities could be benefic if individually adjusted. The physical activity, especially swimming, decreases tics, but the etiopathogenesis of this effect is unclear yet [100-101]. Morning light therapy in one study showed some small but statistically significant decrease in tic severity [102].

In the last year there was found evidence for the median nerve stimulation in the reduction of tics' frequency [103]. The transcranial magnetic stimulation (usually of the supplementary motor area) represents an experimental treatment method whose results are variable and not usually reproducible [104-107].

The algorithm of the therapeutic management of tics is presented in figure 2.

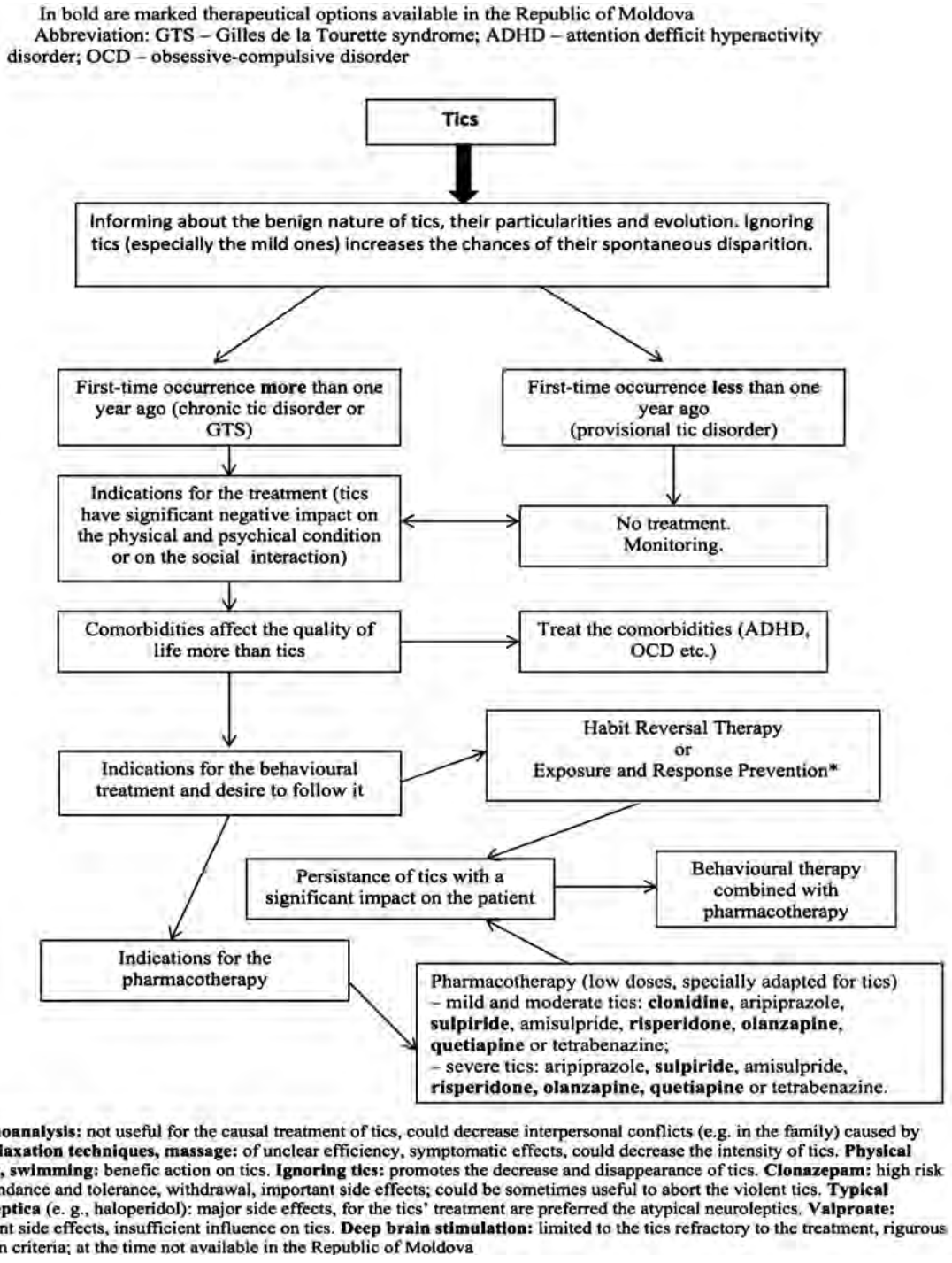

Fig. 2. Algorithm of selection of tics' treatment (adapted from the ESSTS Guidelines Group. European clinical guidelines for Tourette syndrome and other tic disorders. Part II: pharmacological treatment [45]).

Conclusions

The right diagnosis of tics is the key element of their successful therapeutic management. The diagnosis of tics usually requires nothing more than detailed anamnesis and a thorough physical examination of the patient. Only if there is an atypical presentation of tics and another disease is suspected, other investigations could be necessary for a differential diagnosis, e.g., laboratory analysis, brain imaging, electroencephalography, etc. Secondary tics are accompanied by the clinical symptoms of the underlying disease.

In childhood, tics frequently disappear spontaneously in some weeks or months, and do not need treatment. Chronic tics could persist for years and usually decrease at the age of 18-20 years. Only tics with a negative impact on the physical state or affecting social life of the patients require treatment. Providing the psychological support and informing about the benign nature of tics and the probability of their spontaneous resolution, could be much more important than the pharmacotherapy. Social problems could be decreased by explicitly informing the patient's ambience about the origin and particularities of tics.

GTS and tic disorder do not have any psychogenic cause, therefore, psychoanalysis is not useful to treat tics. The psychologist's help is needed to treat the accompanying depression, low self-esteem, and psychosocial problems. The behavioral psychotherapies seem to be efficient in tics, however they need perseverance and could be difficult to practice in everyday life.

The pharmacological treatment of tics includes dopaminergic antagonists (especially atypical neuroleptics, e.g., aripiprazole, quetiapine, risperidone), alfa-adrenergic agonists (clonidine), benzamides (sulpiride, amisulpride), and tetrabenazine. Not all of these drugs are available in the Republic of Moldova. Clonazepam is not the first-intention medication for tics because of the rapid development of dependence and tolerance. However, it could be used as an acute treatment to abort the spontaneous increase of the violent tics during the natural tics' fluctuations. The valproic acid (valproate) is less efficient as the aforementioned drugs and could cause important side effects if administered for a long period, especially on the developing brain of children, as well as teratogenicity when used in women of childbearing age.

The neurosurgical methods consist of the deep brain stimulation of the striatal structures and thalamus. They are indicated for severe tics resistant to all other treatments. Candidates are selected according to rigorous criteria. Deep brain stimulation for GTS is not available in RM yet.

The treatment should be adapted to tics' fluctuations, and sometimes the dosage should be progressively decreased or the drug should be even stopped.

The studies exploring other medication methods (acupuncture, phytotherapy, massage, meditation, etc.) give contradictory results. The physical activity and swimming have a positive influence on tics. Transcranial magnetic stimulation seems to have only a short-term effect on tics. A new electrical wrist device reducing tics was recently elaborated, based on the electrical median nerve stimulation study.

Patients with tic disorder or GTS could have specific psychiatric comorbidities, such as ADHD and OCD. The treatment of these comorbidities could significantly ameliorate quality of life of the patient.

The recommendations referring to the diagnosis and treatment of tics in this paper correspond to the results of the recent scientific studies. The adequate management of tics and their comorbidities assure a good quality of life of the patients and their optimal social integration.

Competing interests

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Abbreviations

DSM-5 – Diagnostic and Statistical Manual, 5th edition; DBS – Deep Brain Stimulation; GABA – gamma-aminobutyric acid; RM – Republic of Moldova; GTS – Gilles de la Tourette syndrome; ADHD – attention deficit hyperactivity disorder; OCD – obsessive-compulsive disorder.

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REVIEW ARTICLE

Hypotension in spinal anesthesia: predictive factors, prevention and volemia's non-invasive estimation methods

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ABSTRACT

Introduction. Evaluation of patient volemia arriving at a medical service today still represents a challenge for specialists, especially in those who need surgical and anesthetic intervention. One of the most common systemic side effect to anesthesia is hypotension. Spinal Anesthesia-Induced Hypotension (SAIH) because of sympathetic blockade is most frequently cited as a complication of subarachnoid anesthesia, its severity being influenced by the patient's volemic state. The aim of this literature review is to analyze if „routine" preanesthetic preloading reduces the incidence of SAIH in patients undergoing spinal anesthesia, also to emphasize the efficacy of preanesthetic assessment of the IVC/Ao (Inferior Vena Cava/Abdominal Aorta) Index measured by ultrasound in determination of patients' volemia.

Material and methods. Narrative literature review. Bibliographic search in the PubMed, NCBI and Google Academic databases, using the keywords: „hypotension inferior vena cava", „hypotension spinal anesthesia", „inferior vena cava/aorta diameter", „preloading hypotension", which were combined with each other. The final bibliography included 40 references.

Results. The principles of perianesthetic volemia management and prevention of arterial hypotension after the administration of the spinal block were detected in different groups of patients: the elderly, adult patients, anesthesiologic assistance in obstetrics and in various types of surgical interventions. Hypotension incidence data in patients with and without preanesthetic volume repletion were detected. At the same time, the effectiveness of the ultrasonographic assessment of IVC/Ao index in assessing patient's volume status was determined. The information was analyzed and synthesized in the article.

Conclusions. The effectiveness of routine preloading in reducing the incidence of arterial hypotension after spinal anesthesia did not prove its benefits in normovolemic patients, and ultrasonographic assessment of the IVC/Ao Index in assessing the volume status appears to be a simple, rapid, non-invasive, cost-effective volume assessment, which does not require the presence of a specialized imagist, being practically devoid of contraindications.

Keywords: hypotension in spinal anesthesia, preanesthetic volume repletion, volume in spinal anesthesia, assessment of volume status, Inferior Vena Cava/Aorta Index.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

Currently, there is no consensus on the methods of estimating the circulating volume and the need for preanesthetic preloading in patients scheduled for spinal anesthesia, arterial hypotension being one of the most frequent adverse reactions, and its severity correlates directly with the patient's volemia.

The research hypothesis

Analysis and synthesis of the available literature for elaboration of perianesthetic management recommendations and their effective-

ness in order to reduce arterial hypotension and diminish adverse effects after spinal block administration.

The novelty added by manuscript to the already published scientific literature

To evaluate the efficacy of ultrasonographic assessment of volume status in optimization of need for preanesthetic volume repletion in patients for elective spinal anesthesia, in order to avoid hypovolemia and hypervolemia, resulting in early and late complications.

Introduction

Spinal anesthesia, frequently used in daily clinical practice, is a safe and reliable method used in various orthopedic, lower abdominal and obstetric interventions.

Although this type of anesthesia is very advantageous due to a number of considerations (rapid onset, cost-effectiveness, ease of administration, postoperative outcome, preservation of respiratory function) [1, 2], arterial hypotension and bradycardia are the most common side effects after induction of spinal anesthesia, with an incidence of PSAH 33% and bradycardia - 13% according to the data given by Carpenter et al. in 1992 [3], and a PSAH about 5.4% according to the data of Hartman et al. [4]; with a higher risk at those with age 50 or more, a sensory level block above Th6, bupivacaine use as a local anesthetic, body mass index (BMI) 30 or more, and those receiving opiate as a premedication [5].

Rachianesthesia also can cause several adverse effects, such as coronary ischemia and delirium [6, 7], directly correlating with the increased mortality rate of patients who developed such intra anesthetic events, according to the data given by Sanborn et al. [8].

Various strategies for the prevention of relative hypovolemia caused by spinal anesthesia, such as empiric preanesthetic volume loading, prophylactic intramuscular or intravenous vasopressors have not proven to be plausible and applicable to all patients [9, 10], cardiac arrest representing the most serious intra anesthetic complication.

High variation in the incidence rate of hypotension and bradycardia after anesthesia is due to different definitions for „hypotension" and „bradycardia", and the various methods of measuring blood pressure and HR (heart rate). In most studies, blood pressure readings were documented manually. However, some authors have shown that automated on-line variable collection, together with an accurate definition of hypotension, can result in more accurate and comprehensive documentation of adverse events compared to manual documentation. This is also true for intraoperative hypotension [11].

At the same time, the definition of arterial hypotension as an absolute value of systemic blood pressure lower than 90/60 mmHg seems to be no longer valid, that being later classified by biometric parameters of blood pressure measurement. Some authors define it as an absolute change in systolic blood pressure less than 90 mmHg or mean blood pressure less than 65 mmHg, others as a relative change, with a decrease in diastolic blood pressure less than 40 mmHg. It may be orthostatic with a drop in systolic pressure of 20 mmHg or more, or a drop in diastolic pressure of 10 mmHg or more at patient's position change [12].

Cardiac arrest (CA) appears as the most serious complication of spinal anesthesia (SA), with the incidence and causes in the perioperative period studied over the years. Most of the literature involves retrospective studies or case reports. Few prospective studies evaluating large numbers of patients have been published [13]. The incidence of CA during regional anesthesia varies in different studies from 1.5-6.4/10000 [14, 15]. Charuluxananan et al. reported an incidence of CA following spinal anesthesia of 2.73/10000 [16].

Theories regarding the mechanism of cardiac arrest after neuraxial block involve a vascular etiology. Initially, it was speculated that sedation caused many of the cardiac arrests during spinal anesthesia [17]. Another reason could be the decreased preload after neuraxial block, resulting in a deviation in cardiac autonomic balance, with subsequent predominance of the parasympathetic system, leading to bradycardia.

Finally, three mechanisms have been proposed: the activation of low-pressure baroreceptors in the right atrium and inferior vena cava, autoregulatory reflexes that involves pacemaker cells of the myocardium, in which the heart rate is proportional to the degree of stretch of these cells [18] and the paradoxical Bezold-Jarisch response, in which mechanoreceptors located in the inferoposterior wall of the left ventricle, when stimulated, can cause bradycardia.

In addition, such conditions as a high level of sympathetic block, sedation, hypoxemia, hypercarbia, and chronic medications (such as beta-adrenergic antagonists) can directly activate vagal tone and contribute to the development and severity of bradycardia. Administration of intravascular fluids, alpha and beta agonists, and vagolytic therapy seems to decrease the frequency and improve survival in cardiac arrest due to neuraxial block [19].

Despite numerous studies have failed to demonstrate a reduction in the occurrence of low blood pressure following spinal anesthesia induction in individuals who have received preoperative volume replenishment [20], preloading still remains a widely used strategy. On the other hand, rapid preloading creates a major risk for cardiac compromised patients due to fluid overload and damage of the endothelium and endothelial glycocalyx [21]. Thus, fluids should be prescribed with the same care as any other medication, and avoid unnecessary administration [22].

Although the determination of volemia is an acceptable and salutary practice in anesthetic management, the difficulty of volume status determination affects the control and prediction of SAIH.

Despite the existence of a wide number of methods for assessing circulating blood volume, none of them is entirely plausible for the requirements imposed by contemporary medicine. Various techniques, such as central venous pressure (CVP) measurement, pulmonary artery catheterization, PiCCO (Pulse index Continuous Cardiac Output), Vigileo are capable of assessing preload as a component of hemodynamic status. However, their use remains a subject of ongoing debate due to financial restrictions, high complication rates, invasiveness, and the length of time required for application [23].

Recent publications in international specialized journals note the interest in studying ultrasound diagnostic methods in volume assessment is of major importance in patients of all age groups, which appear to be simple, rapid, non-invasive, cost-effective, and does not require the presence of a specialized imagist, thus having no contraindications.

Material and methods

In order to obtain the expected results, an initial search of specialized scientific publications was carried out. These were identified through the Google search engine: PubMed, NCBI, SpringerLink, Google Scholar. Articles selection was based on contemporary data regarding the monitoring of patient's hemodynamic and volemic status in perianesthetic period, applying the keywords: „hypotension inferior vena cava", „hypotension spinal anesthesia", „inferior vena cava/ aorta diameter", „preloading hypotension". These keywords were employed in various combinations to optimize search efficiency.

For the advanced selection of bibliographic sources, the following filters were used: full-text articles, articles in English, published in recognized journals, published from 1990 to 2022.

After a preliminary analysis of the relevance of the topic, original articles, randomized clinical trials, meta-analyses and review articles which contained up-to-date information and contemporary concepts regarding patient's volemic status measure and management for elective spinal anesthesia were selected.

The information from the publications included in the bibliography was submitted for analysis, synthesis, systematization, description, and comparative analysis of the results, to emphasize the importance of correct management of patients' volemic status during elective neuraxial blockades.

Only studies that satisfy validity criteria were evaluated and a comprehensive review was based on both: positive outcome studies and those that did not highlight the repletion's benefits.

After excluding duplicate publications and articles that did not meet the purpose of the article, the final bibliography included 40 references.

Results

Carpenter et al. [3] described hypotension with an incidence of 33% in their study. They defined hypotension as systolic blood pressure (SBP) <90 mmHg or, alternatively, as a 10% decrease from baseline in patients with baseline SBP <90 mmHg. Tarkkila and Isola [5] defined hypotension as a drop in SBP of more than 30% of the preanesthetic value or a drop in SBP less than 85 mmHg. They detected episodes of hypotension in 15.3% of patients.

The relatively low incidence of arterial hypotension observed in the study by Hartman et al. (5.4%) may be explained by their strict definition to detect episodes of hypotension with high specificity and, at the same time, an effect of reduced artifacts. Thus, according to their data, an episode of arterial hypotension after spinal anesthesia is defined as a decrease in mean arterial pressure (MAP) of 30% or more from the initial MAP at admission, within 10 minutes after the administration of the spinal block, requiring therapeutic intervention until 20 min after the onset of the decrease [4].

The basic effects of blocking the autonomic nervous system determine the physiological mechanism of action of the neuraxial block on cardiovascular activity. These effects generally increase with involvement of more afferent dermatomes (cephalad) and more extensive sympathectomy and may explain the sudden cardiac arrest sometimes seen during spinal anesthesia [24].

However, the variations in the response of the autonomic nervous system regulatory mechanisms in different patients can explain the different hemodynamic responses occurring after the application of spinal anesthesia [25].

Once analyzed, the risk factors that can cause the occurrence of post spinal anesthesia hypotension (SAIH) showed different precipitating values.

Hartman et al., in their study published in 2002 [4], analyzed the predictive power of hypotension of 13 patient-related variables, 4 directly related to surgery, and 12 anesthesia-related variables (8 variables related to regional anesthesia) as follows:

- variables related to the patient: age, height, weight, body mass index (BMI), sex, physical status of the patient according to the ASA score [26], active cigarette consumption, chronic alcohol consumption (defined as more than three alcoholic drinks per day), chronic heart failure (classification given by the New York Heart Association I-IV), history of preoperative hypertension or hypotension, vascular diseases, endocrine diseases and chronic preoperative antihypertensive treatment (with angiotensin-converting enzyme inhibitors, beta-adrenergic blockers, calcium channel antagonists, diuretics);
- surgical variables: admission status (inpatient/outpatient), emergency or elective surgery, surgical department (orthopedic and trauma surgery, general surgery, urology, gynecology and others) and type of surgical procedure according to the International Classification of Medical Procedures, given by WHO (World Health Organization);
- anesthesia-related variables: oral premedication with 3.8 or 7.5 mg midazolam (yes/no), amount of volume preload with intravenous crystalloid/colloid

given before Spinal Anesthesia (SpA), intravenous sedation after SpA (midazolam, propofol or both) and time interval between SpA puncture and start of surgery;

- variables directly related to spinal anesthesia: type of needle used for spinal puncture (Atraucan, Quincke, Whitacre, Sprotte), spinal needle size (22 to 29 Gauge), spinal anesthesia(SA) puncture site (L1-2 to L5 -S1), number of scoring attempts (from 1 to 4, ≥5), type and dose of local anesthetic (plain bupivacaine 0.5% or hyperbaric mepivacaine 4%), height of sensory block measured 10 minutes after application block by thermal stimulation with cold alcohol spray and local complications after SA puncture (bleeding, paresthesia).

Analysis of the results in Hartman's study revealed the following: a decrease in MAP within 30 minutes after SA induction was recorded in 3074 (99.2%) of 3098 patients. In 46.8% (n = 1450), MAP decreased by 10% to 20%, and therapeutic intervention occurred in 52.9% (n = 767) of this group. In 19.8% (n = 613) of all cases, there was a decrease in MAP of 20% to 30%; 50.4% (n = 309) of these patients underwent therapeutic intervention. In 8.2% (n = 254) of all cases, MAP decreased by more than 30%, but underwent therapeutic intervention 5.4% (n = 166) of these cases. These patients (n = 166) with relevant hypotension, by the definition, were included in the analysis. Evaluation of the samples to determine accuracy revealed no artifacts among the automatically detected events [4].

The following variables were identified by univariate analysis as having an association with a higher incidence of hypotension:

- variables related to the patient: age, weight, height, BMI, chronic alcohol consumption (due to neuropathy due to alcohol, the sympathetic nervous system is affected), the physical status of the patient according to the ASA score (ASA II patients presenting an incidence of 5, 9%, and ASA III of 8% in the univariate analysis, compared to the 3% in patients evaluated ASA I), preoperative history of arterial hypertension (increases the risk 2 times), long-term antihypertensive therapy;
- variables related to surgical intervention: emergency surgical interventions (due to the impossibility of qualitative and detailed assessment of patients), the operating department (general surgery and gynecology interventions presenting a much higher rate of intraoperative hypotension);
- variables related to anesthesia: the colloids administered before the puncture (with significant statistical data in the univariate analysis, but without significance in the multivariate analysis; it is important to note that the administration or not of crystalloids as preanesthetic volume repletion did not in any way influence the incidence of hypotension after spinal anesthesia , similar to the study carried out by Rout et al. back in 1993 [27]), the height of the sensory block above Th6 at 10 minutes after the application of the local anesthetic (due to the risk of blocking the cardioaccelerator fibers in case of advancement) and the frequency of punctures [4].

The association of one of the precipitating factors listed above increases 2-3 times the risk of developing episodes of hypotension after induction of spinal anesthesia.

In contrast, Kyokong et al. 4 years later published a study, where factors associated with hypotension and bradycardia after spinal anesthesia was analyzed, and SAIH was defined as a drop in SBP more than 30% of the initial value and bradycardia as a decrease in HR below 60 beats/min. As a result, he obtained an incidence of hypotension and bradycardia of 36.8% and 4.9%, respectively, the incidence of hypotension in this case being about 4.4 times higher than that of Hartman's study.

The following precipitating factors were detected:

- related to the patient: age and body mass index ≥30;
- related to anesthesia: analgesic level ≥dermatome T4, a prehydration volume less than 500 ml (controversial event by a lot of other recent studies);
- related to surgical intervention: cesarean section [28].

In this context, it is very difficult to determine the definition of SAIH, which will correlate most closely with the real situation of the patient.

Anesthetists consider that the mean, systolic, and diastolic pressure provides valuable information. However, according to Mascha et al. in their study „Intraoperative Mean Arterial Pressure Variability and 30-day Mortality in Patients Having Noncardiac Surgery" [29], diastolic and especially systolic pressures are subject to a considerable distortion depending on the vasomotor state, the measurement site and the type of anesthesia. In contrast, MAP is generally close to aortic pressure in a wide variety of clinical conditions, and close to oscillometric and radial artery measurements. Thus, in the results of his study, MAP values were essentially unchanged when the analysis was limited to radial arterial pressures versus pressures given by noninvasive arterial measurements.

Finally, they found that through SBP that was sustained for more than 10 minutes was associated with a higher 30-day mortality rate when SBP was less than 70 mmHg, but has no association with increased mortality when MAP is greater than 70 mmHg [29].

In the case of the correlation of intra anesthetic hypotension and organ injury (the development of Acute Kidney Injury or Acute Myocardial Infarction), Wasselink et al. in 2018 did a systematic review based on 42 studies, where they tried to highlight the intra anesthetic blood pressure values that create a risk for the postoperative outcome, in the context of the development of organ injury [30].

Based on their results, the reported risks of any end-organ injury after noncardiac surgery began to increase with prolonged exposure (≥10 min) to an SBP <80 mmHg, resulting in a slightly increased risk, with Odds Ratio between 1.0 and 1.4. For shorter durations (<10 min), slightly increased

risks were reported for MAP thresholds of 70 mmHg and lower. The reported risks increased to moderate (OR 1.4 to 2.0) with exposures to MAP <65–60 mmHg for ≥5 min, or any exposure <55–50 mmHg of MAP. High risks (OR > 2.0) were reported for SBP <65 mmHg for ≥20 min, SBP <50 mmHg for ≥5 min, or any exposure <40 mmHg [30].

Although the determination of volume in the patient to be anesthetized would be an acceptable and welcome stage of anesthetic management, the difficulty of determining the volume status in daily clinical practice is one of the causes that we lack control and SAIH prediction.

The use of central venous pressure (CVP) as a measure of patient's volemic status demonstrated a very weak relationship between CVP and circulating blood volume, as well as the inability of the ratio of CVP assessment before and after fluid administration (CVP/ ΔCVP) to predict hemodynamic response to fluid challenge. Therefore, CVP should not be used to make clinical decisions about volume management [31].

The use of other invasive methods of monitoring the hemodynamic status are not suitable in spinal anesthesia, for well-determined reasons previously reported, therefore non-invasive, fast, efficient monitoring methods with an increased degree of sensitivity and specificity are available. One of them would be the determination of the Perfusion Index (PI) by registration of pulse oximetry.

The determinants of the PI are complex and interconnected, involving and reflecting the interaction between peripheral and central hemodynamic parameters, such as vascular tone and stroke volume [32], although it appears to be a useful additional and non-invasive tool for anesthesia monitoring, perioperative and critical care for clinicians, is influenced by too many factors, such as preexisting cardiac arrhythmias, obesity, peripheral perfusion disorders, diabetic angiopathy and neuropathy, etc. The results of several studies support the use of this dynamic plethysmographic index also in the cephalic region when the finger is inaccessible or during states of low peripheral perfusion, and report its clearly superior efficacy compared to the Inferior Vena Cava Distension Index [33]. Toyama et al. in their study „Perfusion index derived from a pulse oximeter can predict the incidence of hypotension during spinal anesthesia for Cesarean delivery", reports that the initial baseline PI value correlated directly with the degree of decrease in systolic and mean arterial pressure (r=0.664, P<0.0001 and r=0.491, respectively P=0.0029). The cutoff PI value of 3.5 identified a risk of spinal anesthesia-induced hypotension with a sensitivity of 81% and a specificity of 86% (P<0.001) [34].

Recent publications in international specialized journals note the interest in studying ultrasound diagnostic methods.

These include the measurement of the Inferior Vena Cava Collapsibility Index (IVCCI) and the Inferior Vena Cava related to the diameter of the Abdominal Aorta Index (IVC/Ao).

The measurement of the Collapsibility Index of the Inferior Vena Cava (IVCCI) is very simple to use, it represents the measurement of the diameter of the Inferior Vena Cava at the end of expiration and the end of inspiration, with the subsequent calculation of the collapsibility index by the following formula:

$$IVCCI = \frac{IVCmax - IVCmin}{IVCmax}$$

Patient being evaluated as hypovolemic when IVCCI > 40%, according to the data given by Davi et al. in 2020 in a study of 100 patients requiring orthopedic surgery and receiving spinal anesthesia, and demonstrated that preanesthetic assessment of IVCCI to optimize fluid therapy can reduce the incidence of SAIH in orthopedic surgery and the need for vasopressors, and, therefore, the association of an IVCCI of over 40% with the development of SAIH [35]. Another study conducted in 2022 by Ting-ting Ni et al. on 90 patients requiring spinal anesthesia, revealed a sensitivity of 83.9%, a specificity of 76.3%, and a positive predictive value of IVCCI of 84% for predicting SAIH at a cutoff value >42%[36]. Likewise Szabo et al. in his study analyzed the predictive value for SAIH of IVCCI at the 50% limit with a low sensitivity of 45.5% but a very high specificity of 90% [37]. The results of the study by Zhang et al. in 2016 on a batch of 90 patients showed the IVCCI cut-off value of 43% and had a sensitivity and specificity of 78.6% and 64.8% [38].

All these studies reveal a very high variability of the results obtained, and small groups of patients included in the study, as well as the need to delimit the patient's inspiration and expiration peak, or other conditions that change the pulmonary pressures during the act of breathing, in order to collect truthful data.

In this context, an alternative is the calculation of the Inferior Vena Cava related to the diameter of the Abdominal Aorta Index (IVC/Ao), in which the inferior vena cava is measured in the subxiphoid region, immediately caudal to the confluence of the hepatic veins with the inferior vena cava (IVC), the anteroposterior diameter is measured longitudinally in M-Mode, while the Aorta is visualized by sliding left, opposite to IVC, and the anteroposterior diameter is measured in M-Mode. The ratio of these 2 measurements represents the IVC/Ao Index, according to the data given by the study of Sridhar et al. performed on 170 patients and published in 2012 [39]. Based on this study, IVC/Ao seems to present a much higher accuracy compared to IVCCI because the aorta is a structure that does not collapse and maintains a relatively constant diameter, regardless of the volume state of the patients. Aortic diameter correlates with body surface area (BSA), patient age, and sex, unlike IVC, which collapses with decreasing intrathoracic pressure during inspiration and re-expands with an increasing pressure during expiration, which reduces its accuracy. Research study states that IVC/Ao is more specific in assessing body fluid status [39].

To reduce doubts about the need for a specialized imagist to perform such measurements as mentioned previously, Durajska et al. in 2014 published the results of a study, where they demonstrate that a 4-hour training is more than enough to make IVC/Ao measurements of a similar quality to a qualified imagist [40].

However, to determine which assessment method IVCCI or IVC/Ao more accurately predicts SAIH, Salama et al. in 2019, European Journal of Anesthesiology (EJA) published the results of a study conducted on 100 patients who benefited from spinal anesthesia, in which both methods were compared simultaneously. According to the study results, the ROC curve revealed that IVC/Ao had a sensitivity of 96%, a specificity of 88%, and a precision prediction power for SAIH of 95% at a cutoff point of less than 1.2, while IVCCI had a sensitivity of 84%, a specificity of 77%, and an accuracy of 84% to predict SAIH at a cutoff point greater than 44.7% [41].

Discussions

The definitions of hypotension used in the previously cited studies are questionable because the authors define hypotension as exceeding a lower safety limit or, choosing the first measure of blood pressure value as the baseline value. Thus, an episode of arterial hypotension after spinal anesthesia defined as a decrease in mean arterial pressure (MAP) of 30% or more from the initial MAP at admission and based on MAP instead of SBP criteria seems to be more accuracy, as MAP is the most important blood pressure variable related to organ perfusion [4].

However, this approach does not take into account individual patient processes. Many variations in the response of the autonomic nervous system regulatory mechanisms in different patients can explain the different hemodynamic responses occurring after induction in spinal anesthesia [25].

There are 3 types of variables that seems to precipitate 2-3 times the risk of development of SAIH after induction of spinal anesthesia:

- patient related: age, weight, height, BMI (≥30) [28], chronic alcohol consumption, patient's ASA score, presence of chronic arterial hypertension with long-term antihypertensive therapy;
- surgical intervention related: the emergency and type of surgical interventions;
- anesthesia related: sensory block level (above T4 dermatome) [28] and the frequency of punctures [4].

In this context, it is very difficult to determine a strong definition of SAIH, which will correlate most closely with the real situation of the patient.

Preloading did not prove its effectiveness in SAIH incidence reduction and could worsen the patient's physical status.

Although the determination of circulating blood volume in patients will significantly simplify anesthetic management, the difficulty of its measurement results in poor prediction of SAIH.

Therefore, CVP measurement cannot be used to make clinical decisions about volume management [31], as well as other invasive monitoring.

Ultrasound diagnostic methods, of major importance in patients of all age groups, appear to be simple to use, fast, non-invasive, cost-effective, free of adverse reactions, and did not need a specialized imagist to perform the measurements [40].

The echographic measurement of IVC/Ao Index instead IVCCI has a greater sensitivity, specificity, and predictive power for SAIH, based on relative constant properties of aorta as structure.

However, there are some limitations in mentioned studies, probably determined by lack of homogeneity and small samples that lead to great variations in results.

Conclusions

Following the literature review carried out on the basis of all the mentioned studies, we can conclude that there are a series of factors with predictive value increasing the risk for SAIH, whether they are related to the patient, the surgical intervention, or the anesthetic management. Some of them can be influenced, and others cannot,at the same time, there are minimally invasive methods, easy to implement, with very high accuracy, which can evaluate the patient's volemic status immediately before the application of anesthesia, and as a result considerably reduce the adverse reactions related to an empirical preloading.

Moreover, the IVC/Ao Index evaluated ultrasonographically seems to be a simple, fast, non-invasive, cost-effective method for volemia evaluation, which determines the patient's volume status with high accuracy and does not require the presence of a specialized imagist, being free of contraindications.

Competing interests

None declared.

Authors' contribution

The authors contributed equally to the search of the scientific literature, the selection of the bibliography, the reading, and analysis of biographical references, the writing of the manuscript and its peer review. All authors have read and approved the final version of the article.

Abbreviations

SAIH - Post Spinal Anesthesia Hypotension; CVP - central venous pressure; PiCCO - Pulse index Continuous Cardiac Output; IVCCI - Inferior Vena Cava Collapsibility Index; IVC/Ao - Inferior Vena Cava related to the diameter of the Abdominal Aorta Index; SBP - Systolic Blood Pressure; MAP - Mean Arterial Pressure.

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CASE STUDY

Compound Angiotrophic Biphasic Myeloid Sarcoma with JAK2 (V617F) and KRAS (G12C) mutations

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ABSTRACT

Introduction. Myeloid sarcomas (MS) are extramedullary manifestations of myeloid neoplasms, associated with conditions like acute myeloid leukemia (AML), myelodysplastic syndromes (MDS), and myeloproliferative neoplasms (MPN). MS presents as tumor masses in various body sites, often expressing myeloid or monocytic markers. This case report details an unusual biphasic MS relapse with a significant "intravascular" component.

Materials and methods. A 59-year-old male with a history of JAK2-V617F positive MDS/MPN underwent allogeneic hematopoietic stem cell transplantation and presented with abdominal pain, skin lesions, and systemic symptoms. Biopsy of colonic masses was performed, and subsequent analysis was carried out.

Results. The biopsy revealed a neoplasm with solid and intravascular components. The solid part was mainly composed of monocytic lineage cells expressing specific markers, with a small population of myeloid blasts. In contrast, the "intravascular" component was mainly myeloid blasts expressing different markers. Genetic analysis uncovered JAK2 (V617F) and KRAS (G12C) mutations. Despite treatment, the disease progressed, and the patient eventually passed away.

Conclusions. Myeloid sarcomas are challenging to diagnose, often being mistaken for large cell lymphomas. They can manifest as isolated extramedullary relapses, with a unique molecular profile. This case stands out due to its biphasic nature, featuring distinct components with differing characteristics, which has not been documented previously in English literature. It underscores the intricate and diverse nature of myeloid sarcomas, emphasizing the need for further research to comprehend their biology and behavior effectively.

Keywords: Compound, Angiotrophic, Biphasic, myeloid sarcoma, JAK2, V617F, KRAS, G12C, AML, acute myeloid leukemia, myelodysplastic syndrome, MDS, myeloproliferative neoplasm, MPN.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

The submitted manuscript introduces a unique case of biphasic myeloid sarcoma with an extensive "intravascular" component, a phenomenon not previously reported in English literature. The distinct spatial, morphological, and immunophenotypic differences between these components represent an unexplored aspect of myeloid sarcomas.

The research hypothesis

The manuscript does not explicitly state a research hypothesis, as it primarily focuses on reporting a rare and novel case of myeloid sarcoma rather than presenting a specific research hypothesis.

The novelty added by manuscript to the already published scientific literature

This manuscript contributes novelty to the existing scientific literature by presenting a previously undocumented case of biphasic myeloid sarcoma with a unique "intravascular" component. The distinct characteristics and spatial separation of these components are unprecedented in the English literature on myeloid sarcomas, highlighting the complex and heterogeneous nature of these neoplasms. This case underscores the need for further research to better understand the biology and behavior of myeloid sarcomas, especially in cases with atypical presentations and features.

Introduction

Myeloid sarcomas (MS), formerly known as chloromas or granulocytic sarcomas, are extramedullary manifestations of myeloid neoplasms including acute myeloid leukemia (AML) and to a lesser extent myelodysplastic syndromes (MDS), and myeloproliferative neoplasms (MPN) [1]. By the World Health Organization's classification definition, MS's are composed of myeloid blasts with or without maturation and must present as a tumor mass that infiltrates and effaces the architecture of the extramedullary tissue [2]. MS can occur at any site, with different sites having different prognoses [3, 4]. The most commonly reported organs are lymph nodes, bones, soft tissues, and central nervous system [5, 6]. Atypical sites such as eyes, gall bladder, testis, and gastrointestinal system have also been reported [3, 7-11]. The majority of MS's typically express myeloid (CD13, CD33, CD34, CD117, PU.1, Myeloperoxidase) or monocytic markers (CD11B, CD14, CD13, CD33, CD64, CD68, CD163, PU.1, Lysozyme) and lack expression of B/T cell markers (CD3, CD5, CD7, CD20, Pax-5) [2, 12]. Here we report an unusual biphasic MS relapse after Allo-SCT with an extensive "intravascular" component previously unreported in literature.

Case Report

A 59-year-old male presented to our institution with abdominal and bilateral lower limb pain in addition to systemic symptoms including fatigue, decreased appetite, and weight loss. The patient had a history of JAK2-V617F positive myelodysplastic syndrome/myeloproliferative neoplasm (MDS/MPN) and underwent allogeneic hematopoietic stem cell transplantation three years before his presentation. Physical examination showed multiple inguinal and bilateral lower limb skin lesions.

Material and methods

Complete blood count and peripheral blood smear were unremarkable except for normocytic anemia. A computerized tomography scan showed partial small and large bowel obstruction due to multiple colonic masses involving the transverse and descending colon, in addition to enlarged mesenteric and inguinal lymph nodes. Laparotomy revealed multiple masses involving all parts of the colon invading into the adjacent organs. Biopsy of the largest mass (7 cm) in the transverse colon was performed. Due to extensive disease, no tumor debulking was done.

Results

Histopathologic examination of the biopsy revealed a neoplasm consisting of both solid and intravascular components which were immunophenotypically and morphologically different (Fig. 1 and 2). The solid component (Fig. 1) was mainly composed of large cells of monocytic lineage which strongly expressed Lysozyme, CD163, CD45, CD68, and PU.1. Myeloid blasts (CD34, CD117) were only focally increased and comprised 3-5% of total cells. In contrast, the "intravascular" component (Fig. 2) was mainly composed of myeloid blasts with high nuclear: cytoplasmic ratios (NC) which strongly expressed CD10, CD34, CD45, partial/heterogeneous CD117, and PU.1, but were negative for CD3, CD20, Pax-5, Lysozyme, myeloperoxidase, and TdT. Next-generation sequencing (NGS) of DNA from the tissue biopsy revealed *JAK2* (V617F) and *KRAS* (G12C) mutations. Fluorescence in situ hybridization (FISH) for *MLL* [11q23], *PML/RARA* [15q22/17q21.1], *CBFB* [16q22], and *RUNX1/RUNX1T1* [21q22/8q22] exhibited normal signal patterns. Fine needle aspiration of an inguinal lymph node revealed a population of myeloid blasts that expressed CD45, CD13, CD33, CD34, CD38, CD71 (dim), CD117, and HLA-DR, and lacked expression of CD14 and CD64. Bone marrow aspiration and biopsy revealed markedly hypocellular marrow with decreased trilineage hematopoiesis and no increase in blasts. The patient was treated with FLAG (fludarabine, cytarabine, and filgrastim) followed by cytarabine and daunorubicin chemotherapy protocols. Despite the therapy, there was disease progression. Therefore, he was referred to hospice and died two months after surgery.

Discussion

MS occurs at an incidence of 2–9% in newly diagnosed AML patients [13, 14]. The prognosis of AML with concurrent MS carries an overall poor prognosis with a 5-year survival rate ranging between 20% and 30% [3]. It can be present upon the initial diagnosis of leukemia, or as a manifestation of relapsed disease after chemotherapy or hematopoietic cell transplantation [15, 16]. MS can be the first manifestation of disease relapse before relapse occurs within the marrow space, as seen in our case. In addition, isolated extramedullary relapse of AML more commonly occurs post allogeneic hematopoietic stem cell transplantation than post chemotherapy [16, 17]. The absence of concurrent leukemia makes the diagnosis of isolated MS a challenging one. MS's are frequently misdiagnosed as large cell lymphomas with a high rate of misdiagnosis that can be up to 75% of cases [18].

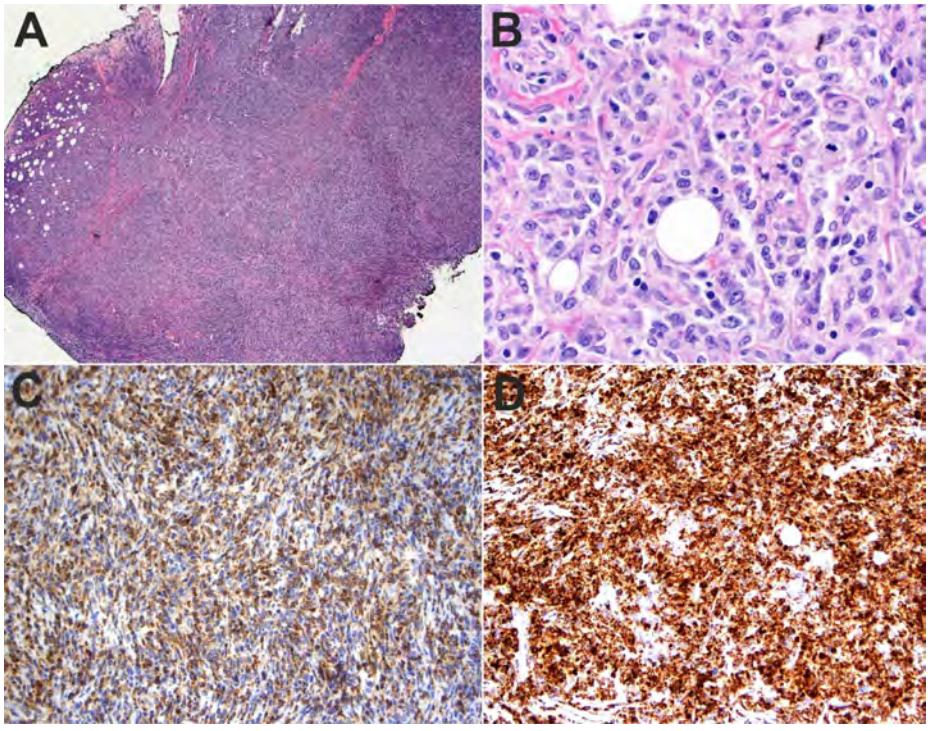

Fig. 1 Angiotrophic Biphasic Myeloid Sarcoma, solid component.

A, H&E (x2) showing the tumor solid component consisting of sheets of cells that efface the colon architecture. B, H&E (x50) a higher magnification of the solid component showing large atypical cells with large irregular nuclei and ample cytoplasm suggestive of monocytic lineage. C, CD163 (x20) and D, Lysozyme/Muramidase (x20) showing that the tumor cells are strongly positive for CD163 and Muramidase immunostains indicating monocytic differentiation. The tumor cells were positive for PU.1 and myeloperoxidase (focally) and negative for CD34 and CD117 (not shown).

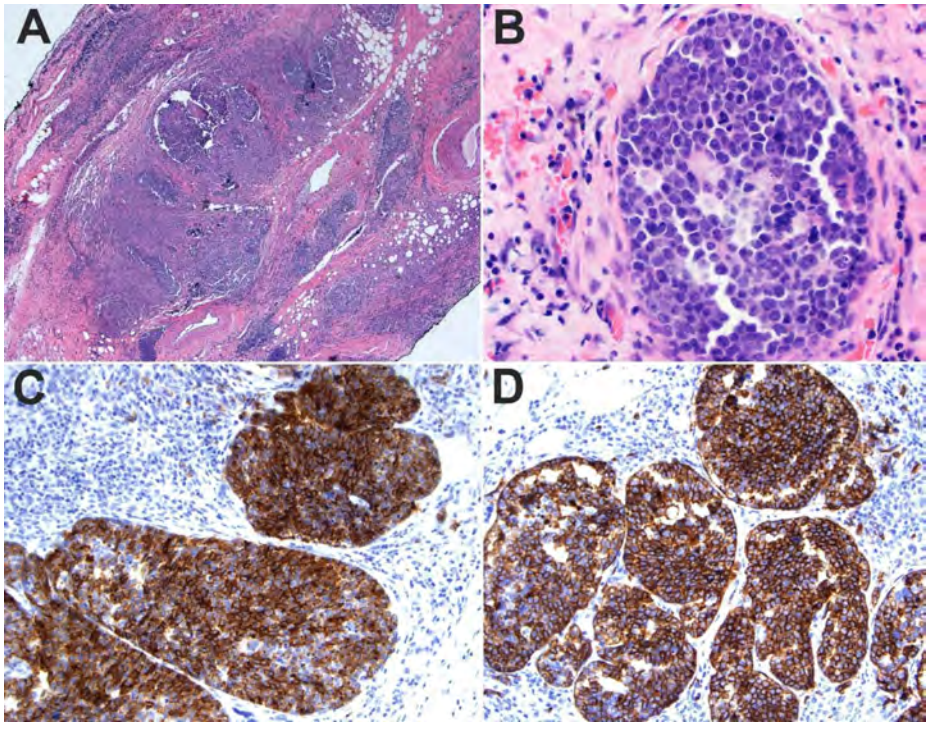

Fig. 2 Angiotrophic Biphasic Myeloid Sarcoma, intravascular component.

A, H&E (x2) showing the Angiotrophic "intra-vascular" component of the tumor cells distinct from the ones identified in the solid component. B, H&E (x50) a higher magnification of the Angiotrophic component showing cells with blast-like cytomorphology and high nuclear: cytoplasmic ratios with a fine/open chromatin pattern. C, CD10 (x20) and D, CD34 (x20) Showing the tumor cells with strong immunoreactivity for CD34 and CD10 immunostains. The myeloid blasts also expressed CD117, PU.1, and CD45 but were negative for myeloperoxidase, CD3, CD20, Pax-5, Lysozyme/Muramidase, TdT, pankeratin, and S100 (not shown).

Similar to AML, molecular events can also be identified in MS. Up to 55% of MS may contain cytogenetic abnormalities with the most common being trisomy 8 and inv(16) [19]. Other events including mutations (such as the common NPM1 mutation), gene rearrangements (such as KMT2A), and copy number variations (such as CBFB gene amplification) have been also reported. The mutation pattern in our case is unique. The finding of the JAK2 V617F mutation in the MS was consistent with the primary malignancy (MDS/MPN) and is proof of the molecular linkage between the two tumors. Although mutations in the KRAS genes are mostly seen in solid malignancies, it can be found in about 4-6% of AML cases [20]. In our case, the presence of those two driver mutations can represent the aggressive clinical behavior that results from acquiring additional driver mutations with progressive neoplastic clonal evolution.

The compound and complex nature of our case is also unique. The two components are distinct spatially, morphologically, and immunophenotypically. Spatially, each of the two components is localized to a particular compartment (solid tissue vs. vascular spaces). Morphologically, the intravascular component shows typical blast cytomorphology (fine/open chromatin with high N/C ratios) in contrast to the solid component, which shows a more differentiated cytomorphology (irregular nuclei with coarse chromatin and a relatively low N/C ratio). Immunophenotypically, the intravascular component expresses myeloid immature/blast markers (CD34 and CD117) with aberrant CD10 expression in contrast to the solid component, which lack those markers and instead express markers of monocytic lineage differentiation (Lysozyme/Muramidase, CD68, and CD163). Aberrant CD10 expression and other lymphoid-associated antigens have been reported to occur in AML in multiple studies [21-23]. Expression of aberrant lymphoid-associated antigens by AML has not been shown to alter the biologic behavior or response to therapy [24]. CD10 expression has been reported to occur with intravascular large B-cell lymphoma (ILBCL) and is usually associated with MUM-1 and BCL-6 expression, reflecting the germinal center origin of some cases [25-28]. In one study on 96 cases, CD10 expression was found in 13% of ILBCL cases [29].

ILBCL is an example of the isolated intravascular growth pattern of tumor cells [30-36]. In this phenomenon, there is a selective intraluminal proliferation of tumor cells in the small to medium-sized vessels due to a possible defect in adhesion molecules and homing receptor of malignant cells. Defects in molecules such as Intercellular Adhesion Molecule-1 and b1 integrin on the tumoral cells can impair the vascular transmigration of extracellular matrix required for tumor mass formation [37]. However, this phenomenon has not been reported to occur with MS. Although this report is limited for being a single case report, to the best of our knowledge, this is the first report in English literature of a compound biphasic myeloid sarcoma with an extensive "intravascular" component that is morphologically and immunophenotypically distinct.

Conclusion

In conclusion, this case report sheds light on the remarkable complexity and diversity of myeloid sarcomas (MS), an area of study that continues to present unique challenges and intriguing phenomena. The presentation of a biphasic MS, featuring both solid and "intravascular" components with distinct characteristics, adds a significant layer of novelty to the existing scientific literature on myeloid neoplasms. This case underscores the ongoing need for in-depth research to better comprehend the biology and behavior of myeloid sarcomas, especially in cases with atypical features and presentations. As we unravel the intricacies of these rare extramedullary manifestations, we move closer to a more comprehensive understanding of their clinical significance and, potentially, improved approaches to diagnosis and treatment.

Competing interests

None declared.

Authors' contribution

SS devised the main conceptual ideas of the project. IA drafted the manuscript. All authors reviewed the manuscript and approved the final version.

Informed consent for publication

Written informed consent was not obtained from the patient for publication of this case report and any accompanying images, as it is not required by the regulations of our institution.

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CASE STUDY

Post-COVID19 pulmonary complications in infants – clinical-imaging approaches

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ABSTRACT

Introduction. COVID-19 infection affects people of any age or gender. It was found that children up to 6 months of age have a major risk of developing a severe form of the infection. Contemporary diagnostic imaging methods of high sensitivity, such as lung CT, allow to establish the degree of lung damage, the volume and the sequelae arising from enduring the COVID-19 infection. The long-term consequences of the COVID-19 infection are still being researched. Pneumonia in the infection of COVID-19 can lead to the long-term development pulmonary fibrosis, atelectasis, bronchiectasis.

Case report. The 5-month-old boy is admitted to the Pneumology Clinic with dyspnea, tachypnea, acrocyanosis, agitation, food refusal. It is known from the anamnesis that at the age of 4 months the child suffered a severe form of the COVID-19 infection. Non-contrast lung CT was performed which suggests fibro-atelectatic changes in both lungs, predominantly in the apical and basal segments.

Conclusions. Infants show an increased vulnerability to develop bronchopulmonary changes after COVID-19. The case is suggestive from a clinical point of view, emphasizing the connection between the form, the evolution of the disease, and the consequences arising from the COVID-19 infection. Fibrotic pulmonary evolutionary changes are suggestive of SARS-CoV-2 virus infection.

Keywords: infants, COVID-19, CT, imaging.

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Key messages

What is not yet known on the issue addressed in the submitted manuscript?

The SARS-CoV-2 infection is a new infection, so there are still a number of uncertainties and equivocal aspects regarding the disease in children. Opinions and approaches to studies are different, sometimes contradictory.

The research hypothesis

Infants are more vulnerable to develop bronchopulmonary complications post-COVID-19.

The novelty added by manuscript to the already published scientific literature

The evolution of pulmonary damage in the context of COVID-19 infection in children deserves special attention. Contemporary imaging methods with a high specificity contribute with certainty to the establishment of the degree of bronchopulmonary damage and complications after enduring the infection of COVID-19.

Introduction

The infection with the SARS-CoV-2 virus caused a pandemic lasting more than 3 years and its worldwide impact was reflected in all fields, leaving long-lasting consequences. AAP (American Academy of Pediatrics) epidemiological data showed that children represent 18.3% of all cases of COVID-19 infection in a study of global statistics [1]. The clinical expression of COVID-19 infection ranges from asymptomatic disease with mild respiratory tract symptoms to severe pneumonia with acute respiratory distress syndrome and multiorgan dysfunction [2]. Compared to adult patients with COVID-19 infection, children have a milder clinical course, fewer radiological changes, and shorter convalescence time [3]. However, bronchopulmonary complications can occur even in cases of asymptomatic infection or mild form of the disease [4].

According to the latest studies presented in the specialized literature, approximately 25% of children who suffered the infection of COVID-19 experience sequelae with various manifestations [5]. Contemporary diagnostic imaging methods of high sensitivity, such as lung CT, allow to establish the degree of lung damage, the volume, and the sequelae arising from enduring the COVID-19 infection. The long-term consequences of the COVID-19 infection are still researched. Pneumonia in the infection of COVID-19 can lead to the long-term development of pulmonary fibrosis and bronchiectasis [6]. Thus, it is important to monitor patients with COVID-19 over time to assess the progression to irreversible fibrotic lung disease and its impact on respiratory symptoms, quality of life, and mortality [7].

Clinical case

We present the clinical case of a 5-month-old boy admitted to the Pneumology Clinic of the Mother and Child Institute in Chisinau.

At the age of 4 months, the child suffered the severe evolution of the COVID-19 infection. He was hospitalized in the specialized section for COVID-19 infections within the Mother and Child Institute. The child presented to the emergency room with a fever of 38.5°C, signs of respiratory catarrh, rare dry cough, tachypnea with 58-60 breaths per minute, SpO_2 – 94%, dyspnea, intercostal draft, acrocyanosis, refusal to eat, sleepiness. From the history of the disease, the child is considered sick for 2 days, with fever up to 38.9°C, difficult breathing, agitation. The reason for referral was the persistent febrile syndrome, which did not respond to the administration of antipyretics. From the epidemiological anamnesis, it is known that the child's mother showed signs of respiratory infection during the last 3-4 days. The objective examination of the child showed a serious general condition, he was apathetic with signs of respiratory failure.

During laboratory examinations, grade I anemia is attested (hemoglobin - 98 g/l, erythrocytes 3.2x106/μL, hematocrit - 26%); leukopenia (leukocytes – 4.4x109/L); lymphopenia (lymphocytes – 27%); accelerated ESR – 11mm/h; elevated liver transaminases (ALT – 82.8 U/l AST – 83.1 U/l); C-reactive protein – negative.

X-ray of the chest confirmed the bronchopulmonary damage with the following conclusion – Bilateral bronchopneumonia in the projection of the upper lobe - S1, S2, S3, signs of obstructive bronchitis with pulmonary hyperinflation (fig. 1 A). Taking into consideration the specialized care and treatment, clinical-imaging evolution with positive dynamics was attested with the reduction of pulmonary infiltration and the involution of clinical signs (fig. 1 B). The patient was discharged home after 6 days with recovery recommendations.

More than a month after suffering COVID-19 infection, the patient goes to the Pneumology clinic with complaints of semi-productive cough, difficult breathing, and loss of appetite. These symptoms surfaced about a week ago. Despite the background of symptomatic treatment, the child's general condition worsens due to toxic-infectious signs, respiratory failure. At admission, the child is agitated, has tachypnea with 53-57 breaths per minute, SpO_2 90-92%,

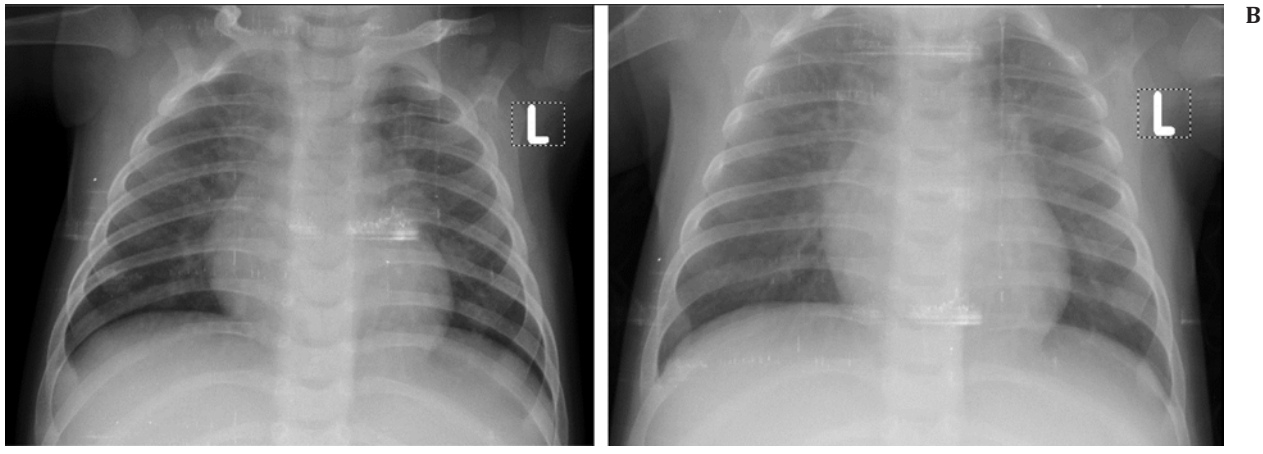

Fig. 1 Chest X-ray

A - chest x-ray at the onset of the disease, show bilateral bronchopneumonia in the projection of the upper lobe - S1, S2, S3, signs of obstructive bronchitis with pulmonary hyperinflation. B - chest x-ray at discharge, with positive dynamics, bronchopneumonia in resorption.

mixed dyspnea, subcostal draft, acrocyanosis, food refusal. Taking into consideration the serious general condition, the complaints and the anamnestic of the disease, the child was admitted to the intensive care unit for specialized treatment. Later, after stabilization of the general condition and clinical signs, a computed tomography of the chest without contrast was performed (fig. 2). The tomographic sections, performed in the pulmonary and mediastinal mode, highlight: consolidation areas by hyperattenuation type, with atelectatic component, located diffusely bilaterally, in the projection S1, S2, S3, S4, S6, S7, S10 of the right lung and in S1, S2, S3, S6, S10 of the left lung, with irregular border, inhomogeneous and with partial visualization of the air bronchogram. Deformation of the anatomical architecture of the lung parenchyma is present. Diffuse bilateral peribronchial thickening is observed, more pronounced at the level of the main and segmental bronchi, associated with narrowing of the lumen of the main and segmental bronchi. Trachea, main and segmental bronchi are permeable. The lung fields are transparent. The costo-diaphragmatic sinuses are free. The pleura shows thin sheets, without fluid collections and pathological thickenings. Heart and major vessels have normal CT appearance. The arterial ligament is calcified. The thymus is placed in the region of the anterior mediastinum, the dimensions correspond for the age, with usual configuration, clearly outlined, homogeneous, with the native density +65UH. Volume formations in both lung fields and in the projection of the mediastinum are not determined. Enlarged mediastinal lymph nodes are not determined. Diaphragm with clear outline, without pathological changes. Tomographic images are suggestive for bilateral polysegmental pneumonic infiltration with severe fibro-atelectatic component, signs of chronic bronchitis.

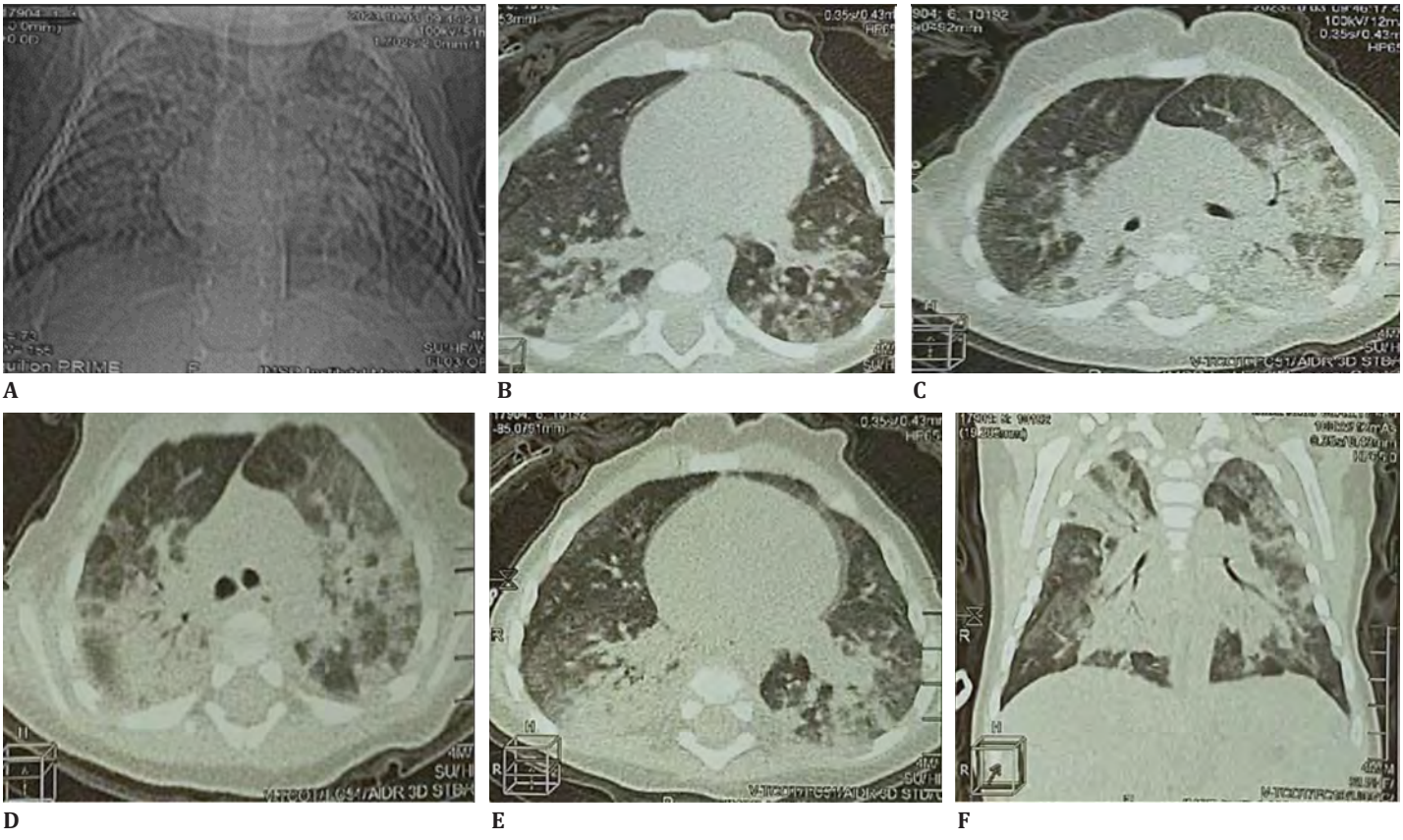

Fig. 2 Non-contrast Computed tomography of the chest organs, infant, one month after COVID-19

A, F – bilateral polysegmental pneumonic infiltration in the upper and lower lobes; B-E – consolidation areas with fibro-atelectatic component, located diffusely bilaterally, in the projection S1, S2, S3, S4, S6, S7, S10 of the right lung and in S1, S2, S3, S6, S10 of the left lung.

Discussion

COVID-19 infection affects people of any age or gender. Among the pediatric population, serious forms of the disease are less common, fewer complications and a shorter recovery period [8]. According to data from specialized literature, children up to 6 months of age have a major risk of developing a severe form of the infection [9]. Young age can be an unfavorable predictor in the development of post-COVID19 schools. These circumstances are also present in the clinical case of this infant with COVID-19 infection. Computed tomography data suggest fibro-atelectatic changes in both lungs, predominantly in the apical and basal segments. Once

triggered, pulmonary fibrosis leaves worrisome sequelae in children, as lung architectural distortions and irreversible lung dysfunction develop [10]. Taking into account that the occurrence of sequelae is unpredictable, the infection of COVID-19 in the pediatric population requires continuous monitoring by multidisciplinary teams with the involvement of pediatric pulmonologists and imaging techniques, especially by pulmonary CT.

Conclusions

Infants showed an increased vulnerability to the appearance of bronchopulmonary changes after COVID-19. The case is suggestive from a clinical point of view, emphasizing the connection between the form, the evolution of the disease, and the consequences arising from suffering from COVID-19 infection. Fibrotic pulmonary evolutionary changes are suggestive of SARS-CoV-2 virus infection.

Authors' contribution

CC conceived and designed the study. RS performed the analysis and drafted the manuscript. SȘ designed the significant revision of manuscript and provided significant intellectual input. All authors revised and approved the final version of the manuscript.

Patient consent

Obtained.

Competing interests

None declared.

Ethics approval

This study was approved by the Research Ethics Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy (Minutes no. 1 from 14.04.2023).

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[Revised May, 2023]

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Hemodynamic instability	7.0%	1.0%	0.034
Prolonged awakening*	11.0%	4.0%	0.19
PONV post-intubation	8.0%	27.0%	0.007
Strong pain on awakening	17.0%	19.0%	1.0

Note: *Unusually slow awaking, after that cerebral concentration of the anesthetic reach the under hypnotic level.
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Data access and responsibility

The corresponding author should indicate that he or she had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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