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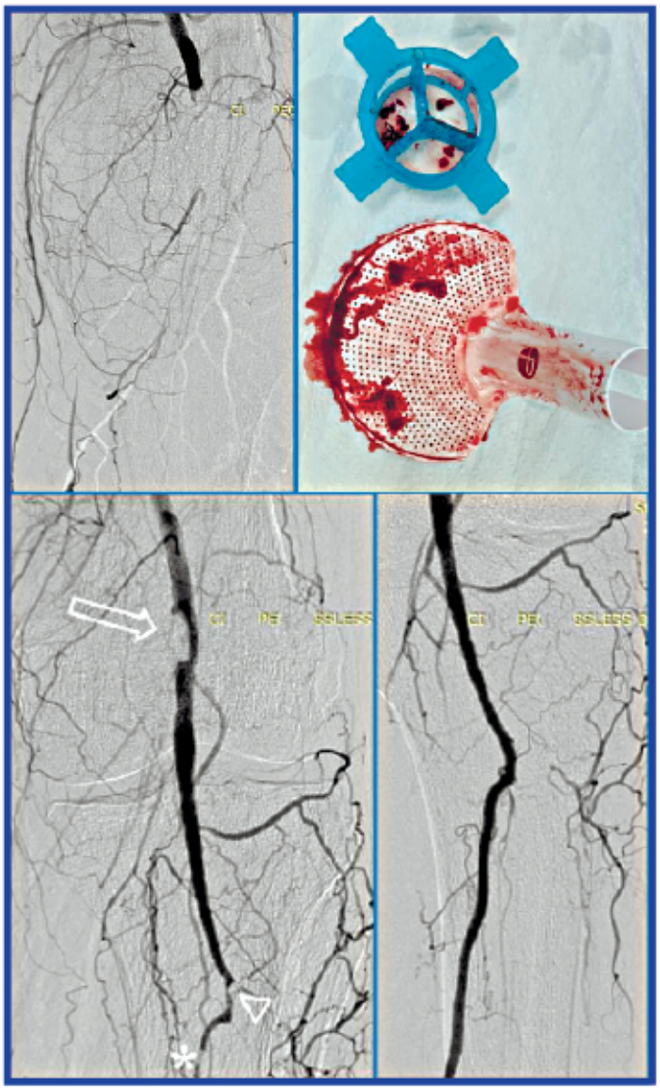

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RESEARCH ARTICLE

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Harmfulness of prooxidants in bronchopulmonary dysplasia in preterm children

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ABSTRACT

Introduction. Oxidative stress can be defined as the imbalance of the redox state of a certain system including living one (organelle, cell, organ/tissue), which excessively produces reactive oxygen and/or reactive nitrogen species (ROS/RNS) that exceed the capacity of the antioxidant defense system, which have the ability to slow down or even prevent the oxidative damage of macromolecules. Oxidative stress is a pathogenic mechanism of a large variety of diseases, including pulmonary one.

Material and methods. 81 preterm born children included in the study were divided into the main group – preterm children with bronchopulmonary dysplasia (BPD), and the control group – preterm children without BPD. The comparison groups were prospectively evaluated clinical, instrumental and laboratory (TPA, prooxidant-antioxidant balance, nitric oxide metabolistes and MDA). Data were statistically analyzed using Microsoft Excel, MedCalc and SPSS and Contingency Table Analysis as a way to evaluate the performance of a diagnostic test.

Results. In preterm children with BPD were found to be decreased by 29% ($p < 0.001$) the prooxidant-antioxidant balance (PAB) and the nitric oxide metabolistes (NO) level by 12% ($p < 0.001$) compared to children in the control group. The assessment of tissue oxidative damage markers revealed a significant 62% ($p < 0.001$) increase in malonic dialdehyde (MDA) content and a 4.86-fold ($p < 0.001$) increase in total prooxidant activity (TPA) in children with bronchopulmonary dysplasia compared to children in the control group. Our study confirms that TPA, PAB, MDA and NO values are reliable markers of hypoxic tissue damage at children with bronchopulmonary dysplasia and can be recommended for assessing the intensity of oxidative stress.

Conclusions. Pulmonary bronchodysplasia is characterized by the imbalance of prooxidant-antioxidant processes with the exacerbation of prooxidant ones that trigger the oxidative/nitrosative stress and the deterioration of vital chemical compounds.

Keywords: bronchopulmonary dysplasia, preterm children, total prooxidant activity, prooxidant-antioxidant balance, malonic dialdehyde, nitric oxide metabolites.

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Key messages

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

Changes in markers of oxidative stress, in particular in preterm children with bronchopulmonary dysplasia have not yet been studied.

The research hypothesis

Preterm infants with pulmonary bronchodysplasia may develop oxidative stress, which may affect their health.

The novelty added by manuscript to the already published scientific literature

The assessment of biochemical markers established a significant increase in oxidative stress in preterm children with bronchopulmonary dysplasia. This was revealed by the prooxidant-antioxidant disbalance and a decrease of the nitric oxide metabolites content, associated with lipid damage as evidenced by the increase in malondialdehyde (MDA) levels.

Introduction

There is increasing evidence linking exposure to increased oxygen concentration and oxidative stress (OS) to the development of chronic bronchopulmonary disease, making the lung of preterm infants more susceptible to various diseases such as respiratory distress syndrome, bronchopulmonary dysplasia (BPD), and persistent pulmonary hypertension. Existing research in the field shows that although BPD is a disease with a multifactorial pathogenesis, the major risk factors are exposure to hyperoxia and the action of reactive oxygen species (ROS) [1, 2].

From conception to birth, mammals (including humans) develop under conditions of physiological hypoxia, with fetal arterial and venous pO_2 values rarely exceeding ~4 kPa (30 mmHg) which constitutes ~4% O_2. Thus, the entire process of organogenesis takes place in hypoxia, despite the fact that fetal hemoglobin has a significantly higher affinity for oxygen compared to that of an adult. The birth process is accompanied by an increase of oxidative stress, and this continues in the first months of life due to exposure to increased concentrations of oxygen provided by breathing. The role of oxidative stress triggered by hyperoxia in development is still unclear, but ROS are known to be involved in signal transduction, they also play an important role in immune function, are important regulators of circulation, activate cellular growth factors, remove dysfunctional proteins by oxidation and are essential for the functioning of cellular organelles [3].

It has been demonstrated that before and at birth in infants there is an increase in signs of oxidative stress, which indirectly reveals the increase in antioxidant capacity with the aim of adapting to the amplification of oxidative processes induced by the spontaneous inhalation of oxygen. At the same time, these adaptive reactions are missing or significantly underdeveloped in premature babies, especially those very premature and those with very low birth weight. Deficiencies of antioxidant protection mechanisms in these children are exacerbated by pulmonary insufficiency, lack of alveolar surfactant, underdevelopment of antioxidant enzymes. Subsequently, premature infants are much more likely to be hyperoxic and will develop oxidative stress following even short-term exposure to high levels of oxygen [4].

As a result of this phenomenon, cellular homeostasis is affected. The balance between the production and excessive accumulation of reactive oxygen species (ROS) in cells and the detoxification capacity due to the lack of endogenous antioxidants is inclined towards the amplification of oxidative processes, which causes tissue damage. Exogenous factors that stimulate ROS production have been shown to have both beneficial and deleterious cellular effects, thus either participating in cellular signaling or causing macromolecular damage [1, 2].

In case of the preterm babies, prolonged exposure to elevated oxygen concentrations can affect and alter the normal development of the lung tissue, triggering developmental disorders such as BPD. Some relevant studies regarding human BPD reported that increased ROS production is associated with impaired lung development [5].

Free radicals have unpaired electrons and are extremely reactive, but at the same time unstable, having low activation energy and short lifetime. It should be noted that they can act as both oxidants and antioxidants (reducers), a phenomenon explained by their ability to donate or accept an electron from other molecules [6].

The generation of ROS occurs in a series of redox reactions, which are the basis of many processes that take place in cells. Under physiological conditions, ROS are produced by the body as part of normal metabolic processes such as the electron transporting chain, Fenton and Haber-Weiss reactions etc. A series of pathological conditions and diseases, including those associated with hypoxia and ischemia/reperfusion, can disrupt the balance between the generation of ROS and the antioxidant system capacity to neutralize them, which causes oxidative stress. The amplification of the levels of intracellular ROS, causes damage of different macromolecules which will alter their function and the cell state [7, 8].

Oxidative stress is often associated with nitrosative stress, due to the interaction of nitric oxide with superoxide radical anion with the formation of peroxynitrite ($ONOO^-$), which, being a highly reactive radical, can cause enzyme inhibition, lipid peroxidation, protein and DNA damage, etc. The multitude of harmful processes initiated by NO metabolites can end with the induction of apoptosis and significant tissue damage [9-11].

Lipid hydroperoxides (LOOH), unsaturated aldehydes (MDA, 4-hydroxy-2-nonenal, 2-propenal or acrolein) and isoprostanes are relatively stable primary products of the lipid peroxidation process [6]. The well-known product of lipid damage produced by oxidative stress, malonic dialdehyde (MDA), is a recognized biomarker of oxidative stress, cell membrane damage, but also tissue and cell oxidative damage [12]. MDA is formed, as a result of the peroxidation process of polyunsaturated FA, either in the presence of a large number of free oxygen radicals from sialic acid and deoxyribose, or from the phospholipid structure of cell membranes [13, 14].

MDA is considered the most mutagenic product, in contrast to 4-hydroxy-2-nonenal, which is considered the most

toxic. There are three mechanisms by which the damaging effect of lipid oxidation products is explained: the damage to the integrity of the cell membrane, the ability to add an additional ROS gene, or the degradation into reactive compounds, which have the potential of damaging DNA, proteins and lipids [6, 7]. The effects of lipid peroxidation in cells are loss of cell membrane properties, inactivation of many membrane receptors, and increased influx of calcium ions. These events alter permeability, membrane electrical potential and intercellular communication [15].

A number of commonly used methods for the assessment of oxidative/nitrosative stress (methods for measuring lipid and protein oxidation end products) are described. Nevertheless, there is an issue that is still addressed – the ability of oxidative/nitrosative stress, measured in plasma, to reflect the tissue processes, along with the need for a simple laboratory method to characterize an oxidative stress „profile" related to growth and maturation in physiological conditions and different diseases [15].

In children with DBP who endure chronic hypoxia due to respiratory impairment, we assume an alteration of these mechanisms. And our study was initiated as a challenge to have answers to these complex questions.

The aim of the research was to evaluate markers of oxidative/nitrosative stress measured in the serum of preterm children with bronchopulmonary dysplasia and to analyze their performance as a diagnostic test.

Material and methods

The research was carried out as part of the doctoral project „Prooxidant and antioxidant status in bronchopulmonary dysplasia in premature children".

To describe the results of the assessment of oxidative stress markers in children with BPD, an analytical analysis based on a cohort study was performed. In this regard, 81 follow-up records of patients admitted to the Institute of Mother and Child (Chisinau, Republic of Moldova) with positive history of preterm births, postnatal oxygen therapy in respiratory distress were documented and analyzed. The patients were examined according to the same protocol, which included the complex examination and contained the information from the outpatient medical record (F112/e), the inpatient medical record (F003/e). Comparison groups were evaluated prospectively, through clinical, laboratory, instrumental examination. The children were divided into main group (children born preterm with BPD) and control group (children born preterm without BPD). Data analysis was performed according to the methodology described in „Basics of Epidemiology and Research Methods" [16].

The biochemical investigations were carried out according to methods adapted by the collaborators of the Biochemistry Laboratory of *Nicolae Testemițanu* State University of Medicine and Pharmacy for the Synergy H1 (Hydrid Reader) microplate spectrofluorometer (BioTek Instruments, USA) and the Power Wave HT spectrophotometer (BioTek Instruments, USA).

For the analysis of markers of interest, venous blood samples (5 mL) were collected, which were centrifuged for 10 minutes at 3000 revolutions/minute. The serum was separated and transferred to Eppendorf tubes and stored at -45°C separately until biochemical testing. All samples were coded.

The prooxidant-antioxidant balance (PAB) was performed by the method described by Toloue Pouya V. *et al.* [17], modified by Pantea V. *et al.* [18]. The method is based on the capacity of the free radicals, peroxides and antioxidants, contained in the blood sample, to interact with the TMB (3,3′,5,5′-tetramethylbenzidine) or TMB cation, that will determine changes of the solution color. PAB values were calculated according to the calibration curve data and expressed in arbitrary units.

Determination of oxidative stress marker – malonic dialdehyde and total prooxidant activity, was performed according to the procedure described by Galaktionova LP. *et al.* [19], and modified by Gudumac V. *et al.* [20]. The method is based on the spectrophotometric identification of the colored trimethine complex, resulting from the interaction of thiobarbituric acid with DAM. The concentration of DAM in the sample is directly proportional to the intensity of the staining. The final result was expressed in μM/L.

Determination of nitrosative stress marker – nitric oxide metabolites, was performed according to the procedure described by Metelskaya VA. *et al.* [21], modified by Gudumac V. *et al.* [22]. The principle of the method consists in the deproteinization of the biological material, the reduction of nitrates into nitrites, the processing of the supernatant with the Griss reagent, and the subsequent measurement of the optical density of the reaction product. The calculation of the nitrite concentration was carried out with the help of the calibration curve, built on the basis of successive dilutions of the standard solution of sodium nitrite and was expressed in μmol/L.

The data were statistically processed by operating electronic computerized assessment techniques of the degree of relationship between the evaluated parameters of the patients in the study groups, using Microsoft Excel, MedCalc (DeLong *et al.*, 1988) and SPSS and Contingency Table Analysis as a way to summarize the performance of a diagnostic test [23-25].

Results

An oxidant is any compound that can accept electrons, including oxygen. On the other hand, a substance that donates electrons, is a reducing agent. The redox reactions are essential to the many processes that take place in cells. For specific biological systems, the terms prooxidant and antioxidant are equivalent in chemistry to the terms oxidant and reductant [15]. Many radicals are unstable and highly reactive. Behaving as oxidants or reductants, they have the ability to give an electron or accept an electron from other molecules, and homeostasis between them is important for optimal functioning of the system [14].

The evaluation of the redox status in the blood of the preterm children with and without BPD revealed the prevalence of oxidative processes in the children of the main group compared to the children of the control group (table 1).

The values of total prooxidant activity (TPA) in children with BPD (41 children) was equal to 138.2±6.1 μM/L, value significantly increased compared to TPA in children without BPD (40 children) equal to 28.4±2.3 μM/L, Fstat = 14.5, p < 0.0001 (table 1, fig. 2).

A 4.86-fold increase (p < 0.001) of the total prooxidant activity was identified in preterm children with BPD compared to those without lung damage, a phenomenon that reveals the intensification of the production and accumulation of prooxidants of different nature, which can amplify the oxidation processes up to the level of oxidative/nitrosative stress (table 1, fig. 2).

Table 1. Values of markers of oxidative stress in children with bronchopulmonary dysplasia

Marker	Control group (n = 40)		Main group (n = 41)		The veracity of differences between groups
TPA (μM/L)	28.4±14.3	100%	138.2±38.9	486%	p < 0.001
PAB (U)	140.3±15.2	100%	99.6±15.8	71%	p < 0.001
MDA (μM/L)	20.4±8.2	100%	33.0±8.9	162%	p < 0.001
NO metabolites (μM/L)	64.9±3.8	100%	57.1±4.8	88%	p < 0.001

Note: TPA – total prooxidant activity; PAB – prooxidant/antioxidant balance; MDA – malonic dialdehyde; NO – nitric oxide. Variables are presented as Mean±SD.

PAB in children with BPD (41 children) is equal to 99.6±2.5 μM/L with minimum value of 65.8 μM/L, median – 101.8 μM/L, maximum – 140.3 μM/L, mode – 76.4 μM/L, compared to PAB concentration in children without BPD (40 children) which have a significant difference between groups equal to 140.3±2.4 μM/L (minimum value of 105.9 μM/L, median – 140.7 μM/L, maximum – 176.8 μM/L, mode – 131.7 μM/L), Fstat = 11.6, p < 0.00001 (table 1, fig. 2).

A major, statistically significant decrease of PAB by 29% (p < 0.001) was identified in preterm children from the group with bronchopulmonary dysplasia compared to children from the control group, which attests to the decrease in the total level of antioxidants with the inclination of the balance towards the formation and accumulation of prooxidants and the definite establishment of the prooxidant status (fig. 1).

Malonic dialdehyde (MDA), in children with BPD (41 children) was 33.1±1.39 μM/L with minimum value of 18.3 μM/L, median – 31.8 μM/L, maximum – 55.4 μM/L, mode – 25.4 μM/L, compared to the MDA level in children without DBP (40 children) which was 20.4±1.3 μM/L (minimum value of 12.9 μM/L, median – 19.1 μM/L, maximum – 66.8 μM/L, mode – 17.3 μM/L), and presents a significant difference between batches (Fstat = 6.5, p < 0.0001) (table 1, fig. 2).

The installation of the prooxidant status in preterm children from the group with bronchopulmonary dysplasia, initiated the atypical oxidation of lipids via the peroxidative pathway, which was manifested by a significant increase of 62% (p < 0.001) in the content of MDA, the final product of the peroxidation of unsaturated fatty acids, mainly from cell membranes phospholipids (fig. 2).

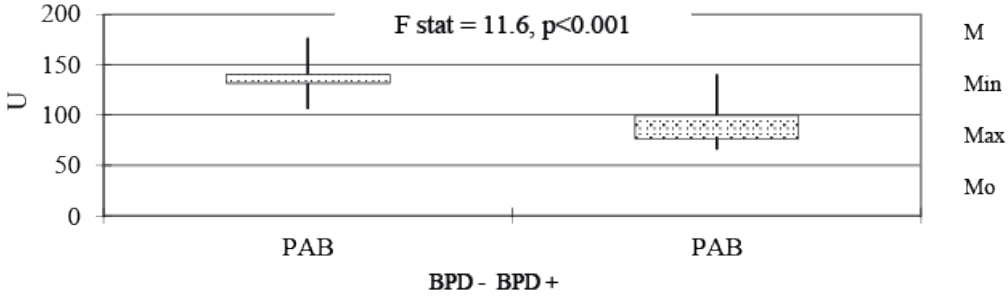

Fig. 1 Values of PAB in children with bronchopulmonary dysplasia, U.

Note: PAB – prooxidant/antioxidant balance; M - mean; Min – Minimum; Max – Maximum; Mo – mod; F stat – F Statistic (Fisher-Snedecor distribution) useful in analysis of variance (ANOVA).

Nitric oxide (NO) in children with BPD (41 children) is equal to 57.13±0.75 μM/L with minimum value of 49.6 μM/L, median – 57.4 μM/L, maximum – 66.7 μM/L, mode – 59.7 μM/L, compared to the concentration of nitric oxide in children without BPD (40 children) which has a significant difference between groups equal to 64.9±0.6 μM/L (minimum values of 55.8 μM/L, median – 65.1 μM/L, maximum – 74.4 μM/L, mode – 59.7 μM/L), Fstat = 7.9, p < 0.00001 (table 1, fig. 2).

A statistically significant decrease in the level of NO metabolites by 12% (p < 0.001) was revealed in premature infants from the group with bronchopulmonary dysplasia compared with children in the control group, which may indicate the use of NO in reactions that cause reactive forms of nitrogen (peroxynitrite, protonated peroxynitrite), thereby contributing to the induction of nitrosative stress and the deepening of the redox imbalance (fig. 2).

We can conclude that preterm children with BPD are characterized by the amplification of ROS/NRS production reactions, the establishment of a prooxidant status and the triggering of OS/NS, which ultimately causes damage to biomol-

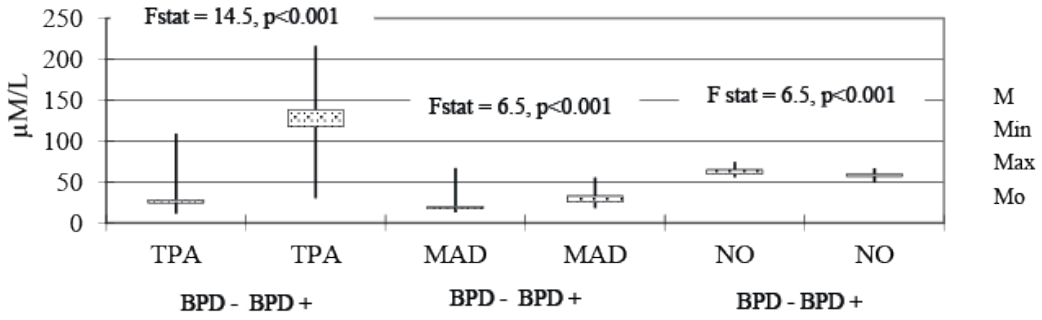

Fig. 2 Oxidative stress system in children with bronchopulmonary dysplasia, µM/L.

Note: TPA – total prooxidant activity; MDA – malonic dialdehyde; NO – nitric oxide; M - mean; Min – Minimum; Max – Maximum; Mo – mod; F stat – F Statistic (Fisher-Snedecor distribution) useful in analysis of variance (ANOVA).

ecules and cellular macromolecular structures (membranes).

Next are presented the derivations of multiple measures using the four outcomes of the 2×2 contingency table for the prooxidant system to assess utility as diagnostic tests in children with BPD.

The analysis using ROC curves (*Receiver Operating Characteristics*) was chosen as a statistical model. These are two-dimensional curves of the values of a diagnostic test that ends with the evaluation of this examination applied to each patient or their comparison [23].

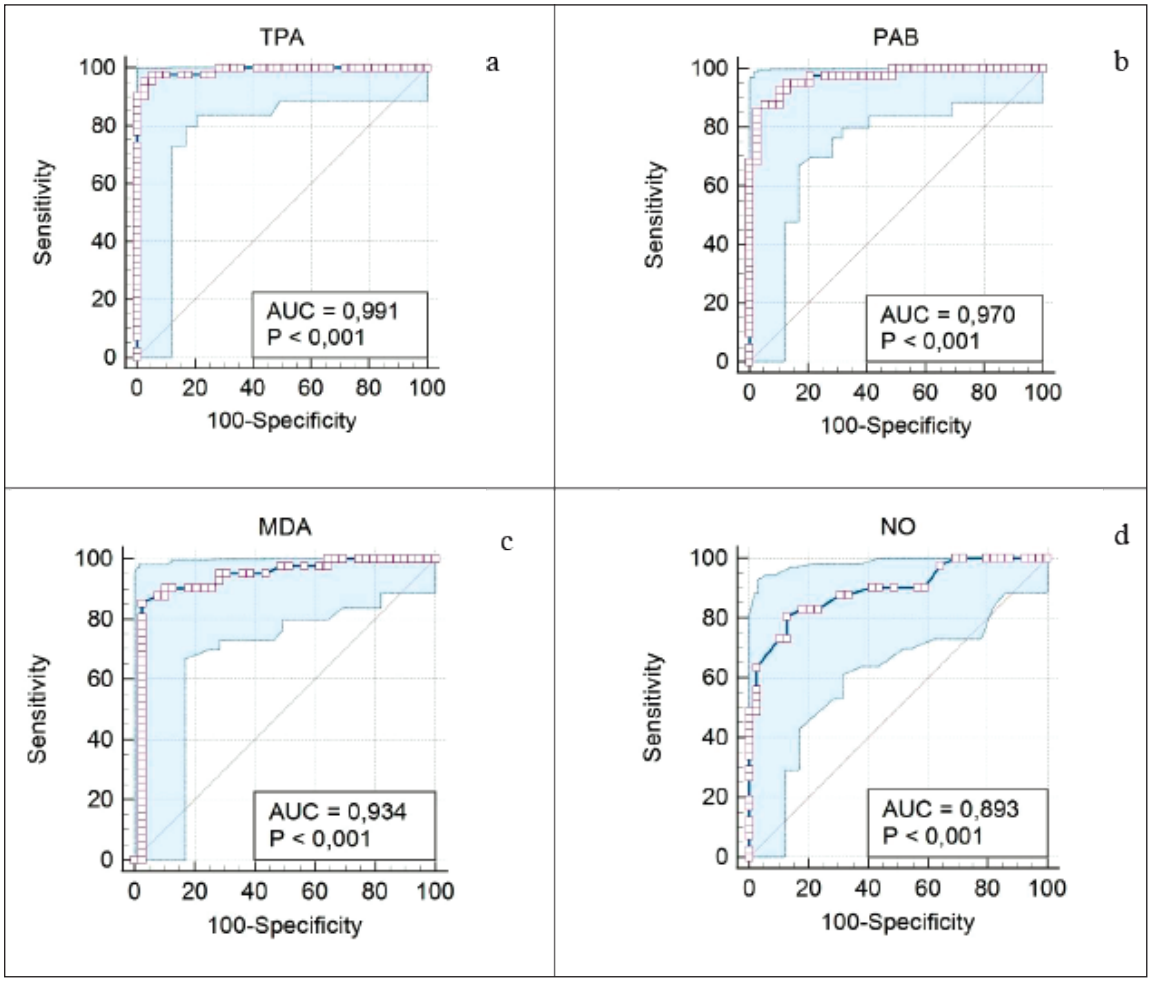

Fig. 3 ROC curve of the intensity of oxidative stress as a function of TPA, PAB, MDA, NO in children with bronchopulmonary dysplasia

Note: a. TPA – total prooxidant activity; b. PAB – prooxidant/antioxidant balance; c. MDA – malonic dialdehyde; d. NO – nitric oxide; ROC - Receiver Operating Characteristics; AUC - Area Under the Curve.

The assessment of the usefulness of determining the TPA, for highlighting children at risk of developing oxidative stress, was carried out by means of the ROC curve, which is an excellent way to compare diagnostic tests (fig. 3).

When the variable under study cannot distinguish between the two groups, i.e., where there is no difference between the two distributions, the area will be equal to 0.5 (the ROC curve will coincide with the diagonal). When there is a perfect separation of the values of the two groups, i.e., there is no overlapping of the distributions, the area under the ROC curve equals 1 (the ROC curve will reach the upper left corner of the plot) (fig. 3). A reflection of the identification of prooxidant processes depending on TPA can be the area located below the level of the ROC curve equal to 0.99 (AUC is equal to 0.991: 95%CI 0.98-1, p = 0.000) (table 2). So, our study confirms the importance of evaluating prooxidant process according to TPA concentration, which has been shown to be a useful test.

Area under the curve of the ROC curve for PAB values was obtained equal to 0.97: 95% CI 0.905-0.999, p = 0.000 (table 2).

Table 2. Area under the curve (AUC) for the values of markers of oxidative stress in children with bronchopulmonary dysplasia.

	AUC	Standard Error	p	95% Confidence Interval	
				Lower Bound	Upper Bound
TPA (μM/L)	0.991	0.008	0.000	0.976	1.000
PAB (U)	0.97	0.016	0.000	0.905	0.995
MDA (μM/L)	0.934	0.032	0.000	0.871	0.997
NO (μM/L)	0.893	0.035	0.000	0.804	0.951

Note: TPA – total prooxidant activity; PAB – prooxidant/antioxidant balance; MDA – malonic dialdehyde; NO – nitric oxide; AUC - Area Under the Curve; p – signification.

In the case of the malondialdehyde test, the minimum sensitivity of the test was of 2.4%: 95%CI, 0.01-0.12 for MDA concentration less than 20 μM/L (characteristic only of a child with DBP). The specificity was also minimal with the highest value reaching only 4.9%: 95%CI, 0.05-0.6, which confirms levels higher than 20 μM/L of MDA in only 20 children from the control group (without DBP), $\chi 2$ = 24.6, p < 0.0001 (fig. 3).

Area under the ROC curve equal to 0.934: 95%CI 0.87-0.99, p = 0.000 values for MDA content (μM/L) in children with BPD (fig. 3, table 2).

And the last demonstration concerns the area under the curve of NO – nitric oxide which was obtained equal to 0.893: 95%CI 0.804-0.951, p = 0.000 (table 2).

Discussions

According to literature data, multiple harmful gestational factors, which can affect the growth and development of the product of conception during the entire intrauterine period, are reported.

During postnatal development, the preterm born child is even more influenced by the convergence of the multitude of endogenous and exogenous factors that have damaged the health status. A dominant focus of the modern experimental studies demonstrates the harmfulness of oxidative stress, as the ultimate goal through the generation of free radicals (FR) and as a result the occurrence of cellular, tissue and organic damage [26, 27].

Our study, based on a prospective evaluation of two groups of children: the main group - preterm infants with BPD and the control group - preterm infants who did not develop BPD, examined clinically, paraclinical and instrumentally, contributes to the study of changes in oxidative stress markers in preterm infants who have developed bronchopulmonary dysplasia, the analysis of these data using various contingency tables and their generalization by performing a diagnostic test.

In multiple series of papers, and a huge variety of laboratory methods and statistical models have been developed and used to measure oxidative stress intensity and its consequences. Researchers such as Ferguson K, Gunko V O, Abiaka C, Machado L in their studies, determine biomarkers using various methods [28-30]. At the same time, all these methods are considered to be quite difficult, since oxidative stress biomarkers are very reactive and have very short half-lives.

Thus, the conducted study is within the limits of the research level, which is of scientific value in terms of the assessed markers of oxidative stress. These markers evaluated in premature children with bronchopulmonary dysplasia compared to those without this lung damage revealed the presence of significant oxidative stress in those enrolled in the main group. Total prooxidant activity increased by 386% (p < 0.001), the balance between prooxidants and antioxidants shifted towards the former (PAB - 29%, p < 0.001) and at the same time the NO content significantly decreased (-12%, p < 0.001). Our research also found lipid damage with the accumulation of the end product of lipid peroxidation – DAM (+62%, p < 0.001).

However, the role of oxidative stress in neonatal lung injury is much more complex and not fully studied. Despite the multiple existing data and different research environments under development, clinical practice is limited in the ability to detect very early preterm newborns who have susceptibility to develop a lung pathology, therefore the markers used currently cannot fully predict the lung damage that may follow in these children. But the detection and monitoring of lung lesions related to oxidative stress, namely through the use of non-invasive methods of detecting different oxidation products, remains to have a predictive and very useful role in the clinical setting.

While a growing body of evidence supports the role of oxidative stress, it appears that the complexity of this multifactorial condition cannot be captured by a single marker. Instead, researchers should move on to develop and validate specific panels of biomarkers that can more reliably predict certain pathological states that evolve with impaired lung function. Early diagnosis and treatment of oxidative stress-related lung diseases may be essential to prevent ad-

verse effects that may spread beyond the neonatal period. Indeed, few of the biomarkers developed to date have been qualified for neonatal lung disease and their analysis has been limited by research settings.

Our study confirms that TPA, PAB, MDA and NO values are reliable markers of hypoxic tissue damage in children with bronchopulmonary dysplasia and can be recommended for assessing the intensity of oxidative stress. Last but not least, the currently available evidence, including our results highlights the need for further studies on a larger scale and with longer follow-up periods to obtain more precise results and allow serial detection of oxidative stress biomarkers.

Conclusions

Oxidative stress is a major contributor to lung injury in preterm children with bronchopulmonary dysplasia, fact confirmed by significantly higher values of total prooxidant activity (4.86 times, $p < 0.001$) and MDA (by 62%, $p < 0.001$) along with concomitant decrease of the prooxidnat/antioxidant and NO metabolites levels in the blood of these children. The phenomenon reveals an increase in the production and accumulation of prooxidants of various nature, which enhances oxidation processes and causes damage to biomolecules and cellular macromolecular structures, membranes in particular.

Thus, our study confirms the importance of evaluating pro-oxidant processes according to the concentrations of TPA, PAB, MDA, NO, which have been demonstrated as useful tests.

Competing interests

None declared.

Patient consent

Obtained.

Authors' contribution

Authors contributed equally to the literature searching, conceptual highlighting of the material as well as writing of the manuscript. The authors read and approved the final version of the manuscript.

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RESEARCH ARTICLE

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Predicting sympathovagal balance using parameters of breathing patterns in abdominal breathing

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ABSTRACT

Introduction. Abdominal breathing is utilized as a non-pharmacological treatment method for various stress-related conditions and autonomic dysfunctions. The objective of the study was to determine the predictors in the modulation of sympathovagal balance, as indicated by the ratio of low frequency to high frequency power of heart rate variability, by utilizing the respiratory pattern parameters recorded during the abdominal breathing model.

Material and methods. The study involved a group of 101 healthy subjects, where the breathing pattern was recorded using a respiratory induction plethysmograph. Heart activity was estimated through electrocardiography, followed by heart rate variability analysis during both resting and abdominal breathing. Eight parameters of the breathing pattern were recorded in the subjects during resting breathing and abdominal breathing, presumed to be predictors of the ratio of low frequency to high frequency power of heart rate variability. Separate predictive models were created for this ratio for both the resting and abdominal breathing types.

Results. The multilinear regression analysis revealed that the primary predictor with the highest predictive power for determining the balance between sympathetic and parasympathetic cardiac influence, as indicated by the low frequency spectral power to high frequency spectral power ratio, in individuals practicing abdominal breathing is Tidal Volume (unstandardized coefficient = 5.007). This was followed by the duration of expiration (coefficient = -3.831) and respiratory minute-volume (coefficient = 4.415), both of which were recorded during resting breathing. In the abdominal breathing model, the most effective predictors were found to be time-related parameters, specifically the frequency of breathing during abdominal breathing (coefficient = -5.953), the duration of the inspiratory phase (coefficient = -4.037), and the duration of the expiration phase (coefficient = -4.194).

Conclusions. Abdominal breathing has the potential to normalize sympathovagal balance by adjusting the duration of inspiration or expiration. Further studies should be conducted to investigate the practical application of breathing pattern parameters in restoring the low frequency to high frequency (LF/HF) ratio, particularly in disorders characterized by elevated sympathovagal balance.

Keywords: abdominal breathing, LF/HF ratio, predictors, breathing pattern.

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Key messages

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

The respiratory parameters involved in the change of sympathovagal balance when resting breathing changes to abdominal breathing. Is this change benefic or no?

The research hypothesis

Parameters of breathing pattern in resting breathing can be predictors of sympathovagal balance in abdominal breathing.

The novelty added by manuscript to the already published scientific literature
Abdominal breathing can normalize sympathovagal balance by modulating the duration of inspiration or expiration.

Introduction

Currently, there is a focus on psychophysiological research in the field of breathing, aiming to understand how various controlled respiratory patterns influence heart rate variability (HRV) [1]. Abdominal (diaphragmatic) breathing, an essential component of protocols that enhance the amplitude of Respiratory Sinus Arrhythmia (RSA), forms the basis of treatment methods for a range of stress-related conditions and autonomic dysfunctions [2, 3].

Respiratory Sinus Arrhythmia (RSA) is characterized by rhythmic fluctuations in heart rate (HR) throughout the respiratory cycle. HR increases during inspiration and decreases during expiration. RSA, as a component of Heart Rate Variability (HRV), is regarded as an indicator of autonomic homeostasis and adaptability [4]. However, despite numerous studies on this subject, much remains unknown regarding the relationship between specific respiratory strategies and RSA [5].

HRV measurements encompass both time and frequency domain variables. Frequency domain HRV metrics include low frequency power (LF), high frequency power (HF), normalized low frequency power (LFn), normalized high frequency power (HFn), and the LF/HF ratio. In healthy adults, the typical resting breathing rate ranges from 9 to 24 breaths per minute [3]. Respiratory sinus arrhythmia (RSA), which is modulated by the parasympathetic nervous system (PNS), occurs within the high-frequency range of 0.15-0.4 Hz [6, 7, 8]. LF serves as a marker of the cardiac sympathetic nervous system (SNS) [8, 9]; however, some studies have not been able to confirm this association [10, 11]. Several studies have suggested that LF is likely influenced by both the SNS and PNS, as well as baroreflex modulation of autonomic flows [11-14].

Previously, the LF/HF ratio was considered an indicator of cardiac autonomic balance, where an increase in the ratio indicated SNS dominance, and a decrease indicated PNS dominance [8]. However, recent studies have demonstrated that the LF/HF ratio may not necessarily reflect SNS or PNS influence [6, 13, 15]. The LF/HF ratio is influenced by various factors, including vagal activity, SNS activity, and respiratory parameters [13, 14], and its interpretation should take into account the individual variations of LF and HF components of heart rate variability [13].

The objective of the study was to identify predictors associated with the modulation of sympathovagal balance, as expressed by the LF/HF index, utilizing respiratory pattern parameters recorded during abdominal breathing.

Material and methods

The study was conducted on a group of 101 subjects from March 2017 to February 2019 at the Department of Human Physiology and Biophysics, *Nicolae Testemitanu* State University of Medicine and Pharmacy. The average age of the individuals included in the study was 33.5 years (ranging from 19 to 60 years old). Subjects with pulmonary and cardiac pathologies were excluded.

All participants signed an informed agreement to be included in this study, which was approved by the Ethical Committee of *Nicolae Testemițanu* State University of Medicine and Pharmacy, with minutes no. 15 dated 11.01.2016.

The recording of breathing patterns was performed using a respiratory induction plethysmograph (RIP) VISURE-SP (RBI instruments, France) to measure movements of the abdomen and thorax [16]. Additionally, the capnograph CapnoStreamTM 20 (Medtronic, USA) was used to record the partial pressure of CO2 in the expired air at the end of expiration (etCO2). The respiratory parameters measured included tidal volumes (Vt), the duration of the respiratory cycle (Tt), respiratory frequency (FR), inspiratory time (Ti), expiratory time (Te), average inspiratory flow (Vt/Ti), respiratory minute volume (MVR), and etCO2. The recording of ECG signals was performed using the computer system Biopac MP-100. The data processing was conducted using the software Kubios HRV Standard (version 3.2.0, 2019), with manual removal of artifacts. The spectral analysis of the RR interval variation involved calculating the power of the components: LF (low frequency power, in ms^2) in the 0.04-0.15 Hz range, and HF (high frequency power, in ms^2) in the > 0.15 Hz range.

The experimental protocol included recording the respiratory signals and ECG in a supine position. During the recording, the subjects were asked to breathe quietly, not talk, and avoid additional movements.

1. Resting period (RR) - for 5 minutes in physical, mental, and emotional rest periods (the first minute was excluded from calculations to exclude artifacts obtained from the application and accommodation movements of subjects in the device's jacket).
2. Abdominal respiration (AR) - the subjects used abdominal (diaphragmatic) breathing. To perform this type of breathing, the movement of the rib cage was restricted using a chest corset.

The statistical analysis included descriptive statistics, multivariate statistics (ANOVA), and regression analysis. The analysis was performed using IBM SPSS Statistics version 22.0 software (Statistical Package for the Social Sciences 22.0, IBM Corp., Armonk, NY, USA).

Results

Our study utilized seven parameters of the breathing pattern as presumed predictors of the LF/HF ratio. These parameters were recorded during resting breathing and abdominal

breathing in the subjects. We developed predictive models for each type of breathing, incorporating these parameters.

Resting respiration. The descriptive analysis of the research group, subjected to statistical analysis (Table 1), revealed the following findings:

- Tidal volume: The tidal volume ranged from 0.27 l to 0.66 l, with an average of 0.466 l. The standard deviation was 0.1012;
- Inspiratory time at rest: The inspiratory time varied between 1.15 s and 2.41 s, with an average of approximately 1.64 s. The standard deviation was 0.3555;
- Duration of free expiration: The duration of free expiration ranged from 1.14 s to 4.64 s. The average duration was 4.64 s, with a standard deviation of 0.8714;
- Total duration of respiratory cycle: The total duration of the respiratory cycle ranged from 2.32 s to 7.05 s. The mean duration was 4.06 s, with a standard deviation of 1.17 s;
- Vt/Ti ratio: The Vt/Ti ratio varied between 0.20 l/s and 0.39 l/s. The mean value was 0.287, with a standard deviation of 0.055;
- Respiratory volume per minute: At rest, the respiratory volume per minute oscillated between 4.49 l and 10.16 l, with a respiratory rate ranging from 8.50 c/min to 24.53 c/min. The average minute respiratory volume was 7,094 l/min, with a standard deviation of 1,591 l;
- Respiratory rate: The average respiratory rate at rest was 15.9 c/min, with a standard deviation of 4.2;
- LF/HF index: At rest, the LF/HF index ranged from 0.18 to 5.80. However, the average LF/HF index was 1.066, with a standard deviation of 1.4459.

Table 1. Descriptive statistics of researched group in resting period.

	N	Minimum	Maximum	Mean	Std. deviation
Vt	15	.27	.66	.4667	.10123
Ti	15	1.15	2.41	1.6383	.35553
Te	15	1.14	4.64	2.4211	.87141
Tt	15	2.32	7.05	4.0593	1.17248
Vt/Ti	15	.20	.39	.2872	.05579
MVR	15	4.49	10.16	7.0935	1.59086
FR	15	8.50	24.53	15.9162	4.20697
CC	15	.70	1.15	.8827	.14144
LF/HF	15	.18	5.80	1.0662	1.44592

Note: Vt – tidal volume; Ti – duration of inspiration; Te – duration of expiration; Tt – duration of respiratory cycle; Ti/Tt – ratio of inspiration in respiratory cycle; Vt/Ti – inspiratory flow; MVR – respiratory minute volume; FR – breathing rate; CC – duration of cardiac cycle; LF/HF – ratio of low frequency power to high frequency power of HRV.

The possible complex interactions between the measured factors argued for the need for multivariate analysis. Consequently, a model (RR model) was developed with the objective of predicting the balance between sympathetic and parasympathetic activity based on the LF/HF ratio. The model incorporated the standardized values of tidal volume, total respiratory cycle time, respiratory frequency, and minute respiratory volume as predictors (Table 2).

Table 2. Model summary for RR model.

Model	R	R squared	Adjusted R squared	Std. error of the estimate
	.880	.75	.684	.56170691

Predictors: (Constant), Zscore (Tt), Zscore (Vt), Zscore (FR), Zscore (MVR)

Dependent variable: Zscore (LF/HF)

Note: Zscore (LF/HF) – standardized score of the ratio of low frequency power to high frequency power of HRV; Zscore (Tt) – standardized score of the duration of respiratory cycle; Zscore (Vt) – standardized score of the tidal volume; Zscore (FR) – standardized score of the breathing rate; Zscore (MVR) – standardized score of respiratory minute volume.

The multivariate analysis conducted on the resting values was able to explain 68.4% of the changes in LF/HF balance. The coefficient of determination (Adjusted R Square) was 0.684, indicating that the proposed model accounted for a significant portion of the variance in the LF/HF variable for resting breathing. The sum of squares was 10,845 out of a possible 14,000, further supporting the model's ability to explain more than two-thirds of the variance. The null hypothesis, which states that no parameter included in the model can predict the LF/HF value for resting breaths better than an arbitrary model, was rejected. This rejection was based on the statistical test result ($F = 8.593$, $p = 0.003$) as shown in Table 3.

Table 3. ANOVA test in RR model.

Model	Sum of squares	df	Mean square	F	Sig.
Regression	10.845	4	2.711	8.593	.003
Residual	3.155	10	.316		
Total	14.000	14			

Dependent variable: Zscore (LF/HF)

Predictors: (Constant), Zscore (Tt), Zscore (Vt), Zscore (FR), Zscore (MVR)

Note: df – degrees of freedom; F – Fisher's coefficient; Zscore (LF/HF) – standardized score of the ratio of low frequency power to high frequency power of HRV; Zscore (Tt) – standardized score of the duration of respiratory cycle; Zscore (Vt) – standardized score of the tidal volume; Zscore (FR) – standardized score of the breathing rate; Zscore (MVR) – standardized score of the respiratory minute volume.

When developing the model, the Backward method was used. Initially, all potential variables were included in the model, and then insignificant parameters were systematically excluded until only the optimal combination of variables remained to form the regression equation and predict the studied outcome. The resulting model, presented in Table 4, included the constant ($B = 3.310E-15$, $p = 1.000$) and the standardized values of MVR ($B = 1.731$, $p = 0.040$), FR ($B = 1.379$, $p = 0.049$), Vt ($B = -1.622$, $p = 0.062$), and Tt ($B = 3.580$, $p < 0.001$). The final model requires attention and possible improvements because it did not include the constant, which is very close to 0. Additionally, the standardized value of Vt was found to be insignificant in this case, as its confidence interval included the value of 0. Therefore, further refinement of the model is necessary.

Based on the model, it was determined that the resting LF/HF value can be predicted using the following equation: LF/HF in resting breathing = Zscore (MVR) × 1.731 + Zscore (FR) × 1.379 – Zscore (Vt) × 1.622 + Zscore (Tt) × 3.580.

Table 4. Coefficients of predictors in RR model.

Model	Unstandardized coefficients		Standardized coefficients	t	Sig.	95.0% confidence interval for B	
	B	Std. error	Beta			Lower bound	Upper bound
(Constant)	3.310E-15	.145		.000	1.000	-.323	.323
Zscore (MVR)	1.731	.733	1.731	2.363	.040	.099	3.363
Zscore (FR)	1.379	.614	1.379	2.246	.049	.011	2.747
Zscore (Vt)	-1.622	.771	-1.622	-2.105	.062	-3.340	.095
Zscore (Tt)	3.580	.703	3.580	5.090	.000	2.012	5.147
Dependent variable: Zscore (LF/HF)							

Note: Zscore (Tt) – standardized score of the duration of respiratory cycle; Zscore (Vt) – standardized score of the tidal volume; Zscore (FR) – standardized score of the breathing rate; Zscore (MVR) – standardized score of the respiratory minute volume; Zscore (LF/HF) – standardized score of the ratio of low frequency power to high frequency power of HRV.

The necessary conditions for linear regression residuals were met by the developed model. The analysis demonstrated an almost normal distribution of residuals and a lack of associations between predictive standardized values and standardized residuals (Fig. 1). Taken together, these findings allow us to consider the model suitable.

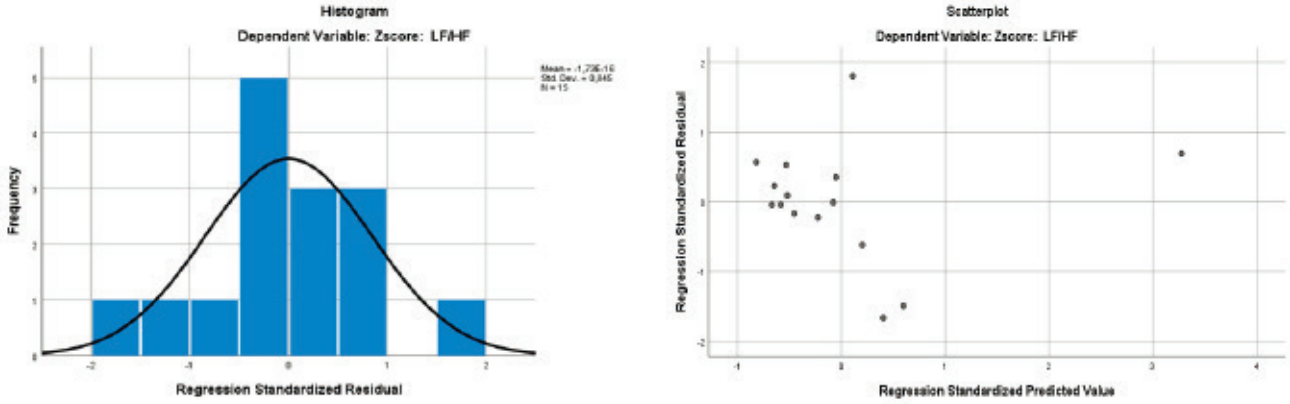

Fig. 1 Histogram (left) and scatterplot (right) of standardized residuals in RR model.
The histogram displays the frequency distribution of residuals. The scatterplot of standardized residuals against standardized predicted values indicates the absence of collinearity.

***Abdominal respiration*.** The effects of physiological parameters recorded during the functional abdominal breathing test were considered as predictors of the LF/HF ratio. To investigate these relationships, an additional model was developed, incorporating the values obtained during resting breathing as well as the newly recorded parameters during abdominal breathing.

The current volume observed in individuals practicing abdominal breathing ranged from 0.37 to 0.64 liters, with an average of 0.496 liters and a standard deviation of 0.085. The duration of inspiration varied from 1.16 to 2.28 seconds, with a mean of 1.68 seconds and a standard deviation of 0.337 seconds. Expiration, on the other hand, had a longer duration than inspiration, ranging from 1.67 to 3.27 seconds. The mean duration of expiration was 2.58 seconds with a standard deviation of 0.43 seconds. The total time of a respiratory cycle ranged from 3.09 to 5.31 seconds, with an average of 4.26 seconds and a standard deviation of 0.67 seconds.

The minute ventilation rate (MVR) measured in the study participants varied between 5.1 and 9.93 liters per minute, with an average of 7.1 liters per minute and a standard deviation of 1.4 liters per minute. Respiratory frequency among patients practicing abdominal breathing ranged from 11.3 to 19.4 breaths per minute, with an average of 14.42 breaths per minute and a standard deviation of 2.34 breaths per minute.

The dependent variable in the current study exhibited an equal ratio ranging from 0.11 to 1.13, with a mean of 0.41 and a standard deviation of 0.24.

The current predictive model aimed to investigate the impact of the measured parameters on the balance between sympathetic and parasympathetic activity, as assessed by the LF/HF ratio, in individuals practicing abdominal breathing. This investigation was conducted using multivariate analysis. The predictive potential of standardized scores for tidal volume, inspiratory and expiratory time, total duration of the respiratory cycle, minute respiratory volume, respiratory rate, and heart rate was evaluated. These measurements were taken at rest and during abdominal breathing (Table 6).

The coefficient of determination (Adjusted R-squared) is 0.61, indicating that the developed model explains more than three-fourths of the variance in the variable of interest,

which is the balance between sympathetic and parasympathetic activity assessed based on the LF/HF ratio in abdominal breathers. The sum of squares was 12.052 out of a possible 14. The null hypothesis, which states that none of the parameters included in the model can predict the balance between sympathetic and parasympathetic activity assessed based on the LF/HF ratio in people with abdominal breathing, was not rejected (F = 3.437, p = 0.094). The Fisher test was statistically insignificant.

Table 5. Descriptive statistics of researched group in resting period and abdominal breathing.

N		Minimum	Maximum	Mean	Std. deviation
VtB	15	.27	.66	.4667	.10123
TiB	15	1.15	2.41	1.6383	.35553
TeB	15	1.14	4.64	2.4211	.87141
TtB	15	2.32	7.05	4.0593	1.17248
MVRB	15	4.49	10.16	7.0935	1.59086
FRB	15	8.50	24.53	15.9162	4.20697
CCB	15	.70	1.15	.8827	.14144
LF/HFB	15	.18	5.80	1.0662	1.44592
Vt	15	.37	.64	.4959	.08540
Ti	15	1.16	2.28	1.6800	.33696
Te	15	1.67	3.27	2.5829	.43274
Tt	15	3.09	5.31	4.2622	.67374
MVR	15	5.10	9.93	7.1024	1.47426
FR	15	11.30	19.40	14.4200	2.33703
CC	15	.72	1.10	.8640	.11783
LF/HF	15	.11	1.13	.4184	.24757

Note: VtB – tidal volume; TiB – duration of inspiration; TeB – duration of expiration; TtB – duration of the respiratory cycle; MVRB – respiratory minute volume; FRB – breathing rate; CCB – duration of the cardiac cycle; LF/HFB – ratio of low frequency power to high frequency, all recorded in breathing at rest. Vt – tidal volume; Ti – duration of inspiration; Te – duration of expiration; Tt – duration of respiratory cycle; MVR – respiratory minute volume; FR – breathing rate; CC – duration of cardiac cycle; LF/HF – ratio of low frequency power to high frequency, all recorded in abdominal respiration.

Table 6. Model summary for AR model.

Model	R	R squared	Adjusted R squared	Std. error of the estimate
	0.928	0.861	0.610	0.62418749

Predictors: (Constant), Zscore (CC), Zscore (LF/HFB), Zscore (MVRB), Zscore (Te), Zscore (Ti), Zscore (TeB), Zscore (VtB), Zscore (FR), Zscore (Vt)

Dependent variable: Zscore (LF/HF)

Note: Zscore (CC) – standardized score of the duration of cardiac cycle; Zscore (LF/HFB) – standardized score of the ratio of low frequency power to high frequency; Zscore (MVRB) – standardized score of the respiratory minute volume; Zscore (Te) – standardized score of the duration of expiration; Zscore (Ti) – standardized score of the duration of inspiration; Zscore (TeB) – standardized score of the duration of expiration; Zscore (VtB) – standardized score of the tidal volume; Zscore (FR) – standardized score of the breathing rate; Zscore (Vt) – standardized score of the tidal volume.

Table 7. ANOVA test in AR model.

Model	Sum of squares	df	Mean square	F	Sig.
Regression	12.052	9	1.339	3.437	.094
Residual	1.948	5	.390		
Total	14.000	14			

Dependent variable: Zscore (LF/HF)

Predictors: (Constant), Zscore (CC), Zscore (LF/HFB), Zscore (MVRB), Zscore (Te), Zscore (Ti), Zscore (TeB), Zscore (VtB), Zscore (FR), Zscore (Vt)

Note: df – degrees of freedom; F – Fisher's coefficient; Zscore (LF/HF) – standardized score of the ratio of low frequency power to high frequency power of HRV; Zscore (CC) – standardized score of the duration of cardiac cycle; Zscore (MVRB) – standardized score of the respiratory minute volume; Zscore (Te) – standardized score of the duration of expiration; Zscore (Ti) – standardized score of the duration of inspiration; Zscore (TeB) – standardized score of the duration of expiration; Zscore (VtB) – standardized score of the tidal volume; Zscore (FR) – standardized score of the breathing rate; Zscore (Vt) – standardized score of the tidal volume.

The coefficient of determination was significantly reduced after adjusting for the larger number of independent variables included in the prediction model for assessing the balance of sympathetic and parasympathetic activity based on the LF/HF ratio in subjects using abdominal respiration. In order to avoid including ineffective and unnecessary variables in the calculation model, the Backward method was also employed. Consequently, the coefficients presented in Table 8 were obtained.

As shown, the regression model was optimized by including constant values and standardized scores of Vt, Te, MVR, and LF/HF recorded during restful breathing, as well as standardized values of Vt, Ti, Te, FR, and CC recorded during abdominal breathing. Among all the variables included, the final multiple regression model for this specific scenario was represented by the equation:

LF/HF in people with abdominal breathing = Zscore (VtB) × 5.007 - Zscore (TeB) × 3.831 - Zscore (MVRB) × 4.415 + Zscore (LF/HFB) × 1.428 - Zscore (Vt) × 0.728 - Zscore (Ti) × 4.037 - Zscore (Te) × 4.194 - Zscore (FR) × 5.953 - Zscore (Vt) × 0.705.

In this final model, there are variables whose predictive power raises doubts due to statistical insignificance and the inclusion of the value 0 within the 95% confidence interval. However, their predictive value can be further explored in future research involving larger numbers of participants.

Table 8. Coefficients of predictors in AR model.

Model	Unstandardized coefficients		Standardized coefficients	t	Sig.	95.0% confidence interval for B	
	B	Std. error	Beta			Lower bound	Upper bound
(Constant)	-4.933E-15	.161		.000	1.000	-.414	.414
Zscore (VtB)	5.007	1.156	5.007	4.330	.007	2.034	7.979
Zscore (TeB)	-3.831	1.087	-3.831	-3.526	.017	-6.624	-1.038
Zscore (MVRB)	-4.415	1.116	-4.415	-3.957	.011	-7.284	-1.547
Zscore (LF/HFB)	1.428	.427	1.428	3.340	.021	.329	2.526
Zscore (Vt)	-.728	.360	-.728	-2.023	.099	-1.653	.197
Zscore (Ti)	-4.037	1.097	-4.037	-3.681	.014	-6.856	-1.218
Zscore (Te)	-4.194	1.237	-4.194	-3.391	.019	-7.374	-1.014
Zscore (FR)	-5.953	1.815	-5.953	-3.280	.022	-10.617	-1.288
Zscore (CC)	-.705	.283	-.705	-2.492	.055	-1.431	.022
Dependent variable: Zscore (LF/HF)							

Note: Zscore (VtB) – standardized score of the tidal volume in RR; Zscore (TeB) – standardized score of the duration of expiration; Zscore (MVRB) – standardized score of the respiratory minute volume; Zscore (LF/HFB) – standardized score of the ratio of low frequency power to high frequency;.Zscore (Te) – standardized score of the duration of expiration; Zscore (Ti) – standardized score of the duration of inspiration; Zscore (FR) – standardized score of the breathing rate; Zscore (Vt) – standardized score of the tidal volume; Zscore (CC) – standardized score of the duration of cardiac cycle.

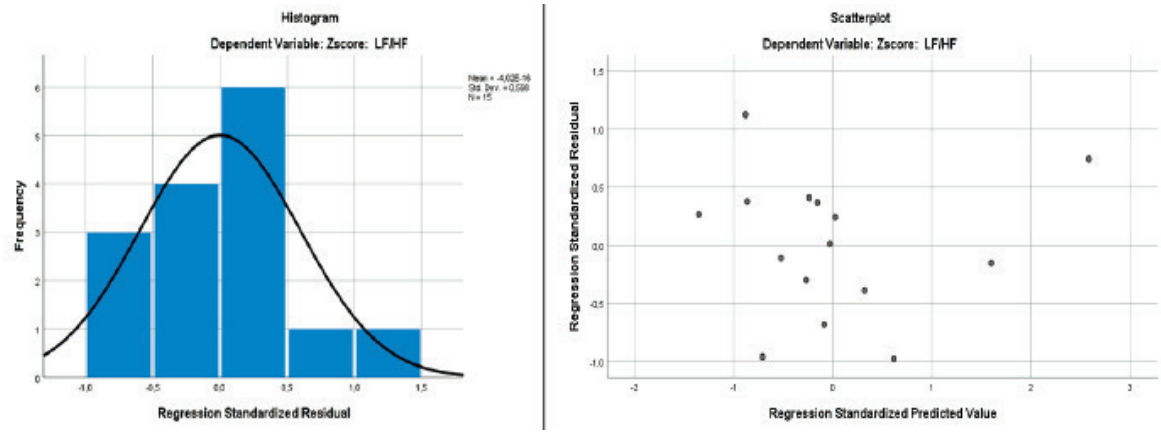

Fig 2. Histogram (left) and scatterplot (right) of standardized residuals in AR model.
The histogram displays the frequency distribution of residuals. The scatterplot of standardized residuals against standardized predicted values reveals no collinearity.

The residuals of the linear regression model satisfied the necessary conditions. The observed distribution exhibited a slight right skewness and a random scatter without any discernible pattern (Fig. 2). These characteristics indicate that the developed model is optimal for predicting LF/HF in individuals with abdominal breathing based on the provided data.

Discussion

The present study documents that PR parameters, measured during both resting breathing and abdominal breathing, can predict sympathovagal modulation in healthy individuals undergoing breathing pattern re-education. Based on the obtained results, we determined that Vt has the greatest predictive power for assessing the balance between sympathetic and parasympathetic activity, as measured by the LF/HF ratio in individuals practicing abdominal breathing. The unstandardized coefficient for Vt is 5.007, followed by Te (B = -3.831) and MVR (B = 4.415), both measured during resting breathing. Consequently, we can predict that decreasing Vt or increasing MVR during resting breathing may lead to a reduction in the LF/HF ratio during abdominal breathing. This can be explained by an accentuation of parasympathetic influences and a decrease in sympathetic influences. However, these findings are not immediately evident due to the general lack of change in HRV. Further studies incorporating longer periods of abdominal breathing may reveal more pronounced alterations in HRV.

The LF/HF ratio observed during the abdominal breathing pattern can also be predicted by the PR parameters measured during abdominal breathing. The most effective predictors are found to be the PR time parameters, includ-

ing the frequency of breathing in the abdominal breathing pattern (FR) with a coefficient of -5.953, the duration of the inspiratory phase (Ti) with B = -4.037, and the duration of the expiratory phase (Te) with B = -4.194. Increasing FR along with an increase in Ti or increasing FR along with an increase in Te would lead to a reduction in the LF/HF ratio, thereby improving the sympathovagal balance.

Therefore, we can assume that individuals with higher MVR at rest and correspondingly higher frequency in abdominal breathing may experience a decrease in the sympathovagal balance during abdominal breathing.

In conclusion, by modulating these two parameters of the breathing pattern, namely MVR at rest and the total duration of a respiratory cycle (which influences the frequency of breathing), during normal breathing in healthy individuals, we can potentially enhance the sympathovagal balance.

Conclusions

The statistical analysis data presented in this study enable us to propose a hypothesis that certain volume and time parameters of the breathing pattern have the potential to predict changes in the ratio between sympathetic and vagal tone of the heart. Specifically, abdominal breathing has shown the ability to restore or normalize the sympathovagal balance by modulating the duration of inspiration or expiration.

To gain a deeper understanding of the practical applications of breathing pattern parameters in restoring the LF/HF ratio, particularly in disorders characterized by an elevated sympathovagal balance

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. 15 from 11.01.2016).

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RESEARCH ARTICLE

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The impact of imuheptin and imupurin on cytokine profile and antioxidant status in rat model of inflammation

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ABSTRACT

Introduction. Insects, throughout evolution, have developed a huge arsenal of active compounds, which they use to defend themselves against enemies and diseases, at the same time in recent years insects have shown great interest as a source of food rich in biologically active substances. Research in recent decades has shown that insects produce a variety of proteins and peptides with antibacterial, antifungal, antiviral, immunomodulatory, anti-inflammatory, antioxidant, antitumor, hepatoprotective, antithrombotic, antihypertensive and detoxifying activity during or after contact with the microbial agent or unfavourable factor.

Material and methods. The anti-inflammatory effect of imuheptin and imupurin was investigated in a rat model of subacute inflammation induced by subcutaneous implantation of felt discs. The intensity of the exudative and proliferative phase of inflammation, cytokine profile (TNFalpha, IL-6, IL-10), ceruloplasmin and antioxidant enzymes (superoxide dismutase, catalase, glutathione reductase, glutathione peroxidase and glutathione-S-transferase) in the serum of rats were evaluated.

Results. Imuheptin and imupurin reduced the level of pro-inflammatory cytokines (TNF-alpha, IL-6) and increased that of the anti-inflammatory cytokine (IL-10), as well as ceruloplasmin, glutathione reductase and glutathione peroxidase in subacute inflammation. Additionally, imupurin significantly increased the level of catalase and imuheptin that of glutathione-S-transferase.

Conclusions. Imuheptin and imupurin determined a moderate effect of inhibiting the exudative and proliferative processes, compared to the reference preparation - dexamethasone, but with a favourable effect on the cytokine profile, decreasing the level of pro-inflammatory cytokines (TNF-alpha, IL-6) and increasing the level the anti-inflammatory one (IL-10), as well as the modulation of antioxidant enzyme activity.

Keywords: imuheptin, imupurin, inflammation, cytokine, antioxidant status.

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Key messages

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

At the moment there is limited data on the anti-inflammatory properties and the mechanism of achieving the anti-inflammatory potential of the entomological preparations – imuheptin and imupurin.

The research hypothesis

Preparations of entomological origin (imuheptin and imupurin) through the content of biologically active substances will improve the evolution of the inflammatory process and restore the imbalance of the pro- and antioxidant systems in rat model of felt-pellet-induced granuloma formation.

The novelty added by manuscript to the already published scientific literature

The ability of imuheptin and imupurin to reduce the level of pro-inflammatory cytokines and increase the level of IL-10 with anti-inflammatory functions, as well as to modulate the activity of antioxidant enzymes in subacute inflammation was revealed.

Introduction

The current arsenal of preparations for the treatment of inflammatory processes consists of non-steroidal, steroidal, and disease-modifying antirheumatic drugs (DMARDs) with good efficacy, but safety issues require the research of new substances with anti-inflammatory properties, possibly with different mechanisms and increased safety profiles. Currently, there is a varied and documented basis of methodological recommendations for the *in vitro* and *in vivo* study of the anti-inflammatory properties of new substances that allow determining the influence of the investigated substances on inflammatory processes with the elucidation of the mechanisms and peculiarities of action [1-4].

Insects have become an object of research due to their ability to survive in adverse environmental conditions, including infectious factors and those that produce inflammatory processes. Bioactive compounds such as phenols, flavonoids, terpenes, saponins, sugars, alkaloids, glycosides and fatty acids, identified in a wide variety of insects, have demonstrated biological properties including antioxidant, anti-inflammatory, antiproliferative, cytotoxic, analgesic, immunomodulatory, antidiabetic, cardioprotective, antihypertensive, antimicrobial properties. Analysis of literature data demonstrated that a number of extracts, peptides, and synthetic analogues exhibite anti-inflammatory properties [5-8].

Previous preclinical and clinical studies of preparations of entomological origin obtained from *Lymantria dispar* at different stages of development (entoheptin, imuheptin, imupurin, adenoprosine) have shown hepatoprotective, immunomodulatory, anti-inflammatory properties [9, 10]. The purpose of the study was to determine the influence of preparations of entomological origin (imuheptin and imupurin) on the exudative and proliferative processes of subacute inflammation.

Materials and methods

This experimental study was conducted in the Department of Pharmacology and Clinical Pharmacology and the Biochemistry Scientific Laboratory of *Nicolae Testemiţanu* State University of Medicine and Pharmacy. Albino rats were purchased from the Animal House of *Nicolae Testemiţanu* State University of Medicine and Pharmacy. The animals were allowed standard access to food and water. Rats were housed at room temperature under conditions of 12 h of light and 12 h of dark. The experimental procedures involving rats were approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy, Minutes No. 78 from 22.06.2015. The entomological preparations obtained from insects of the order *Lepidoptera*, the genus *Lymantria* at the pupal stage (imupurin) and at the egg and pupae stage (imuheptin) were produced by Arena Group SA, Romania. Dexamethasone was purchased from KRKA d.d., Slovenia.

Adult male Wistar rats (180-330 g) were used for the study. They were randomly divided into the following groups: intact (n=8) – no manipulations, only saline (0.9% NaCl) solution intraperitoneally was administered; control (n=6) – felt pellets were implanted, saline (0.9% NaCl) solution intraperitoneally was administered; standard (n=9) - felt pellets were implanted, the steroid anti-inflammatory drug dexamethasone was administered; treatment 1 (n=7) - imuheptin was administered; treatment 2 (n=9) – imupurin was administered. In all animals, except intact group, subacute inflammation was induced by implanting felt pellets, weighing 26±1 mg, in the groin region of the animal's body on the right and left sides (1st day). The intervention was performed in aseptic conditions, under general anaesthesia with sodium thiopental (50 mg/kg intraperitoneally). Substances of entomological origin (imuheptin, imupurin) were administered daily internally for seven days, in doses of 500 mg/kg, dexamethasone (the reference preparation) – in a dose of 2.5 mg/kg intraperitoneally. On the 8th day, under general anaesthesia, the pellets were extracted together with the formed granulation tissue, weighed wet, and then dried at 60°C to constant weight.

The degree of the exudative reaction was assessed calculating the difference between the weight of the wet and the dry granuloma, and the percentage of inhibition of the exudative phase. Proliferative reaction was evaluated calculating the difference between the weight of the dry granuloma formed and the initial weight of the pellet, as well as the percentage of inhibition of the proliferative phase. To calculate the percentage of inhibition of the exudative and proliferative phases, the formula was used:

$$Pi = \left(1 - \frac{Mt}{Mm}\right) * 100$$

where: *Pi* – the percentage of inhibition;
Mt – wet/dry weight of granuloma in treated group;
Mm – wet/dry weight of granuloma in control group [1, 3].

The level of TNF-α, IL-6 and IL-10 was determined in the serum of rats by the ELISA method, using Invitrogen kits, ThermoFisher Scientific Inc, USA. The activity of catalase, superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPO), glutathione-S-transferase (GST) and ceruloplasmin (CP) was determined according to the methods described by Gudumac V. *et al.* [11, 12].

***Statistical analysis*:** The results were statistically processed using the functions of the computer program SPSS (version 25.0) and the basic indicators of descriptive statistics were determined – mean and standard deviation. The differences between the groups were analyzed using One –Way ANOVA, followed by *post hoc* Bonferonni test. The significance threshold set was for the 95% confidence interval.

Results

The data presented in table 1 showed that the initial weight of subcutaneously implanted felt pellets was almost identical in all groups. After extracting the pellets with the granuloma formed around them, it was found that their weight increased significantly in all groups, which proves the development of the inflammatory process. Thus, the wet granuloma weight in the control group was 315.1±32.0 mg (increased 11.2 times), in the dexamethasone group 196.1±10.0 mg (increased 7 times), in the imuheptin group 273.1±24.2 mg (increased 9.7 times), and with imupurin 267.5±34.4 mg (increased 9.5 times). Thus, we can state that the implantation of the felt pellets caused a marked exudative inflammatory reaction. In order to assess the influence of the investigated preparations on the exudative phase, the percentage of inhibition was calculated (tab. 1). Dexamethasone caused an inhibition of the exudative process by 38%, imuheptin by 13%, and imupurin by 15%. The analysis of the weight of the dry granuloma revealed that in the control group, it was 90.4±12.0 mg or 3.2 times higher than the initial weight of implanted pellet, which reveals the presence of a marked proliferative process. In the group treated with dexamethasone, the dry granuloma weight was 49.9±6.1mg or 1.8 times higher than the initial weight, but significantly reduced compared to the control group. In the group treated with imuheptin, the weight of the dry granuloma was 73.4±10.7 mg or 2.6 times higher than the initial one, and in the group with imupurin 73.0±16.7 mg or 2.6 times higher. The intensity of the proliferative process was analyzed, calculating the percentage of inhibition, which for dexamethasone was 45%, for imuheptin 19% and for imupurin 20%. These data confirm that dexamethasone essentially reduced the proliferative inflammation, and preparations of entomological origin showed a moderate effect. Based on the results obtained, we can conclude that dexamethasone effectively inhibited the exudative and proliferative phases in subacute inflammation, and the entomological preparations mainly decreased the proliferative phase.

Table 1. The effects of imuheptin and imupurin on the exudative and proliferative phase of subacute inflammation in rats

Treatment	Initial weight of pellets	Wet weight	The percentage of inhibition of exudative phase	Dry weight	The percentage of inhibition of proliferative
Control, saline solution	28.1±0.4	315.1±32.0		90.4±12.0	
Dexamethasone 2.5mg/kg	28.1±0.9	196.1±10.0*	38%	49.9±6.4*	45%
Imuheptin 500 mg/kg	28.1±1.0	273.1±24.2	13%	73.4±10.7	19%
Imupurin 500 mg/kg	28.2±0.9	267.5±34.4	15%	73.0±16.7	20%

Note: Values expressed as mean ± SD, SD – standard deviation; the results were analyzed using Oneway ANOVA followed by Bonferroni multiple comparison test; *- P < 0.05 was used to indicate statistical significance when compared to control

At the same time, in the control group with subacute inflammation, the level of TNF-alpha increased compared to the intact group (48.68±10.77 pg/ml) and constituted - 72.67±20.19 pg/ml (P1-2<0.05); increased IL-6 level was observed - 37.57±1.69 pg/ml (P1-2<0.05) compared to the control group (33.75±0.57 pg/ml); as well as the decrease in IL-10 content - 15.28±2.36 pg/ml (P1-2<0.05) compared to the control group, where the level was 32.35±13.39 pg/ml (table 2). Dexamethasone caused a significant decrease in TNF-alpha level and IL-6 level, also slightly increased the IL-10 level. Preparations of entomological origin decreased the level of TNF-alpha and that of IL-6. Imuheptin, and especially imupurin, increased the content of IL-10, a cytokine with anti-inflammatory properties, compared to the control group (table 2). Thus, the steroid anti-inflammatory mainly decreased the level of pro-inflammatory cytokines (TNF-alpha, IL-6), and preparations of entomological origin restored the ratio between pro-inflammatory (TNF-alpha, IL-6) and anti-inflammatory (IL-10) cytokines.

Table 2. The influence of imuheptin and imupurin on cytokines and ceruloplasmin level in rats serum with felt-pellets induced granuloma

Treatment	TNF - alpha, pg/ml	IL-6, pg /ml	IL-10, pg /ml	CP, mg/L
Intact (no pellets were implanted)	48.7±10.8	33.7±0.6	32.3±13.4	470.4±87.0
Control, saline solution	72.7±20.2 §§	37.6±1.7§§	15.3±2.4 §§	390.7±78.9
Dexamethasone 2.5 mg/kg	43.3±6.5*	34.2±1.4*	21.2±5.6§§	323.8±42.5§§
Imuheptin 500 mg/kg	46.1±10.9*	35.1±1.5*	23.3±6.6	539.7±68.5*/**
Imupurin 500 mg/kg	46.2±12.9*	34.1±1.0*	27.4±4.2*	501.5±66.4*/**

Note: Values expressed as mean±SD, SD – standard deviation; the results were analyzed using One Way ANOVA followed by Bonferroni multiple comparison test; TNF alpha - tumour necrosis factor alpha ; IL – interleukin; CP - ceruloplasmin; §§ - P<0.05 was used to indicate statistical significance when compared to intact group; * - P<0.05 was used to indicate statistical significance when compared to control group; ** - P<0.05 was used to indicate statistical significance when compared to dexamethasone group.

In felt-pellets-induced granuloma, a decrease in the level of ceruloplasmin was found - from 470.41±87.0 in the intact group to 390.68±78.96 mg/L (P>0.05) in the control group. Dexamethasone caused an even more pronounced reduction in ceruloplasmin levels. Imuheptin and imupurin significantly increased the content of ceruloplasmin compared to the control group with subacute inflammation (table 2). Withal, a tendency to decrease the activity of catalase, SOD and GPO and to increase GR was found in the control group, without essential changes in GST. Dexamethasone virtually restored the activity of catalase and SOD, the level of these enzymes being comparable to that of the intact group, and increased the activity of enzymes of the glutathione system (GR, GPO, GST). Imuheptin, administered to animals with inflammation, reduced SOD activity and restored catalase activity compared with the control group, and increased GR, GPO and GST activity. Compared to the control group, imupurin increased the activity of catalase and decreased that of SOD and GST, simultaneously increasing GR and GPO levels (table 3).

Discussion

The screening of the anti-inflammatory properties in the previous research, namely formaldehyde-induced paw oedema allowed us to find that the drugs of entomological origin (entoheptin, imuheptin, imupurin) do not prevent inflammation but had an anti-inflammatory activity comparable to that of diclofenac. The comparative analysis between the anti-inflammatory potential of entoheptin, imuheptin, imupurin and diclofenac revealed that entoheptin possesses the strongest anti-inflammatory activity, achieving complete healing in 48 hours, followed by diclofenac and imuheptin. Imupurin showed the weakest anti-inflammatory action, but it was more intense than in the group of untreated animals [9, 10].

Table 3. The influence of imuheptin and imupurin on antioxidant enzymes in rats serum with felt-pellets induced granuloma

Treatment	Catalase μM/s.L	SOD c/u	GR, nM/s.L	GPO, nM/s.L	GST, nM/s.L
Intact (no pellets were implanted)	19.7±1.7	918.1±45.7	64.8±18.9	430.8±90.3	24.5±12.9
Control, saline solution	15.9±3.2	905.3±49.2	80.4±21.9	380.3±42.3	24.8±10.3
Dexamethasone 2.5 mg/kg	18.3±2.4	944.9±79.3	99.2±33.5	531.7±116.8	50.2±13.4$^{§§/*}$
Imuheptin 500 mg/kg	20.4±2.9	865.0±96.6	127.6±21.7§§	551.8±96.8*	34.3±8.2
Imupurin 500 mg/kg	31.8±9.5$^{§§/*/**}$	888.9±135.9	150.8±65.7$^{§§/*}$	535.5±100.6*	21.1±9.5**

Note: Values expressed as mean±SD, SD – standard deviation; the results were analyzed using One Way ANOVA followed by Bonferroni multiple comparison test; SOD - superoxide dismutase; GR - glutathione reductase; GPO - glutathione peroxidase; GST - glutathione-S-transferase; §§ - P<0.05 was used to indicate statistical significance when compared to intact group; *** -** P<0.05 was used to indicate statistical significance when compared to control group; **** -** P<0.05 was used to indicate statistical significance when compared to dexamethasone group.

Insects include the largest number of species and play an important role in the terrestrial ecosystem and have been considered a useful natural resource as food, especially due to their protein and fatty acids. Some studies have shown that insects not only have a high protein content, but micronutrients and bioactive peptides with various pharmacological effects, including anti-inflammatory, antioxidant, antimicrobial and antitumor activity. Wasps (*Vespa orientalis*) have a major protein content, and the aqueous extract of *Vespa affinis* has demonstrated antioxidant effects by activating the antioxidant enzymes glutathione-S-transferase (GST) and catalase (CAT) [13-16].

Oxidative and inflammatory processes are closely related, so antioxidants annihilate free radicals that damage cells and lead to inflammation. Several studies have shown that antioxidant and anti-inflammatory peptides have protective effects against reactive oxygen species (ROS) and can contribute to a significant reduction in oxidative stress levels. *Tenebrio molitor*, *Schistocerca gregaria* and *Grylodes sigillatus* have been shown to be a rich source of bioactive peptides with antioxidant and anti-inflammatory properties, which have shown high antiradical activity and an ability to chelate iron ions and inhibit the activity of lipoxygenase and cyclooxygenase-2 [16, 17].

Subacute and chronic inflammation is a response to prolonged stimulation of proinflammatory factors on tissues and is characterized by leukocyte infiltration at the site of inflammation, fibrosis, and granuloma formation. The mechanism of chronic inflammation is attributed, in part, to the release of ROS from activated neutrophils and macrophages, excessive cytokine production, dysregulation of cell signalling, and loss of barrier function. This overproduction causes peroxidation of membrane lipids, which leads to tissue damage by damaging macromolecules. ROS cause or extend inflammation by stimulating the release of cytokines (IL-1beta, TNF-alpha, INF-alpha), which stimulate the recruitment of additional neutrophils and macrophages [18].

Subcutaneous implantation of felt pellets causes the formation of granulomatous tissue. This granulomatous tissue is due to the accumulation of macrophages, neutrophils and lymphocytes around the foreign particles, followed by the proliferation of fibroblast cells. The implanted felt pellets stimulate the immune system to produce interleukins and antibodies that stimulate the proliferation of lymphocytes and the accumulation of cells around them. Initially, exudative processes develop through the transudation of liquid and a marked increase in the weight of wet felt pellets. Steroidal and nonsteroidal anti-inflammatory drugs are shown to reduce granuloma size and transudate by inhibiting the production of proinflammatory mediators (inflammatory cytokines, leukotrienes, and prostaglandins), inhibiting cell (leukocyte) infiltration, and preventing fibroblast proliferation and collagen fibre production and mucopolysaccharide synthesis. A similar effect was demonstrated by dexameth-

asone in our study. Imupurin showed a lower ability, compared to dexamethasone, to reduce the exudative and proliferative processes. Possibly, unlike the steroid anti-inflammatory, the preparation of entomological origin develops a slower effect due to its immunotropic properties on cellular immunity - modulation of T-lymphocytes [18, 19].

Implantation of felt discs causes an exudative and proliferative reaction and an increase in the level of pro-inflammatory cytokines TNF-alpha, IL-1beta and IL-6, products that characterize the function of macrophages (activation, infiltration). The administration of indomethacin, a non-steroidal anti-inflammatory, causes a decrease in the mass of the granuloma and the level of IL-6, with an increase in TNF-alpha, without changing that of IL-1beta [20].

When foreign bodies, such as the implantation of felt discs, penetrate the skin, the production of nitric oxide occurs under the action of nitric oxide synthase. Subsequently, the cascade of proinflammatory mediators and cytokines is activated which includes cyclooxygenase 2, interleukins IL-1β and IL-6, and TNF-alpha. These pro-inflammatory mediators and cytokines cause the activation of the classical inflammatory pathway, nuclear factor NF-κB and mitogen-activated protein kinase (MAPK) triggering an uncontrolled inflammatory response. The use of wasp venom suppressed the production of nitric oxide and reduced the mRNA expression of IL-1β, IL-6 and TNF-α [21].

Glucocorticoids (GCs) play an important role in the regulation of the inflammatory and immune response, acting on most types of immune cells. Glucocorticoids can: regulate the phenotype, survival and functions of monocytes and macrophages; exhibits anti-apoptotic effects that promote the survival of anti-inflammatory macrophages; improve the phagocytic activity of macrophages; stimulates the clearance of neutrophils; inhibits the release of pro-inflammatory mediators (cytokines, chemokines, etc.) and reactive oxygen species; regulate the maturation, survival and migration to lymph nodes and the functionality of dendritic cells. Glucocorticoids inhibit transcription factors that control the synthesis of proinflammatory mediators and cells, including macrophages, eosinophils, lymphocytes, mast cells, and dendritic cells. Another important effect is the inhibition of phospholipase A2, responsible for the production of pro-inflammatory mediators. Glucocorticoids inhibit the genes responsible for the expression of cyclooxygenase-2, iNOS and proinflammatory cytokines. Concomitantly, GCs produce an increase in lipocortin and annexin A1, with subsequent reduction in the synthesis of prostaglandins and leukotrienes [22-24].

The plasma level of ceruloplasmin, considered an acute-phase inflammatory plasma protein, produced predominantly by hepatocytes and activated monocytes and macrophages, increases in response to inflammation, trauma, or infection. Ceruloplasmin production by myeloid cells is induced by interferon-γ (IFN-γ) and tumour necrosis factor-alpha (TNFα). The ferroxidase activity of ceruloplasmin inhibits the ferrous ion-mediated production of reactive oxygen species with the manifestation of antioxidant activity. Ceruloplasmin also exhibits ferroxidase-dependent bactericidal activity. The increase in the plasma level of ceruloplasmin during the acute phase reaction suggests a possible anti-inflammatory function of the antioxidant, bactericidal and ferroxidase activity. Ceruloplasmin, due to its antioxidant activity, prevents the carbonylation of proteins by reactive oxygen species in inflammatory diseases. The anti-inflammatory action of ceruloplasmin, most likely, is determined by its synthesis by infiltrated macrophages at the site of inflammation and, less so, by the modulation of the T-cell response. Thus, the prevention of oxidation and tissue damage can be considered the basic mechanism of ceruloplasmin, generated by macrophages recruited to the site of inflammation [25].

Most organisms use aerobic cellular respiration to produce energy for their functioning, but this process is also accompanied by side effects caused by metabolic products in the form of free radicals. Living organisms use exogenous and endogenous antioxidants to defend themselves against the harmful effects of free radicals, and studies on the antioxidant activity of substances of plant, animal, entomological, or biological origin have captured the interest of researchers for many years. [26]. Antioxidants are compounds capable of counteracting the effects of oxidative processes in cells or exogenous systems, reacting in particular with reactive oxygen or nitrogen species or with other free radicals or unstable molecules generated during normal metabolic oxidative reactions. Antioxidant systems include enzymes (SOD, catalase, GP, GR, GST) and non-enzymatic substrates (glutathione, coenzyme Q, ascorbic acid, retinols, tocopherols, flavonoids, carotenoids, etc.). Antioxidants are found in products of vegetable, animal, or entomological origin, in food supplements. Antioxidant capacity is the general ability of organisms or compounds to interact with free radicals and prevent their harmful effect. The antioxidant effect includes the protection of cells and cellular structures against the effect of free radicals, especially oxygen and nitrogen [26-28].

The enzymes of glutathione metabolism - glutathione reductase (GR), glutathione peroxidase (GPO) and glutathione-S-transferase (GST) constitute a group of antioxidants that ensure the protection of cells against ROS and RNS action, also against lipid peroxidation products. The main role in the degradation of hydroperoxides belongs to the GPO/GR enzyme system. GR has a variable distribution in organs and intracellular organelles and ensures the maintenance of the optimal level of glutathione (GSH) by reducing oxidized GSH (GSSG). The enzyme reduces the need for the new synthesis of GSH from amino acids. GR function is in constant correlation with GPO and GST, enzymes that oxidize GSH in peroxide reduction processes [29, 30].

Glutathione peroxidase catalyzes the cleavage of hydrogen peroxide and organic peroxides by using GSH and converting it to GSSG. This enzyme is in competitive relations, due to its different intracellular localization, with catalase and SOD in the neutralization of excess hydrogen peroxide and organic peroxides, which ensures the efficient functioning of these enzymes. Glutathione peroxidase in mitochondria and peroxisomes works in tandem with catalase, and in the cytoplasm with SOD, which ensures, together with

non-enzymatic antioxidants, the protection of subcellular structures and the modulation of the oxygen activation process by deregulating the formation of the hydroxyl radical (OH•). Glutathione-S-transferase catalyzes the conjugation of GSH with electrophilic organic compounds, an important detoxification reaction of exogenous products and the neutralization of endogenous substances within the physiological processes of metabolic waste elimination [29, 30].

Insect antioxidant systems are of crucial importance in defence mechanisms against xenobiotics that produce endogenous reactive oxygen species (ROS) in insects. Increased levels of radicals from xenobiotics, such as plant secondary metabolites, are associated with oxidative stress in the midgut tissues of lepidopteran larvae. Xenobiotics (prooxidant substances, heavy metals, pesticides) and their metabolism are associated with the production of free radicals, which react with various biomolecules and affect cellular functions. These radicals are removed by innate antioxidant defence systems, including antioxidant enzymes and various antioxidant compounds. Deficiency of the antioxidant defence system leads to increased ROS, which interacts with many cellular biomolecules, including proteins, lipids, enzymes, carbohydrates, and DNA with their damage. Insects, in order to overcome the toxic effects of SRO, have developed a complex antioxidant mechanism consisting mainly of the enzymatic action of glutathione peroxidase (GPX), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase, and glutathione transferases (GST). In insects, GSTs are involved in the transformation of many insecticides, and their overexpression is responsible for the development of resistance against those insecticides. Glutathione-S-transferases present selenium-independent glutathione peroxidase activity and can remove highly reactive electrophilic components, lipid hydroperoxides (DAM, trans-4-hydroxy-2-nonenal), generated by ROS-initiated lipid peroxidation. After exposure to xenobiotics, increased levels of DAM have been correlated with a variety of tissue and cell membrane damage in animals [15, 31, 32].

The anti-inflammatory properties of edible insects have been evaluated *in vivo* and in cellular models. *In vivo*, studies have revealed a reduction in circulating cytokine levels elevated by various stressors after administration of different insect extracts. An increase in cytokine levels was found only at high doses of *Hermetia illucens* administered to healthy fish, without being confirmed by inflammatory events on histological analysis. In studies of healthy subjects, circulating levels of TNF-α have been shown to be reduced, data that must be reviewed because they may have had a reduced level of inflammation [32]. Levels of NF-κB, the transcription factor regulatory genes involved in inflammatory responses, were decreased in cell and animal models. At the same time, the levels of TLR4, whose stimulation leads to the activation of NF-kB, were not affected. Some studies have shown activity in reducing the production of NO in macrophages, a radical involved in the modulation of inflammation and immunity. In conclusion, evidence from cellular and animal models supports an effect on reducing inflammatory cytokines by modulating NF-kB levels, without affecting immunoglobulins [33, 34].

Conclusions

Imuheptin and imupurin showed a moderate inhibitory effect, predominantly of proliferative processes compared to dexamethasone, which essentially diminished inflammation's exudative and proliferative phases. Imuheptin and imupurin reduced the level of pro-inflammatory cytokines (TNF-alpha, IL-6) and increased that of anti-inflammatory cytokines (IL-10). The studied entomological preparations increased the ceruloplasmin level and restored the activity of catalase and glutathione peroxidase with the increase of glutathione reductase activity in subacute inflammation. Due to the effects mentioned earlier, the researched entomological preparations – imupurin and imuheptin have an anti-inflammatory potential, which requires a more in-depth study to determine the mechanisms of anti-inflammatory action and the pathological conditions where these effects would be beneficial.

Competing interests

None declared.

Authors' contribution

IG conceived and participated in the study design, performed the experiments and statistical analysis, and drafted the manuscript. NB participated in the study design and helped drafted the manuscript. VG had contribution to acquisition and interpretation of data, and helped drafted the manuscript. All the authors reviewed the work critically and approved the final version of the manuscript.

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* and approved by the Research Ethics Committee of *Nicolae Testemiţanu* State University of Medicine and Pharmacy, Minutes No. 78 from 22.06.2015.

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RESEARCH ARTICLE

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Clinical effectiveness study of the new diagnostic score of acute appendicitis in the elderly

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ABSTRACT

Introduction. Acute appendicitis is among the three most frequent surgical diseases. The lifetime likelihood of developing acute appendicitis is around 7%. The incidence of acute appendicitis reduces with age after adolescence. Several studies evaluated the relevance of the current scores to the general population, mostly children, but a limited number of studies have studied the elderly population. This study aims to assess the clinical effectiveness of the new diagnostic score for the elderly population in comparison to both the Alvarado score and the non-standardized score.

Material and methods. In order to evaluate the effectiveness of the diagnostic score of acute appendicitis, we examined 78 patients who were admitted to emergency unit of the *Saint Archangel Michael* Municipal Clinical Hospital during 2018-2021 with the presumptive diagnosis of acute appendicitis. Of all patients admitted, Acute Appendicitis was confirmed on pathological examination in 22 (28.2%) patients. The average age of patients was 73.5±13.5 years (minimum - 60 years, maximum - 87 years). The ratio of males to females was 1:1.6.

Results. Comparative evaluation of the new diagnostic score of acute appendicitis and the non-standardized clinical-echographic examination for acute appendicitis diagnosis showed better performance indicators of the new diagnostic score of acute appendicitis compared to the non-standardized clinical method for acute appendicitis diagnosis. The high sensitivity of the new diagnostic score of acute appendicitis was statistically demonstrated ($\chi 2 = 4.32$; $p < 0.05$), a lower rate of missed acute appendicitis cases in the new diagnostic score of acute appendicitis ($\chi 2 = 4.32$; $p < 0.05$), the „grey area" is lower in the new diagnostic score of acute appendicitis ($\chi 2 = 5.28$; $p < 0.05$), than in the non-standardized diagnosis. It was shown to have a lower rate of acute appendicitis cases in the „grey area" of the total number of acute appendicitis cases ($\chi 2 = 4.9$; $p < 0.05$). Benchmarking indicators such as specificity and diagnostic accuracy showed no statistically significant differences. At the same time, a definite increase in specificity and accuracy was observed for the new diagnostic score of acute appendicitis compared to non-standardized clinical diagnosis.

Conclusions. Diagnosing acute appendicitis in elderly patients remains challenging due to the numerous potential diagnoses with similar clinical manifestations that are observed in this patient population. It was necessary to utilize clinical risk-scoring systems that could aid in the prompt identification of patients with acute appendicitis. This study concludes that the *New diagnostic score* has higher clinical efficiency in diagnosing acute appendicitis in elderly patients. It has a sensitivity of up to 93.15%, compared to the unstandardized clinical method and the Alvarado diagnostic score, and is independent of „risk factors" such as obesity and atypical vermiform appendix localization.

Keywords: acute appendicitis, elderly, diagnostic score.

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Key messages

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

The epidemiology and outcomes of acute appendicitis in elderly patients are very different from the
younger population. Elderly patients with acute appendicitis have higher mortality, higher perforation rate, lower diagnostic accuracy,

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longer delay from onset and manifestation of symptoms, higher rate of postoperative complications, and a higher risk of colon and appendix cancer. Therefore, it is necessary to develop a new diagnostic and remedial approach to treating acute appendicitis in elderly patients.

The research hypothesis

We aim to develop a new diagnostic score for elderly patients and apply it in parallel with the non-standardized diagnosis.

The novelty added by manuscript to the already published scientific literature

The clinical efficacy of the new score was evaluated in comparison with the Alvarado and a non-standardized diagnostic score.

Introduction

Acute appendicitis (AA) is among the three most commonly occurring acute surgical diseases. The likelihood of experiencing AA in a lifetime is approximately 7%. The incidence of AA reduces with age after adolescence [1]. Approximately 15% of patients over 60 years of age who present with acute abdominal pain to the Emergency Department receive a final diagnosis of acute appendicitis, which is half as common as in younger patients [2]. Nevertheless, the epidemiology and outcomes of acute appendicitis in elderly patients differ significantly from those of the younger population. First, despite the decrease in incidence, acute appendicitis in elderly patients is marked by significantly higher mortality, which is 8% in the category of patients over 60 years of age, compared to less than 1% among younger patients. A large observational study of 164,579 patients with acute appendicitis, age greater than 60 years was a significant risk factor for mortality by multivariate analysis [3].

All the data suggest that older patients are more likely to have complicated appendicitis with perforation or abscessing compared with other age groups. The rate of complicated appendicitis ranges from 18% to 70% [2, 4-6] (compared to a rate of 3 % and 29% among patients under 60 years). The reason for this major risk of perforation could be the vascular sclerosis that the vermiform appendix develops in elderly patients and the narrowing of the lumen by the phenomenon of fibrosis. In these patients, the muscle layers are infiltrated with fat, so having a fragile structure they have a tendency toward early perforation [7]. These findings together with the delay in diagnosis and treatment could explain a more aggressive evolution of the disease in this population category.

Another finding among the elderly population, who develop acute appendicitis, is the lower rate of correct preoperative diagnosis compared to the younger population [8], with a reported diagnostic accuracy (defined as the percentage of appendices removed with a histological diagnosis of acute appendicitis out of the total number of appendectomies performed) of 64% in patients over 60 years of age versus 78% in other age groups [9]. Moreover, in the vast majority of included studies, the mean time from symptom onset to admission was longer in older patients than in younger patients [9-11].

Focusing on appendectomy, compared with young patients, elderly patients are burdened with higher postoperative mortality, higher postoperative morbidity [12], longer length of hospital stay [13], lower rate of laparoscopic appendectomy, and a higher risk of being subjected to high-throughput investigations [14-15].

In a large Swedish study that included more than 117,000 patients, the mortality rate after appendectomy was strongly influenced by age, with a threefold increase for each decade of age, reaching more than 16% in nonagenarians. Finally, the complication rate in elderly patients with negative appendectomy was significantly higher than in younger patients (25% vs 3%) [16].

This raises the question of whether existing clinical scoring systems have sufficient diagnostic accuracy for the diagnosis of acute appendicitis in elderly patients?

The Alvarado score is the most widely studied. Its validity in adult and pediatric patients was summarized in a recent meta-analysis [17] that included 5960 patients in 29 studies. According to Ohle *et al.*, the performance of the score depends on the cutoff value: a clinical cutoff score of < 5 can be applied to „exclude" appendicitis with a sensitivity of 99% (95% CI 97–99%) and a specificity of 43% (36–51%).

According to the Jerusalem guidelines [18], in adult patients the Alvarado score (with a cut-off score < 5) is sensitive enough to exclude acute appendicitis, but is not specific enough in the diagnosis of acute appendicitis.

However, the Alvarado score was developed based on the pattern of presentation of clinical and laboratory variables of a young population (mean age 23.4 - 25.9) [19]. Considering that the complication rate in elderly patients with negative appendectomy is significantly higher than in younger patients (25% vs 3%, $p < 0.05$) [20], the preoperative diagnosis in these patients must be as accurate as possible.

Although computer tomography (CT) with intravenous (IV) contrast is associated with lower rates of negative appendectomy [21]. Ultrasound (US) is clearly inferior to CT in sensitivity and negative predictive value for appendicitis, however, it may be equally useful for excluding appendicitis [22, 23], while CT is especially useful if the appendix is not visualized by US.

The New diagnostic score (DS), which we aimed to compare with the Alvarado score, is a diagnostic score that includes 10 parameters: the positive Kocher symptom- 1 point; positive Blumberg symptom in the right iliac region - 2 points; positive Bartomier-Michelson symptom - 1 point; the presence of nausea and/or vomiting - 1 point; leukocytosis in Complete Blood Count (CBC) 10 x 10^9/l and more - 1 point; ultrasound determination of Vermiform Appendix (VA) with a diameter greater than 7 mm is estimated at 2 points; VA incompressibility - 1 point; thickening of the peri-appendiceal tissue - 1 point; coprolite in the VA lumen - 1 point; the presence of ultrasound signs of another acute non-appendiceal pathology of the abdominal cavity and/or ultrasound detection of a compressible VA less than 7 mm in diameter - „minus" 3 points.

In this scoring system, the total score varies between -3 and 10 points. When obtaining a score below 2 points, the diagnosis of AA is excluded. If adding up the points of the positive clinical and laboratory criteria of AA, a result of 6-7 points will be obtained, and then the diagnosis of AA will be established. In this case, an additional ultrasound will not be necessary, because even the identification of another acute pathology with / or without signs of inflammation of vermiform appendix on UST („minus" 3 points), will not affect the result and the interpretation of the New DS application algorithm, because the final score will be 3 or more points, which definitely indicates that the patient has AA. The patient diagnosed with AA will later undergo urgent surgical treatment.

If the sum of the points of the clinical and laboratory criteria of the New DS will be less than 4 points, an ultrasound of the abdominal cavity will be performed with the additional inclusion of ultrasound signs of AA. If following a general ultrasound examination, the sum of AA points will constitute < 2 points, the diagnosis of AA will be excluded.

When following the general ultrasound evaluation of signs of AA, the number of points will be 3 or more, the diagnosis of AA will be very likely and appendectomy will be indicated.

Material and methods

There were prospectively analyzed 78 cases (patients), who were admitted to the Emergency Department of *Saint Archangel Michael* Municipal Clinical Hospital in 2018-2022 with the diagnosis of acute appendicitis (AA). Of all hospitalized patients, AA was confirmed on histological examination in 22 (28.2%) patients.

The average age of patients was 73.5±13.5 years (minimum - 60 years, maximum - 87 years). The ratio of males to females was 1:1.6. Demographic data of patients, including age, sex, duration of hospitalization, and histopathological reports of appendectomy materials were recorded. Analyzing the obtained data, we note that the structure of distribution by sex and age in this group of patients is comparable to that in the group of patients in which the new DS was developed.

The time from the debut of complaints of abdominal pain to hospitalization was: in 9 (11.5%) people - less than 6 hours, from 6 hours to 24 hours - in 28 (35.9%) patients, from 24 hours to 48 hours - in 22 (28.3%) patients, more than 48 hours - in 19 (24.3%) patients.

After data processing, the patients admitted to the study group had the concomitant pathologies noted in table 1.

Table 1. Patient demographic data and characteristics

	Associated medical conditions		№	%
1.	Hypertension		37	47.4
2.	Coronary heart disease		20	25.6
3.	Diabetes		11	14.1
4.	Obesity		5	6.4
5.	Dyscirculatory encephalopathy		14	17.9
6.	Urolithiasis		7	8.9
7.	Chronic duodenal ulcer		4	5.1
8.	Chronic gynecological pathologies without exacerbation	Uterine myoma	3	3.8
		Uterovaginal prolapse	2	2.6
		Pelvic inflammatory disease	2	2.6

Statistical analysis

The SPSS 18 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Numerical data were presented as mean ± standard deviation. The one-sample Kolmogorov–Smirnov test was used to assess the distribution of numerical data. The independent sample t-test was used when the distribution was normal and the Mann-Whitney U test was used for the non-normal distribution. A chi-square test was used to compare between groups. Values with a P value < 0.05 were considered to be statistically significant.

Results

New DS in AA implementation results

In this group of patients, the non-standardized clinical and ultrasonographic diagnosis of AA was used as the main diagnostic method, which was performed by the doctor on call, based on professional knowledge and skills, in the absence of a mandatory research standard and an algorithm for interpreting the obtained data, which is largely subjective. In the Emergency department, the non-standardized diagnosis of AA was made based on clinical, laboratory, and ultrasonographic investigations. The method of diagnosis and the clinical-therapeutic tactic used for all groups of patients were methodologically similar to this diagnostic method (New DS) (Table 2), and its algorithm, which corresponds to all the training principles of the diagnostic algorithm (Fig. 1).

All patients were examined using the same diagnostic equipment. The non-standardized clinical and ultrasonographic method included the use of a general clinical examination, laboratory investigations (CBC, urinalysis), ultrasonography (USG) of the abdominal cavity.

The diagnosis and management of AA patients were specified directly by the on-call surgeon. The general clinical examination was performed in all 78 (100%) patients and consisted of collecting anamnesis, and patient complaints to determine the symptoms of AA with their subsequent interpretation.

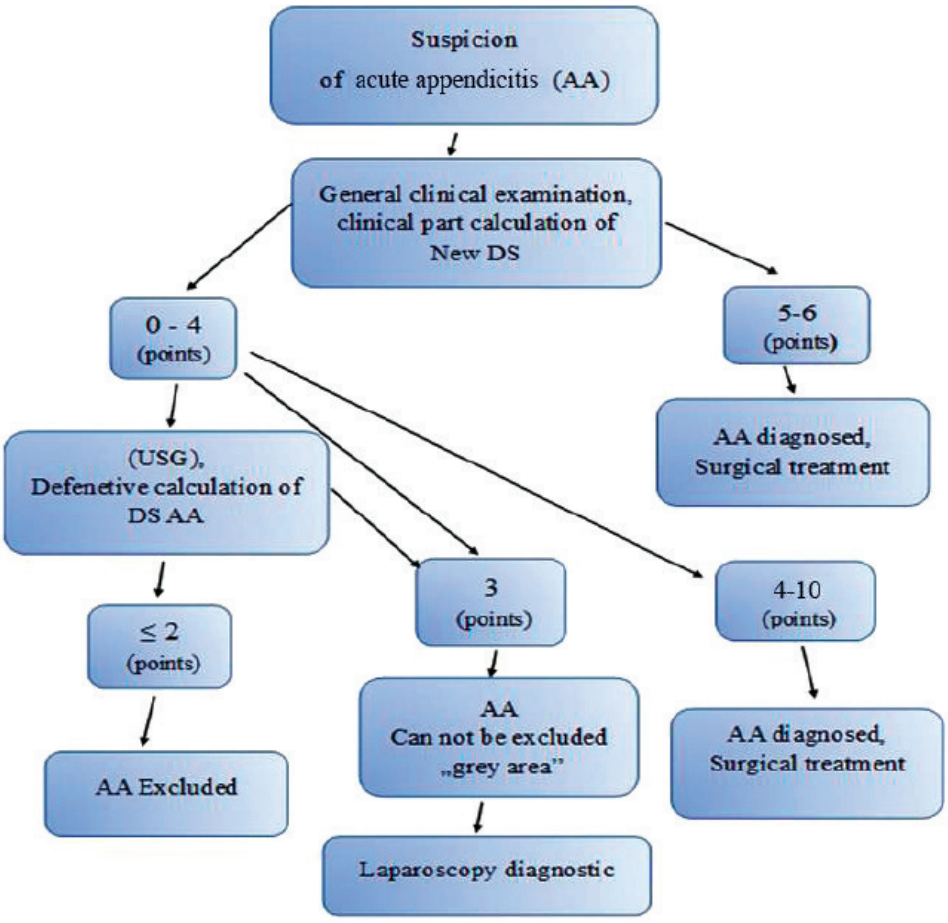

Fig. 1 Use of algorithm of New DS. Diagnostic score 0-2 (points) - Acute Appendicitis excluded; DS 5-6 (points) - Acute Appendicitis diagnosed; DS 3-4 (points) - ultrasound examination; Diagnostic score 4-10 (points) - Acute Appendicitis diagnosed; Diagnostic score 3 (points) - laparoscopy diagnostic recommended.

Table 2. The New Diagnostic Score.

No.	Criterion	Assessment	Score
1.	Kocher symptom	Positive	1
2.	Nausea /vomiting	Present	1
3.	Blumberg symptom in right iliac region	Positive	1
4.	Bartomier-Michelson symptom	Positive	1
5.	Leukocytosis	>10 × 10^9/l	1
6.	Ultrasound: VA unchanged and /or other pathology	Determined	-3
7.	Ultrasound: increased VA diameter > 7mm	Determined	2
8.	Ultrasound: thickening periappendicular tissue	Determined	1
9.	Ultrasound: VA Incompressibility	Determined	1
10.	Ultrasound: coprolite in VA lumen	Determined	1
	Total	Max Min	10 -3

Note: *VA - vermiform appendix; the total score is a sum of criteria points. Minimal Score (-3), maximum (10).*

Patients in the study group presented the following complaints (Table 3).

Table 3. Diagnostic criteria of patients

No.	Diagnostic criteria	Patients No.	%
1	Abdominal pain	78	100
2	Nausea	30	38.5
3	Vomiting	41	52.5
4	Kocher's symptoms	32	41.0
5	Gynecological anamnesis	7	9
6	Intestinal disorders (constipation)	64	82.9
7	Dysuria	37	47.4
8	Local tenderness (pain) (on palpation in the right iliac region)	78	100
9	Blumberg's symptom in the right iliac region	78	100
10	Bartomier-Michelson's symptom	56	71.8
11	Rovzing's symptom	44	56.4
12	Sitkovsky's symptom	54	69.2
13	Obraztsov's symptom	3	3.8
14	Coupe's symptom	2	2.5
15	Voscresenscky symptom	2	2.5
16	Hyperthermia >37.4°C	65	83.3
17	Tenderness on palpation of the anterior rectal wall	3	3.8
18	Leukocytosis >10×10^9/l	78	100
19	Deviation of the leukocyte formula >74%	78	100
20	Hematuria, leukocyturia	78	100
21	Free fluid in the abdominal cavity	78	100
22	US signs of unchanged VA or other pathologies of the right iliac region	43	55.1

Note: *US – ultrasound*; VA - *vermiform appendix.*

Results of laboratory examinations of patients

In examined patients, the increase in the number of leukocytes in complete blood count (CBC) >10x10^9/l was detected in 70 (89.7%) patients. Left shift of (increased neutrophil ratio) more than 74% was found in 58 (74.3%) patients.

Left shift of (increased granulocytes ratio) more than > 6% was found in 47 (60.25%) examined patients. The absence of pathological changes (leukocyturia, hematuria, bacteriuria) in the urinalysis was observed in 44 (56.4%) of the examined patients. Leukocyturia/hematuria was found in 34 (43.5%) cases.

Results of ultrasound

The following ultrasonographic signs were recorded in the examined patients:

- AV diameter increase > 7 mm was determined in 30 (38.4%) patients.
- AV incompressibility during compression was observed in 28 (35.8%) patients.
- Positive „Target" symptom was detected in 39 (50%) of the examined patients.
- Coprolite in the VA lumen was detected in 5 (6.4%) patients.
- Thickening of the peri-appendiceal tissue was detected in 16 (20.5%) of the examined patients.

Free liquid in the abdominal cavity was detected in 28 (35.8%) patients.

Increased blood flow in the VA wall during Doppler examination was observed in 17 (21.7%) patients.

In 23 (29.4%) of the examined patients, ultrasound signs of unchanged AV or other pathologies of the right lower quadrant of the abdomen were detected. The distinctive feature of establishing the diagnosis through a DS is that the surgeon has the possibility of interpreting the results of investigations and symptoms in three categories: positive, negative, and doubtful, which, in our opinion, largely depends on personal qualification and experience. Laboratory diagnosis consisted of CBC and urinalysis, which were performed in all patients included in the clinical trial. In 13 (16.6%) patients, additional biochemical blood analysis was performed (amylase level, urea, creatinine, serum protein level, and bilirubin). Blood glucose analysis was performed in 56 (71.7 %) patients.

Examination of the ultrasound signs of AA can confirm or deny the diagnosis, as well as exclude abdominal surgical pathologies of the gallbladder and pancreas, and some gynecological pathologies.

Overall radiography of the abdomen was performed in 19 (24.3%) patients. Additionally, a gynecologist consulted 12 (15.3%) patients.

Following the examination, the patients were divided into three groups: the first group of patients, who underwent emergency surgery for AA; the second group of patients - who „accumulated" insufficient data to exclude or to confirm AA, and in our proposed algorithm for the implementation of the New DS was designated by us as a „grey area", and the third group of patients - in which the diagnosis of AA was excluded.

In the group of patients, in which AA was excluded, ulcer disease was diagnosed, chronic duodenal ulcer in exacerbation - 2 cases, urolithiasis, right renal colic - 1 case, acute pancreatitis - 2 cases, pelvic inflammatory disease - 2 cases, myxomatous node necrosis - 1, cr. Right ovarian cancer - 1 case, cancer. of cecum - 1, and functional bowel disorders - 3 (3.8%) cases.

Patients who did not „accumulate" enough data to exclude and confirm AA, 3 (3.8%) were admitted to the hospital, where they were evaluated and monitored dynamically for 72 hours. In all these patients, the diagnosis of AA was excluded.

Diagnostic laparoscopy was performed in 11 (14.1%) patients, of which 6 (5.1%) patients subsequently underwent laparoscopic appendectomy. From this group (laparoscopy + laparotomy) in 5 (6.4%) patients the diagnosis of AA was confirmed histologically. „Negative" appendectomy due to intraoperative overdiagnosis of AA was performed in one case. Based on the results of diagnostic laparoscopy, AA was excluded in 5 (6.4%) patients. The pathologies diagnosed by diagnostic laparoscopy were destructive acute appendicitis (AA) in 5 (6.4%) patients, simple acute appendicitis in 1 (1.2%) case, terminal ileitis in 1 patient, necrosis of the mammary nodule in 1 patient, ovarian cancer on the right in 2 patients, functional disorders of the intestine in 1 patient.

Patients in whom the diagnosis of AA was established based on the results obtained from the non-standardized diagnosis, underwent emergency surgical treatment, and laparoscopic appendectomy. At the histopathological examination, the diagnosis of AA was confirmed in 39 (73.5%) of the number of patients initially operated on – 53 (100%) cases. 14 (26.8%) people were found to have undergone „negative" appendectomy. Of 59 (75.6%) patients who underwent appendectomy (initial or after diagnostic laparoscopy), the diagnosis was confirmed in 44 (74.5%) and non-destructive forms of AA were established in 15 (26.4%) patients.

Following the analysis of the „negative" appendectomy protocols, it was demonstrated that in 8 (53.3%) cases, the non-destructive form of VA inflammation was diagnosed by the surgeon intraoperatively, but the appendectomy was still performed due to the surgical approach in the already present right iliac region. In 7 (46.7%) cases, an intraoperative hyper-diagnosis of AA was found, but it was not histologically confirmed.

Overall, AA was histologically confirmed in 45 (57.6%) patients, and in 33 (42.4%) patients, this diagnosis was excluded. Typical VA localization was observed in 31 patients (68.8% of the total number of operated patients). The atypical location of the VA was observed in 14 (31.2%) patients.

New DS implementation results

In parallel with the non-standardized clinical-paraclinical diagnosis of AA, in the group of patients under study, an assessment based on certain criteria of AA symptoms was

performed based on the New DS. According to the New DS, the following diagnostic criteria were recorded in the study group: positive Kocher symptom in 9 (11.5%) patients; nausea and/or vomiting in 41 (52.5%) patients; the positive Shchetkin-Blumberg symptom in the right iliac region in 25 (32%) patients; positive Bartomier-Michelson symptom in 17 (21.7%) patients; leukocytosis >10 x 10^9/l - in 39 (50%) patients.

The ultrasound data obtained in the patients of this study group showed that the determination of signs of another pathology and/or VA without signs of inflammation was detected in 20 (25.6%) patients; volume increase of VA diameter greater than 7 mm in 21 (26.9%) patients; AV incompressibility was determined in 19 (24.3%) patients; coprolite in the VA lumen - in 4 (5.1%) patients; thickening of the peri-appendiceal tissue - in 16 (20.5%) patients.

Considering that the indications for surgical treatment were established based on New DS, appendectomy was performed in 30 (38.2%) patients from the study group. With the sum of New DS scores >3, surgical intervention was performed in 27 (90%) cases, and histologically AA was confirmed in 21 (70%) patients. In 7 (8.9%) cases from this group of patients, based on the New DS, the diagnosis of AA was excluded, respectively, surgical treatment was avoided. Subsequently, those patients no longer requested specialized medical help.

Patients who accumulated 2 points – 6 (7.6%) cases, were assigned to the „grey area" of the New DS, of which 3 patients underwent diagnostic laparoscopy and subsequent appendectomy through laparotomy in one case. AA was histologically confirmed in 1 patient. The others – 4 (57.2%) patients, avoided appendectomy, AA being excluded. None of the patients with excluded AA required further medical attention.

Out of 41 (52.7%) patients with total New DS results < 2, surgery was performed in 31 (75.6%) patients. Histological AA was confirmed in 5 (12%) patients. The other 10 (24.4%) patients were not operated on, AA being excluded.

When evaluating the effectiveness of the New Diagnostic Score, the following results were obtained: sensitivity - 94.1%, specificity - 73.8%, precision - 79.6%, the size of the „grey area" - 7.6%, the proportion of AA in the „grey area" of the total amount of AA - 4.3%, the proportion of undiagnosed AA cases - 5.9%.

Comparative evaluation of the effectiveness indicators of the New DS. The comparative analysis of the New DS and the non-standardized diagnosis demonstrated the superiority of the respective indicators and the effectiveness of the diagnostic score. The high sensitivity of the New Diagnostic Score was statistically demonstrated ($\chi 2 = 4.32$; $p < 0.05$), a lower rate of missed AA cases in New Diagnostic Score ($\chi 2 = 4.32$; $p < 0.05$), the „grey area" is smaller in New SD ($\chi 2 = 5.28$; $p < 0.05$) than by non-standardized diagnosis. A lower rate of AA cases in the „grey area" of the total AA cases was demonstrated ($\chi 2 = 4.9$; $p < 0.05$).

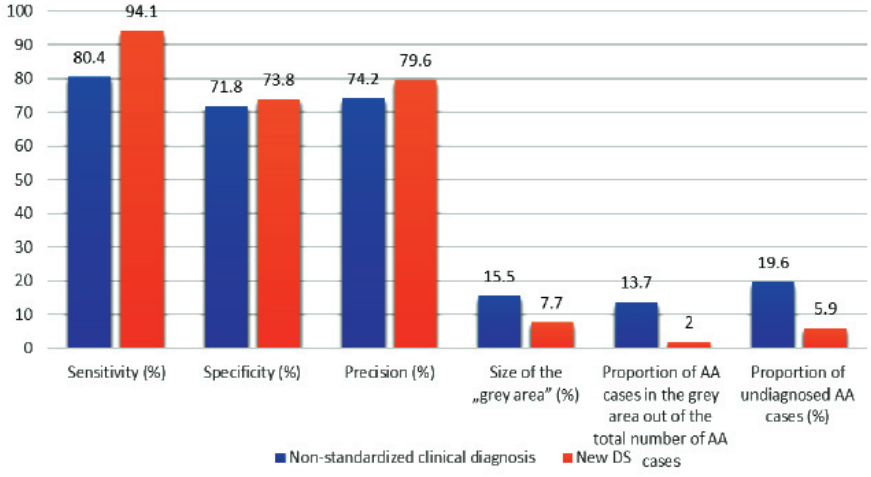

Fig. 2 Comparison of Non-standardized diagnosis and New DS in the diagnosis of acute appendicitis.
DS - Diagnostic Score; AA - Acute Appendicitis; „grey area" - Situations where diagnosis using new DS is not possible.

Based on these data, the comparative evaluation indicators such as specificity and diagnostic accuracy did not show significant statistical differences. At the same time, a definite increase in specificity and accuracy is noted in the case of New DS compared to the non-standardized clinical diagnosis.

Risk factors in the diagnosis of AA and evaluation of their impact on the effectiveness of the New DS

In specialized literature, it is indicated that it is difficult to diagnose AA using the clinical method and unstandardized DS in female patients, at a young age, in patients with atypical VA localization, obesity, and in geriatric patients. This leads to false positive and false negative diagnoses of AA [24 - 27]. We consider these circumstances as risk factors for the clinical diagnosis of AA. Considering the fact that New DS is based on clinical data, we conclude that this criterion may affect its performance indicators.

We considered necessary to study the effectiveness of the New DS in the presence of the indicated risk factors. We evaluated the performance indicators of New DS in the subpopulation with risk factors - obesity, atypical location of VA.

Table 4. Effectiveness of the New DS analysis according to risk factors

No.	Indicator of performance	General	Atypical location of VA	p*	Obesity BMI >25 kg/m^2	p*
1	Sensitivity (%)	93.15	92.2	>0.05	93.8	>0.05
2	Specificity (%)	73.06	72.3	>0.05	68.3	>0.05
3	Precision (%)	78.8	77.8	>0.05	79.5	>0.05
4	The size of „the grey area" (%)	7.5	8.3	>0.05	5.5	>0.05
5	The proportion of ADA from the „grey area" out of the total number of AA (%)	2	0	>0.05	3.1	>0.05
6	The ratio of undiagnosed cases of AA (%)	5.84	0	>0.05	3.1	>0.05

Note: *VA - vermiform appendix; BMI - body mass index; p - coefficient; ADA -acute destructive appendicitis; AA - acute appendicitis; „grey area" - Situations where diagnosis using new DS is not possible.*

Thus, the use of the New Diagnostic Score for the selected subpopulations did not demonstrate statistically significant differences in performance indicators compared to the universal population. This fact indicates the possibility of the universal application of the New DS developed by us on the population of elderly and senile patients, excluding people with central nervous system injuries, as well as obese patients. The sensitivity of New DS in typical localization of VA was 94.1% and in the atypical one - 92.2%. Thus, the atypical location of the AV does not affect the sensitivity of the New DS.

Comparative evaluation of the clinical efficacy of New DS and DS Alvarado.

According to the Jerusalem guidelines for the diagnosis and management of acute appendicitis in the general population, which recommend the use of scoring systems for the exclusion of AA in elderly patients compared to the low-probability score - DS AA Alvarado, we performed an analysis of the effectiveness of the New DS.

Few studies have evaluated the applicability of existing appendicitis diagnostic scores in the elderly population [28, 29]. A retrospective study of 96 patients over 65 years of age demonstrated that the use of the Alvarado scoring system, with a cut-off of 5, maintains reliability in elderly patients. In fact, the vast majority of patients with morpho-pathologically confirmed appendicitis (86.6%) had an Alvarado score ranging from 5 to 8 and 40% a score of 5 or 6. According to these data, Alvarado scores ranging from 5 to 10 should correspond to an increased risk of appendicitis in the elderly. Another retrospective study performed on 41 patients aged over 65 years presented an area under the curve (AUC) of the Alvarado score for this population of 96.9% with 100% negative and positive predictive values of the two cut-off points of 3 and 6 [30]. In the absence of high-quality evidence dedicated to the elderly, the multitude of experts could not make a strong recommendation; The Alvarado score is suggested for excluding but not diagnosing appendicitis in elderly patients, with a conditional recommendation based on low-quality evidence.

Another Diagnostic Score of acute appendicitis Tzanakis did not include the analysis performed, since, according to the structural-comparative analysis of it and its application algorithm, a very important diagnostic concept is missing in its structure, namely the presence of the „grey area", due to which, according to the data of the specialized literature, an unacceptably high number of non-destructive forms of AA was admitted (54% of operated patients), which is currently a very low indicator of the clinical efficiency of AA diagnosis.

Also, a comparative evaluation of the clinical effectiveness of the original clinical score with the RIPASA, Christian, Lintula scores, which are not focused on the diagnosis of destructive forms of AA, being developed on the basis of retrospective studies, without using statistical methods to calculate the diagnostic efficiency (MStA), was not performed.

In accordance with the recommendations of the National Clinical Protocol for the diagnosis and treatment of acute Appendicitis adopted and approved by the Moldovan *Nicolae Anestiadi* Association of Surgeons, all examined patients, according to DS AA Alvarado, were assigned as follows:

- 0-4 points (AA is unlikely) – 31 (39.7%) patients;
- 5-6 points (AA is possible and the patient needs observation) – 20 (25.6%) patients;
- 7-8 points (AA is probable) – 22 (28.2%) patients;
- 9-10 points (AA confirmed and the patient needs urgent surgical treatment) – 5 (6.4%) patients.

At the same time, we recommend that patients with score results of 7-8 and 9-10 points to be combined into one group, because the formulation of the algorithm for patients of these groups is ambiguous, AA being diagnosed in both cases, constituting 27 (34.6%) patients.

In 27 (34.7%) patients with a score between 7-10 points, surgery was performed in 18 (66.7%) cases. In 9 (33.3%) patients the diagnosis of AA was excluded without surgical intervention. Histologically AA was confirmed in 12 (44.4%) patients.

In 20 (25.6%) patients, with a score of 5-6, 17 (85%) patients underwent surgery, of which in 3 (15%) patients, the diagnosis of AA was excluded without surgery. Histologically AA was confirmed in 6 (30.0%) patients.

In 31 (39.7%) patients, with a score of 0-4, 26 (83.8%) patients underwent surgery; in 5 (16.2%) patients, the diagnosis of AA was excluded without surgery. Histologically AA was confirmed in 9 (29.0%) patients.

For a comparative evaluation of New DS with DS Alvarado with the help of the PASW Statistics 18 program, ROC analysis was performed with the construction of the corresponding curves (Fig. 3).

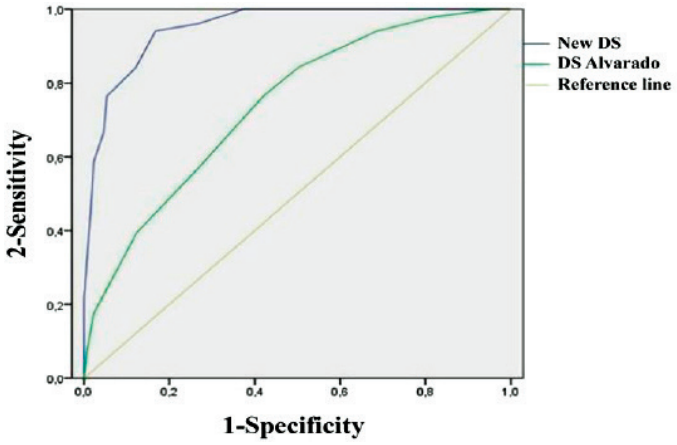

Fig. 3 ROC - curves for compared DS.
The area comparison curves of New DS and Alvarado DS,

The area under the curve for New DS was found to be statistically significantly higher compared to DS Alvarado and amounted to 0.95, which corresponds to the excellent quality indicator of the statistical model.

Table 5. ROC metrics (area under the curve) of New DS and Alvarado DS

Diagnostic scores	The area under the curve	95% - confidence interval	
New DS	0.952	0.924	0.981
Alvarado DS	0.739	0.662	0.816

Note: The area comparison curves of New DS and Alvarado DS

As a result of the comparative evaluation of New DS and Alvarado DS, it was observed that New DS has significantly higher sensitivity, specificity, and accuracy, and the number of undiagnosed AA cases compared to Alvarado DS is lower. If Alvarado's AA DS had been used in undiagnosed AA cases, there would have been 1.6% perforated, gangrenous, and complicated AA.

The size of the „grey area" and the weight of the „grey area" of AA, out of the total number of AA in New DS, was significantly smaller than in DS Alvarado ($p < 0.001$). The comparative evaluation of the main performance indicators New DS and DS Alvarado is shown in Fig. 3.

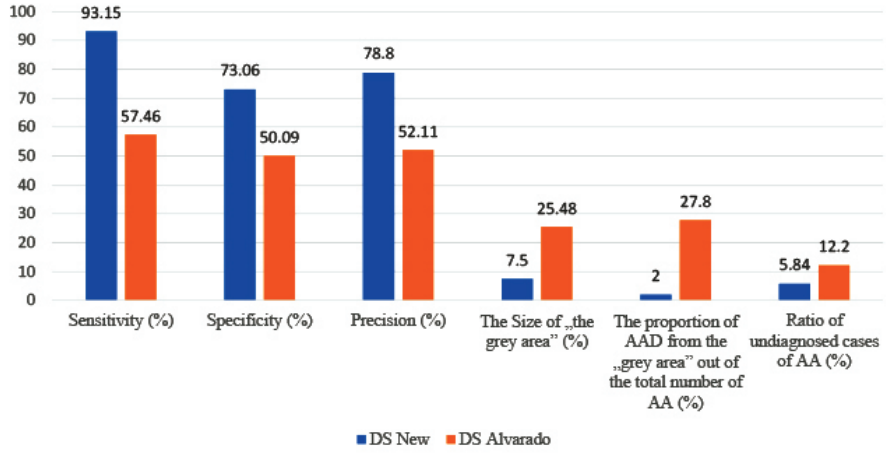

Fig. 3 The comparison of the performance of New DS vs Alvarado DS
ADA - acute destructive appendicitis; AA -acute appendicitis; „grey area" - Situations where diagnosis using new DS is not possible.

Thus, New DS showed greater clinical effectiveness in diagnosing AA in the elderly compared to the non-standardized clinical method and DS Alvarado, a lack of dependence on „risk factors" for diagnosing AA, such as obesity and atypical location of VA.

Discussion

This study evaluated the acceptability of the Alvarado scores and the developed New DS in determining the diagnosis of AA in elderly patients.

Early diagnosis of AA is quite laborious in elderly people, having a high mortality and morbidity rate. In a study conducted in Finland, the data of 164,579 patients who underwent appendectomy surgery were examined over a 20-year period, and mortality increased 39 times in patients over 60 years. Similarly, the same study determined that negative appendectomy increased four-fold and mortality increased 10-fold. In the literature, the rate of negative appendectomy in geriatric patients ranges from 17% to 31% [3-4]. In the current cohort, the negative appendectomy rate was 28.3%, which is in accordance with the specialized literature.

Due to increasing life expectancy, diseases previously associated with the younger population, including AA, have an increasing incidence among elderly patients [6]. Although the lifetime risk of AA is 7% for the general population, this rate may increase to 10% among the elderly population [2]. As in most diseases, the clinical diagnostic process of AA is more difficult in the geriatric population than in the young. This is due, in part, to altered pain sensations due to impaired nerve conduction as a result of aging and the atypical picture of classical AA [6]. Since a delayed diagnosis will increase the mortality and morbidity of AA, international guidelines and evidence-based medicine guidelins recommend the use of clinical scoring systems in the initial evaluation process of patients [15].

The Alvarado score [17-19] being a 10-point scale based on indications, symptoms, and laboratory data, is one of the

most widely used and evaluated scoring systems for the assessment of AA. A score of 5 or 6 points on the Alvarado scale is considered compatible with the diagnosis of AA; a score of 7 or 8 suggests a plausible diagnosis of AA; and a score of 9 or 10 indicates a very likely diagnosis of AA. This diagnostic score was designed to assist clinicians in clinical decision-making by objectively determining which patients should be monitored and evaluated and which should be operated on. The limited research that assessed the relevance of the Alvarado score in the elderly population, retrospectively analyzing 96 patients over 60 years of age, using the Alvarado score system with a cut-off value of 5, demonstrated high efficacy in the elderly [17]. In another study, the Alvarado and Lintula scores were compared in elderly patients undergoing appendectomy, and the former was found to be a more useful predictive tool, with an AUC value of 96.9% [18]. Another research, however, demonstrated that the Alvarado score is ineffective in elderly people [5].

There are, however, some limitations to our study. First, the results obtained by us cannot be generalized to the general population, since they were obtained from a single center. Second, because this study was retrospective, the results may have been influenced by inadequate or erroneous data from hospital records. Another disadvantage is the small group of patients.

Conclusions

The use of the diagnostic score in the elderly will raise the quality of care, reduce the amount of time it takes to diagnose a similar case and as a result - lead to a reduction in complications and mortality in acute appendicitis. The study of the efficacy of new AA DS by comparative evaluation with the traditional non-standardized clinical diagnosis of AA and Alvarado AA DS demonstrated higher clinical efficiency in diagnosing AA with sensitivity up to 93.15% compared to the non-standardized clinical method and Alvarado AA DS and also does not depend on „risk factors" for AA diagnoses such as obesity and atypical location of AV, due to which we recommend wide application in medical practice.

Competing interests

None declared.

Patient consent

Obtained.

Ethics approval

This study was approved by the Research Ethics Committee of *Nicolae Testemiţanu* State University of Medicine and Pharmacy (Minutes No. 25, from 21.11.2016).

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RESEARCH ARTICLE

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Early results of endovascular treatment using percutaneous vacuum-assisted thromboaspiration in acute lower limb ischemia

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ABSTRACT

Introduction. Open balloon thrombectomy and embolectomy remain the preferred initial option in the management of acute lower limb ischemia (ALI), but various endovascular techniques have become accessible and are growing in popularity. The aim of the study was to assess our early experience with percutaneous vacuum-assisted thromboaspiration using the Penumbra/Indigo® system for non-traumatic ALI.

Material and methods. The study group comprised 13 patients with ALI who received treatment between September 2022 and June 2023; with 7 (53.8%) being males. The median age was 71 years (25%-75%IQR 62.5-77.5). ALI cases were classified according to the Rutherford scale: grade I – 2 (15.3%), grade IIA – 7 (53.8%), and grade IIB – 4 (30.7%). In 10 (76.9%) cases, ischemia was classified as "acute-on-chronic." The occluded native vascular segment, determined through preoperative computer tomography angiography (n=4; 30.7%), duplex scanning (n=5; 38.4%), or both examinations (n=4; 30.7%), were as follows: superficial femoral artery (n=7) and popliteal artery (n=2). In two patients, thrombosis of the below-knee femoropopliteal bypass with autogenous vein was identified, while two others presented with femoral artery stent thrombosis. An embolic etiology of ALI was observed in 4 (30.7%) cases, and thrombotic etiology in 9 (69.2%) cases. Endovascular access was established via the ipsilateral common femoral artery (n=10), crossover (n=2), or brachial artery (n=1). Thromboaspiration was carried out using dedicated CAT6™ and CAT8™ catheters.

Results. The technical success rate of vacuum-assisted thromboaspiration was 92.3%. Subsequent angiography revealed accompanying occlusive-stenotic lesions in all instances, necessitating transluminal angioplasty, and in 8 (61.5%) patients, additional stenting was required. Catheter-directed thrombolysis was utilized as an adjunct in one patient. There were 2 (15.3%) instances of distal embolization, both addressed within the same surgical session. Survival and limb salvage rates at the 30th-day follow-up stood at 100%.

Conclusions. Utilizing the Penumbra/Indigo® system, percutaneous vacuum-assisted thromboaspiration appears to be a safe and effective minimally invasive technique for treating ALI. This method allows for the concurrent correction of coexisting chronic peripheral arterial lesions.

Keywords: acute lower limb ischemia, balloon embolectomy, endovascular treatment, vacuum-assisted thromboaspiration.

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Key messages

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

Since open thromb/-embolectomy and catheter-directed thrombolysis remain the first-choice options in the treatment of acute lower limb ischemia (ALI), the role of other endovascular techniques, including percutaneous aspiration thrombectomy, is not yet well defined.

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The research hypothesis

We assume that percutaneous vacuum-assisted thromboaspiration using Penumbra/Indigo® device and dedicated catheters (CAT™) can be suitable for ALI of multiple etiology.

The novelty added by manuscript to the already published scientific literature

In the context of the first experience applying percutaneous vacuum-assisted thromboaspiration using Penumbra/Indigo® system in the Republic of Moldova, we identified that this is a safe and effective minimally invasive technique for the treatment of non-traumatic ALI of various causes: embolism, native arteries or stent thrombosis, acutely occluded femoropopliteal bypass with autogenous vein conduit; as well as for "acute-on-chronic" ischemia. The method allows simultaneous correction of coexisting chronic peripheral arterial lesions. Intraoperative distal embolization occurs rarely and can be solved during the same surgical session.

Introduction

Acute lower limb ischemia (ALI) is characterized by a sudden decrease in arterial perfusion of the pelvic extremity, potentially threatening the viability of the respective anatomical segment and requiring urgent evaluation and treatment [1]. ALI remains one of the most frequent vascular surgical emergencies, being associated with a high rate of amputation of the affected extremity and a mortality rate surpassed only by that recorded in cases of ruptured abdominal aortic aneurysm [2, 3].

Embolism, in-situ thrombosis of the native artery, stent or vascular graft thrombosis, arterial trauma, or a complicated peripheral aneurysm (sac thrombosis or distal embolization) stand among the common etiological factors of ALI [2, 4]. Atrial fibrillation and mural intracardiac thrombosis that develop after myocardial infarction are presently more frequently noted sources of peripheral embolism [4]. Concurrently, a significant proportion is attributed to iatrogenic embolism, which arises intraoperatively during percutaneous transluminal angioplasty (PTA) for peripheral arterial disease of the lower extremities – an endovascular intervention that is frequently performed in the daily practice of vascular surgery services [5]. In the same context, acute stent thrombosis, particularly at the level of the femoropopliteal artery, is diagnosed in over 6% of cases [6].

Restoring the patency of the arterial lumen as swiftly as possible, ideally within the initial 6-8 hours following the onset of ALI, is crucial for preserving the limb and constitutes the primary objective of treatment [4]. Conventionally, this is achieved through open surgery – thromb/embolectomy using the Fogarty balloon catheter. The technique is characterized by its surgical simplicity, cost-effectiveness, speed, and accessibility, and it is largely clinically effective, particularly in cases of embolic ALI where a single arterial segment is obstructed, especially above the knee [3, 7]. However, the same intervention doesn't yield a similar technical success in the presence of organized embolic masses situated within small-caliber arteries or when embolism occurs against the backdrop of peripheral arterial disease – a situation known as "acute-on-chronic" ischemia [7]. By the way, the latter is being increasingly registered in recent studies dedicated to peripheral arterial embolism [1]. Advancing the balloon catheter towards the infrapopliteal vessels, especially in diabetic patients where distal occlusive-stenotic lesions are characteristic, may encounter difficulties [8, 9]. Also, it's important to take into account that the procedure is typically carried out blindly in the vast majority of cases, without the option of separately guiding the catheter towards the lumen of each calf artery (tibial or peroneal arteries). The routine practice of performing fluoroscopic-assisted balloon thrombectomy or intraoperative angiography is still limited [3, 7, 8, 10]. In the clinical circumstances mentioned above, the extraction of thrombotic masses with the Fogarty catheter often remains incomplete, with the documented rate of residual thrombosis in small-caliber (distal) arteries varying between 36% and 86% [3].

Nonetheless, even in the absence of pre-existing atherosclerotic lesions, the indirect surgical embolectomy using a balloon catheter can be linked to the migration of thrombotic masses (resulting in distal embolization or, conversely, propagation in the proximal direction) or harm to the arterial wall (such as dissection or perforation), delayed pseudoaneurysm or arteriovenous fistula formation, and diffuse arterial narrowing due to intimal proliferation [5, 11]. Therefore, in cases of ALI, more intricate surgical interventions for limb revascularization might frequently be necessary: open procedures (like endarterectomy or bypass surgery) or hybrid approaches (combining open surgery with endovascular techniques) [12].

In an effort to address the aforementioned deficiencies in open surgical ALI treatment over the past two decades, specialized medical companies have introduced new technologies and devices for percutaneous revascularization. Consequently, the current array of endovascular treatment methods applicable to ALI patients encompasses: catheter-directed thrombolysis; ultrasound-accelerated

thrombolysis; percutaneous mechanical thrombectomy involving rheolytic or fragmentation (rotational) techniques; pharmacomechanical thrombectomy; simultaneous angioplasty combined with thrombolytic irrigation (SATI technique); as well as manual percutaneous aspiration thrombectomy or the utilization of devices offering continuous automatic thromboaspiration [4, 7, 13]. To the latter group is also attributed the Penumbra Indigo® (Penumbra Inc., Alameda, CA, USA) – a device designed for extracting thrombotic masses/emboli from the lumen of peripheral vessels through percutaneous vacuum-assisted aspiration, applicable in cases of ALI and venous thrombosis. The initial data reported up to this point for the utilization of the Penumbra Indigo® system in ALI seem promising, but the overall evidence remains limited. The objective of the current study was to present the preliminary results of our initial experience with the application of percutaneous vacuum-assisted thromboaspiration in patients with non-traumatic ALI resulting from infrainguinal occlusions.

Material and methods

The study was carried out in the University Vascular Surgery Clinic, Chair of General Surgery-Semiology No.3 of the *"Nicolae Testemiţanu"* State University of Medicine and Pharmacy (Department of Vascular Surgery, Institute of Emergency Medicine, Chişinău), during the period September 2022 – June 2023. Informed consent was acquired from all subjects encompassed by the study. The data from the electronic register of prospective records of patients who underwent revascularization surgery for ALI were evaluated, and cases treated with the application of percutaneous thromboaspiration using the Penumbra Indigo® system were selected for further analysis. The mentioned research received approval from the institutional Ethics Committee, within the project dedicated to the study of acute ischemia of the extremities (No.1 of 16.02.2021).

Features of Penumbra Indigo® system. As per the manufacturer's specifications, the Indigo® device (Fig. 1A) comprises the subsequent components: Penumbra Engine® aspiration source, Penumbra Engine canister, Indigo aspiration tubing equipped with a valve switch for system activation and deactivation, Indigo Separator™, and Indigo CAT™ mechanical thrombectomy catheters.

The aspiration source is capable of providing a pure, continuous vacuum (-29 in Hg / 736.6 mm Hg / 98.2 kPa / 0.96 Atm), enabling the elimination of thrombi from the lumen of vessels with various diameters. This capability is also attributed to the availability of an extensive array of catheters with different diameters and lengths, designed to be tapered and resistant to collapsing (Fig. 1B): CAT3, CAT5, CAT6, CAT8 (including various tips: STR / TORQ / XTORQ), CAT D, CAT RX, or CAT7 and CAT12 – the latter two being of a newer generation.

Technical aspects of percutaneous vacuum-assisted thromboaspiration. Following the establishment of endovascular access and the placement of a 6F or 8F sheath,

Fig. 1 General aspect of Penumbra Indigo® system (A) and the dedicated CAT6™ and CAT8™ mechanical thrombectomy catheters (B).

digital subtraction angiography (DSA) was conducted to confirm the location and extent of arterial occlusion. The guide-wire was advanced through the occlusive lesion, and subsequently, the dedicated thromboaspiration catheter was guided towards the proximal end of the lesion. After the catheter tip engaged the thrombus, the suction pump was activated, with a wait time of approximately 90 seconds to enable the creation of negative pressure. Subsequently, the suction tube switch was turned on, and the catheter was gradually withdrawn. Confirmation of thrombi aspiration was visual (by observing the presence of thrombotic masses in the canister after defoaming its contents), and also through DSA. In the presence of co-existing or underlying hemodynamically significant occlusive-stenotic lesions, their simultaneous endovascular treatment was applied, depending on the assessment of the operating surgeons.

Definitions and data interpretations. As per the guidelines, acute limb ischemia was defined with symptom duration less than 2 weeks [1]. The degree and clinical categories of ischemia were evaluated in line with the widely

acknowledged Rutherford ALI classification system, incorporating criteria such as sensory loss, motor deficit, prognosis, and Doppler signals: gr. I (viable), gr. IIA (marginally threatened), gr. IIB (immediately threatened), gr. III (irreversible).

As primary endpoints of the study, the technical and clinical success of thromboaspiration were assessed, with secondary endpoints encompassing the rates of complications, primary patency, limb salvage, and 30-day mortality. Technical success was defined as restoring antegrade blood flow with near/complete aspiration of the embolus/thrombus and maintaining patency in at least one run-off vessel. For interpreting the technical results, the adapted classification for Thrombo-aspiration In Peripheral Ischaemia (TIPI), modified from the Thrombolysis in Myocardial Infarction (TIMI) classification, was employed [14]. Success was indicated by an increase of at least ≥1 point in comparison to the baseline score: 0 (no recanalization of the thrombotic occlusion), 1 (incomplete or partial recanalization of the thrombotic occlusion with no distal flow), 2 (incomplete or partial recanalization of the thrombotic occlusion with any distal flow), and 3 (complete recanalization of the thrombotic occlusion with normal distal flow).

Clinical success was defined as a post-interventional relief of ALI symptoms and an upward shift of at least one grade in the Rutherford classification. Complications/adverse events were categorized using the CIRSE classification system: gr.1 (can be resolved within the same session), gr.2 (requires prolonged observation <48 h), gr.3 (prolonged hospital stay >48 h or additional post-procedure therapy), gr.4 (causes permanent mild sequelae), gr.5 (causes permanent severe sequelae), and gr.6 (results in death) [15]. Primary patency was determined as a target lesion without haemodynamically significant stenosis (>50%) or re-occlusion on duplex scanning, conducted on the first postoperative day and at one month.

Continuous variables are presented as medians with interquartile range (25%-75%IQR), while categorical variables are represented as percentages.

Results

Patient data and ALI characteristics. The study group comprised 13 patients with ALI resulting from unilateral occlusion of the femoropopliteal arterial segment; 7 (53.8%) were males. Subjects' ages ranged from 43 years to 83 years, with a median value of 71 (25%-75%IQR 62.5-77.5) years. The right lower extremity was affected in 8 (61.5%) cases. All patients exhibited characteristic ALI symptoms (classic "6 P's"), occurring over 72 (25%-75%IQR 24-96) hours after onset (4-302). Thrombosis (n=9; 69.2%) or embolism (n=4; 30.7%) was identified as the etiological factor of ALI. According to the Rutherford classification, cases were distributed as follows: gr. I – 2 (15.3%), gr. IIA – 7 (53.8%), and gr. IIB – 4 (30.7%). In 10 (76.9%) instances, ischemia was categorized as "acute-on-chronic". Among comorbidities, the following were notable: arterial hypertension (n=13), chronic heart failure (n=13), ischemic heart disease (n=9), normo- (n=1) or tachysystolic (n=5) atrial fibrillation, diabetes mellitus (n=3), and chronic obstructive pulmonary disease (n=2). Each patient presented with at least three chronic diseases.

All patients were on chronic anticoagulant/antiplatelet treatment, while 6 (46.1%) were receiving antiarrhythmic medication. In 3 (23%) cases, a recurrent episode of ALI was identified, with the patients having undergone previous open embolectomy at the same limb level. Two (15.3%) patients had a history of superficial femoral artery stenting for chronic occlusive-stenotic lesions, and two others had undergone femoropopliteal below-knee autogenous vein bypass. Laboratory data revealed leukocytosis (n=6), hyperfibrinogenemia (n=6), mild anemia (n=4), and thrombocytopenia (n=2). All patients received therapeutic doses of anticoagulants upon admission: sodium heparin (n=10; 76.9%) or enoxaparin (n=3; 23%).

The occluded arterial segment determined by preoperative computer tomography angiography (n=4; 30.7%), duplex scan (n=5; 38.4%), or both examinations (n=4; 30.7%) included the superficial femoral artery (n=7) and popliteal artery (n=2). Thrombosis of the femoropopliteal below-knee bypass was identified in two patients, while two others experienced acute stent thrombosis of the superficial femoral artery.

Results of the application of thromboaspiration and adjuvant endovascular techniques.

Patients underwent revascularization through percutaneous mechanical thromboaspiration using the Penumbra/Indigo® system as a primary or salvage intervention within 9 (25%-75%IQR 2.5-48) hours after hospitalization (2-96). In 11 (84.6%) cases, thromboaspiration was performed with local anesthesia, and in two others – under spinal anesthesia. Endovascular access was established via the ipsilateral common femoral artery (n=10), crossover (n=2), or brachial artery (n=1). DSA before thromboaspiration revealed thrombotic occlusion with no distal flow (TIPI score = 0) in all instances. Thromboaspiration was conducted using dedicated CAT6™ (n=3) and CAT8™ (n=10) catheters as the initial choice, depending on the diameter of the targeted vessel. Technical success (TIPI score = 2-3) was achieved in 12 out of 13 (92.3%) cases. In one patient with bypass thrombosis, despite recanalization of the autologous venous conduit and subsequent PTA for infrapopliteal occlusive lesions, restoration of distal flow was not possible. Consequently, a decision was made to opt for catheter-guided thrombolysis.

Control angiography following aspiration revealed associated infrainguinal occlusive-stenotic lesions in all cases, necessitating the use of adjunctive techniques: PTA, and in 8 (61.5%) cases – additional stenting of the femoropopliteal segment (Fig. 2). Distal embolization was identified during thromboaspiration in two cases, resulting in a perioperative complication rate of 15.3%. It's important to note that both instances were readily resolved during the same operative session through repeated aspiration (gr.1 CIRSE).

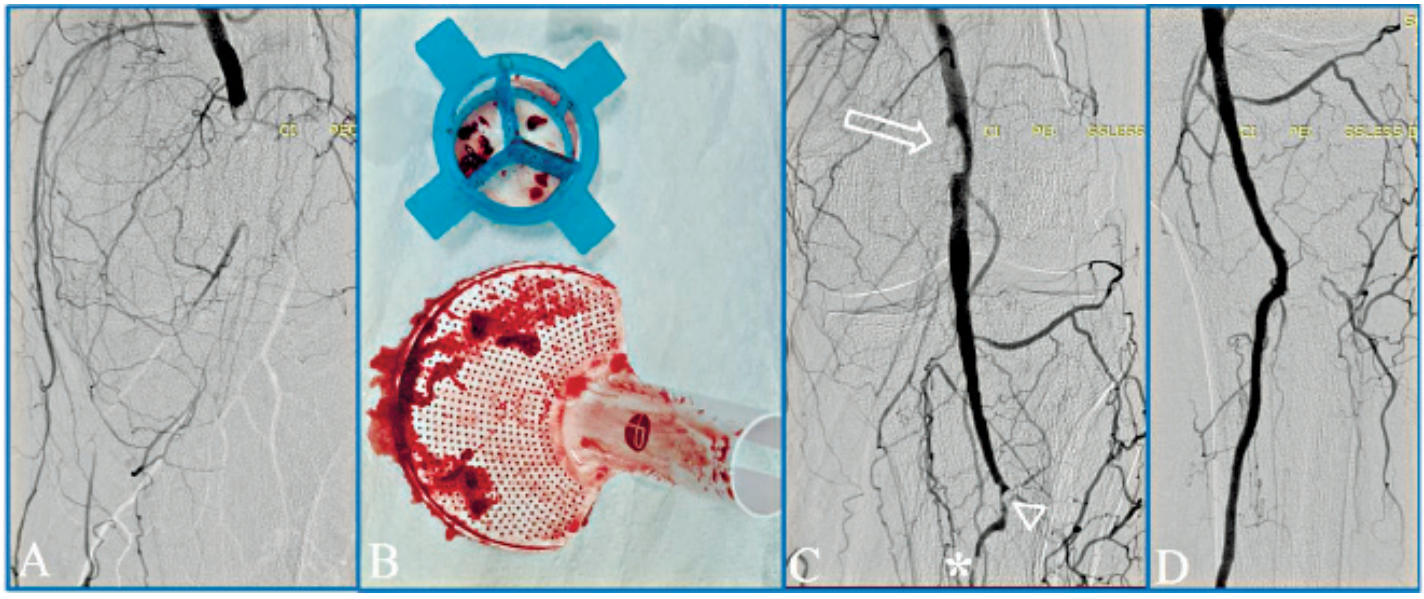

Fig. 2 Angiographic images captured during percutaneous thromboaspiration using the Penumbra/Indigo® system and the extracted thrombotic masses.

Note: (a) – popliteal artery occlusion at diagnostic angiography; (b) – thrombotic masses aspirated into the canister of Indigo® device; (c) – persistent intraluminal embolus (arrow), intraoperative distal embolization (arrowhead) and concomitant chronic arterial lesion (asterisk) identified after initial thromboaspiration; (d) – restoration of blood flow at the completion angiography, after iterative aspiration followed by percutaneous transluminal angioplasty.

The duration of surgical interventions ranged from 60 minutes to 160 minutes, with a median value of 120 (25%-75%IQR 72.5-120) minutes. The volume of intraoperative blood loss (aspirated into the canister) ranged from 260 ml to 480 ml. No patients required postprocedural blood transfusion. The clinical success rate was 92.3%. Follow-up duplex scanning confirmed the preservation of primary patency in all cases. There were no instances of death within the 30-day period, and the limb salvage rate was 100%.

Discussion

Despite its multiple shortcomings and potential perioperative complications, open thrombectomy with the Fogarty catheter remains the standard approach for ALI caused by embolism [1]. In contrast, the present study reflects the results of the initial experience of percutaneous vacuum-assisted thrombectomy using the Penumbra Indigo® system for ALI in the Republic of Moldova. In all four of our cases considered with embolic etiology, coexisting occlusive-stenotic lesions were identified after percutaneous thromboaspiration, necessitating adjunctive treatment – PTA or stenting. It can be assumed that in these cases, the standard approach might have overlooked the associated lesions with significant hemodynamic impairment, potentially affecting clinical outcomes or necessitating additional surgery. Moreover, three patients had previously undergone surgery for embolism, potentially exposing them to the associated risks of repeated procedures. Generally, considering the substantial percentage of cases presenting with "acute-on-chronic" ischemia (76.9%) and the dominance of arterial thrombosis (69.2%), the conventional open approach within the studied group likely would have necessitated complex revascularization interventions instead of simple balloon thromb-/embolectomy. Given the patients' advanced age and the elevated prevalence of comorbidities, we hold the view that bypass surgery might have been linked to notably higher morbidity rates compared to those observed following the application of the percutaneous technique.

The current guidelines suggest catheter-directed thrombolysis as an alternative to surgery in ALI, with both methods demonstrating comparable clinical outcomes [1, 3, 16]. However, thrombolysis has somewhat more limited indications, being recommended for less severe ALI cases (Rutherford grade IIA); and the rate of complications such as intracranial hemorrhage, major bleeding requiring surgery or transfusion, distal embolization, and compartment syndrome remains high (13%-30%) [11, 14, 17]. Furthermore, the approach demands meticulous monitoring in the intensive care unit with the requirement for subsequent angiography; it is time- and resource-intensive, and the reported high rate of technical success (up to 90%) can be attained only after the accumulation of extensive personal experience [16, 18]. Other previously mentioned endovascular techniques are also associated with a certain percentage of specific complications: hemorrhages, distal embolization (pharmaco-mechanical thrombectomy), hemolysis and renal failure (rheolytic thrombectomy), vessel injury (ultrasound-enhanced thrombolysis) [4, 19].

Among the endovascular techniques potentially associated with reduced periprocedural risks, percutaneous

thromboaspiration stands out as a treatment option for ALI patients. In 1978, Horvath *et al.* first proposed the use of intra-arterial catheter aspiration to address iatrogenic embolism related to PTA [5]. Manual aspiration thrombectomy was subsequently described by Snidermann *et al.* in 1984 and successfully implemented using sheaths and catheters by Starck *et al.* in 1985 [11, 14, 20]. Traditionally, this procedure involves the use of a large-bore catheter and manual (50 ml) syringe aspiration, making it a cost-effective and widely available approach with a high technical success rate, ranging between 87% and 96% [21, 22]. Manual thromboaspiration has long been used as a complementary technique to thrombolysis, but current viewpoints suggest that it can also serve as the primary choice, with thrombolysis reserved for cases of treatment failure [10]. However, the method has several limitations. One of the primary concerns is the occurrence of sudden pressure changes during aspiration due to the inability of manual suction to maintain a stable (negative) pressure, which could potentially lead to distal embolization or the movement of the clot in a proximal direction [4, 7]. Additionally, the occurrence of arterial spasm, iatrogenic intimal dissections, or thrombosis is not negligible, mainly due to the necessity for multiple catheter movements [11, 20, 22].

Several devices for automatic thromboaspiration have recently been introduced, among which is the Penumbra Indigo® system. Initially, in 2005, the Penumbra mechanical thrombectomy system, utilizing vacuum aspiration as its primary mechanism of action, became available for revascularizing occluded intracranial vessels in patients with acute ischemic stroke [2, 14]. Following a successful initial experience in treating stroke, acute pulmonary embolism, and renovisceral occlusions, another device was launched in 2014 – the Penumbra Indigo® aspiration thrombectomy system, specifically designed for peripheral applications [5, 14]. The summarized early outcomes, reflecting the experiences of conducting peripheral thromboaspiration for ALI using the Penumbra Indigo® system across various medical centers, in addition to our own data, are outlined in Table 1.

Table 1. Concise synthesis of early outcomes of percutaneous thromboaspiration for ALI using the Penumbra Indigo® system from international experience.

Author (year)	Number of cases	(Assisted) technical success rate	Limb salvage rate at 30 day
Gandini *et al.* (2015) [8]	3*	100%	100%
Baumann *et al.* (2016) [18]	33	(53.9%)†	-
Saxon *et al.;* PRISM trial (2017) [11]	79‡	(96.2%)	97.5%
Kwok *et al.* (2018) [19]	15	53.3%	100%
Lopez *et al.* (2020) [16]	43	51%	88.4%
Farhat-Sabet *et al.* (2020) [5]	4	100%	100%
de Donato *et al.;* INDIAN trial (2021) [14]	150	88.7% (95.3%)	99.3%
Zied *et al.* (2021) [21]	19	(94.7%)	100%
Rossi *et al.* (2021) [12]	33	70% (90%)	87.8%
Present study (2023)	13	(92.3%)	100%

Note: *treated with Penumbra system (non-dedicated); †for above-the-knee occlusions; ‡52 cases – treated with Penumbra system (non-dedicated), 27 cases – treated with Indigo® system.

Percutaneous aspiration thrombectomy is suitable for patients with anticipated difficulty during surgical embolectomy (morbidly obese, previous groin surgery) or an anticipated need for adjuvant endovascular procedures [21], as well as in cases when catheter-directed thrombolysis is contraindicated [11, 14]. As in the case of the application of many other techniques, the success of the method is determined by the selection of appropriate cases. More acute thrombus, presumably softer and more malleable, has a higher probability of successful removal [12, 18]. Despite the delayed referral of the patients in the current study (median value of ALI onset–presentation time being 72 hours), we obtained a high clinical success rate, presumably due to the predominance of atherothrombotic cases with well-developed collateralization.

The location of the arterial occlusion is considered a factor that can influence the technical success of the method. In the below-knee segments, the technique has a higher reported rate of success because the lesions are more often iatrogenic, shorter, and there is better concordance between the diameter of the vessel and the catheter [21]. Vice versa, a lower success rate in the above-knee lesions may be explained by a larger mismatch between the vessel size and the thrombectomy catheter size [18]. Therefore, the use of larger catheters, even in smaller vessels, is favored and believed to provide better thrombus removal [16].

Vacuum-assisted thromboaspiration minimizes the risk of endothelium injury and potential iatrogenic distal embolization, which are typical for open surgery [5, 11]. The reported rate of the latter during endovascular interventions is 1-5%, while in some studies it can be as high as 24% [22]. However, the actual frequency of this complication is believed to be much higher, estimated at 30-50%; fortunately, it often remains clinically silent [20, 22]. The open surgical approach in such cases, using the Fogarty balloon catheter in the below-knee arterial segment, could be ineffective due to the difficulty in directing the catheter into crural and foot arteries, and therefore does not appear to be an equivalent alternative to endovascular treatment [8]. In our study group, two (15.3%) cases of distal embolization were

identified. However, it should be noted that these occurred during our initial practical experience when the full range of dedicated catheters was not yet available. Fortunately, both complications were ultimately resolved within the same intervention by repeating the aspiration procedure. Jung Guen Cha *et al.* reported a similar rate of distal embolization (16.7%). It's important to mention that the authors used a Penumbra aspiration catheter and a simple syringe instead of an automated device [3]. Overall, the rate of perioperative complications associated with the use of vacuum-assisted thromboaspiration is acceptable, ranging from 2% to 14%, being basically non-device specific [3, 14, 16].

Under the aspect of interventional technique, the use of access sheaths with removable check-flow valves that can be replaced, or rotating hemostatic valves, is considered beneficial. The last one allows for easier introduction of the catheters without damage to the tip, as well as removal of the clot intact when "corked" at the end of the aspiration catheter [11]. Even if its use remains at the discretion of the operating surgeon, the Indigo Separator™ allows thrombus disruption at the tip of the catheter ensuring its patency and makes possible fragmentation of the clot with cleaning the lumen without catheter removal [11, 14]. In general, it is believed that better results are related to a more precise technique, including the 1:1 sizing of the CAT™ catheter to the target vessel diameter in all cases, the application of the Separator™ in almost half of the cases, and the use of more than one catheter per case when necessary [14].

Judicious use of suction control by switching the on/off position of the tube valve and intermittent application of vacuum-assisted aspiration became important tricks for minimizing blood loss [11, 16]. By the way, the volume of hemorrhage in our study was comparable to the amounts described by other authors – on average 240 ml (up to 600 ml) [14].

To the disadvantages of percutaneous aspiration thrombectomy compared to catheter-directed thrombolysis can be attributed a potentially higher risk of traumatic injury to the endothelium and the inability to infuse lytic agents into collaterals or run-off arteries that are too small for an aspiration catheter [3]. However, it is important to mention that in the case of failure of vacuum-assisted thromboaspiration as an initial approach, the possibility of resorting to thrombolysis, embolectomy, or bypass surgery is not ruled out [14, 18]. In a similar context, unsuccessful open thromb-/embolectomy does not exclude the possibility of subsequent application of percutaneous thromboaspiration [3, 23].

Currently, other automated systems for percutaneous aspiration thrombectomy in ALI are also available, such as *ClearLumen-II* (ClearLumen-II, Groupates), which includes pulse spray thrombolysis; *Aspirex* (Straub Medical AG), which provides fragmentation of the thrombus by the spinning steel helix; or *ThromCat XT* (Spectranetics International, Leusden, The Netherlands), which implies rotational thrombectomy [7, 9]. More recently, the *Indigo® Lightning™ 7* system, followed by the *Lightning Bolt™ 7* and *Lightning Flash™,* have been implemented, providing intelligent computer-aided mechanical aspiration. Nevertheless, due to the limited evidence regarding the comparative efficacy of the respective methods, at the moment, it cannot be concluded that one of the thromboaspiration techniques is obviously superior in cases of ALI.

In conclusion, the Indigo® system represents a modern, minimally invasive technology with high effectiveness and a low rate of complications, suitable for both primary treatment of ALI and salvage or secondary therapy. The system setup is straightforward and doesn't necessitate the use of adjunctive devices or thrombolytic agents. Advantages of the technique include immediate restoration of blood flow and the absence of the need for protection filters. The availability of dedicated catheters with different diameters allows for reaching clots in very distal arteries, overcoming a limitation present in other techniques [8, 14]. The diverse clinical scenarios suitable for applying this technique to ALI patients, as also confirmed in our study (embolism, atherothrombosis, stent or bypass thrombosis, "acute-on-chronic" ischemia), highlight the wide applicability of the method. These factors could potentially contribute to a shift in the treatment trend for ALI in the near future.

Conclusions

Percutaneous vacuum-assisted thromboaspiration using the Penumbra/Indigo® system appears to be a safe and effective minimally invasive technique for treating ALI, providing the opportunity for concurrent correction of concomitant chronic peripheral arterial lesions.

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. 1 of 16.02.2021).

Authors' contributions

AP performed data collection and drafted the manuscript. VC participated in study design, performed statistical analysis, interpretation of data, and helped drafting the manuscript. DC conceived the study, interpreted the data and helped drafting the manuscript. All the authors reviewed the work critically and approved the final version of the manuscript.

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REVIEW ARTICLE

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Venous and arterial endothelium: markers of dysfunction and pathophysiological significance

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ABSTRACT

Introduction. Endothelial dysfunction is a result of complex pathogenic interface involving inflammation, oxidative stress, disorders of endothelization and hemostasis etc., in both arteries and veins, leading to a lot of cardiovascular diseases. Identifying markers with high predictive value has an important diagnostic and prognostic significance.

Material and methods. To create this review article, we conducted a thorough search for relevant references that are current, specific, and aligned with the goals of the article. We utilized databases such as PubMed, MEDLINE, Google Scholar, and Cochrane, going as far back as the year 2000 to gather the necessary information. The identified articles were structured based on the main objectives, comprehensively analyzed, and the key findings have been critically exposed.

Results. A few main markers endothelial dysfunction were revealed, which reflect axial pathogenic events such as inflammation, endothelium lesion and reendothelization, inherent hemostasis disorders and prothrombotic risk. Likewise, some distinct morphophysiological traits of arterial and venous endothelium are disentangled, as well as markers having common and distinct predictive power of endothelial dysfunction in arteries and veins.

Conclusions. Multi-marker panel is a reliable tool for prediction of endothelial dysfunction in both arteries and veins, as well as the risk of inherent consequences. Noteworthy, majority of markers are common for arteries and veins, but some, like C-reactive protein and von Willebrand factor should be treated distinctly.

Keywords: venous and arterial endothelium, markers of endothelial dysfunction.

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Key messages

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

Currently, there is not a comprehensive algorithm of vascular endothelium dysfunction assessment using circulating biomarkers which refer to most important pathogenic pillars: inflammation, reendothelization and hemostasis disorders for both arteries and veins.

The research hypothesis

A segregation of leader markers of endothelium dysfunction of arteries and veins based on their pathophysiological role in triggering and worsening of vascular dyshomeostasis.

The novelty added by manuscript to the already published scientific literature

Some markers of endothelial dysfunction should be distinctively assessed in regard to their predictive power in arteries and veins, such as C-reactive protein and von Willebrand factor. Likewise, soluble receptor of protein C is a stronger predictor of thrombosis risk linked to endothelial dysfunction in comparison to its ligand.

Introduction

Vascular endothelium (VE) plays a versatile role in general, and especially, cardiovascular homeostasis control. Since the discovery of nitric oxide (NO) as an endothelium derived factor having a key role in vascular tone regulation, a lot of data are still accumulated available to depict a multi-facet caliber of VE. To note in this regard the property of VE to prevent platelet adhesion and prothrombotic state activation, circulating white cells passage, pathological vascular remodeling and atherogenic plaque progressing [1-3]. Likewise, VE influences angiogenesis and arteriogenesis, processes that have a dichotomic significance, meaning they can have both positive and negative effects, the last being touchy linked to cancer growth and cancer spreading [4].

The VE functions in veins and arteries are basically common, although some differences exist, such as: (i) venous endotheliocytes express more amount of NO; (ii) endothelial junctions in arteries appears to be tighter; (iii) expression of endothelial vascular growth factor (VEGF) and of receptors against von Willebrand factor is higher in the endotheliocytes of arteries [5]. Remarkably, large veins versus arteries have a greater capacity to trigger and sustain an inflammatory response. Likewise, in veins the formation of thrombus is much faster than in arteries due to lowered blood flow, and the red blood thrombus does not need von Willebrand factor.

Despite the fact that both arteries and veins express a lot of common markers, the genes families inherent to endotheliocytes of arteries and veins are not totally similar. Arterial endotheliocytes express more opulently ephrinB2 gen, but in veins predominates EphB4 gen [6]. Conceptually is admitted that hemodynamic stress of the blood flow might change the phenotype and morpho-functional support of vessel behavior regardless a certain identical embryonal pattern. Thus, the increased blood flux can induce an arterial phenotype of venous endothelial cells.

Noteworthy, the VE injury associated by various models of dysfunction becomes an important pathogenic pillar of many homeostasis disorders, which in veins are mostly manifested by high risk of thrombus formation (e.g. deep venous thrombosis), and in arteries by vascular remodeling developing in a field of atherogenic event and artery reactivity disturbances. Artery endothelial dysfunction is viewed as a pivotal tool of so dangerous cardiovascular disorders like acute myocardial infarction, stroke, and arterial hypertension. Endothelial damage associating the coronary angioplasty triggers the process of in-stent re-stenosis qualified as a pattern of pathological vascular remodeling resulting in the angioplasty benefic lost.

Taken together these arrangements underline the real importance of early endothelial dysfunction detection for prediction of cardiovascular homeostasis impairment as well as for disentangle of main therapeutic targets. Therefore, a multi-marker strategy or multi-marker panel is applied as a feasible algorithm containing markers which are referring to most important pathogenic interfaces of the endothelial dysfunction and its imminent pathological consequences.

Material and methods

In order to build this review article, the searching of needed references concerning actuality, specificity, relevance and matching to article goal was projected on databases of PubMed, MEDLINE Google Scholar and Cochrane with the depth of the relating up to the year 2000. The found articles were structured in regard to main objectives, comprehensively analyzed, and the principal coagulated entities have been critically exposed.

Results and discussion

Conceptually the multi-marker algorithm is built from a puzzle of various pathogenic mechanisms leading to VE injury and dysfunction whose most important expression is NO lack. In arteries these phenomena are closely linked to atherogenesis and inducing endogenous and exogenous factors. Leader mechanisms taken as intelligible objectives for seeking feasible markers and predictors of VE dysfunction and its repercussions are inflammation, oxidative stress, endothelial reendothelization potency, decreases expression and activity of endothelial nitric oxide synthase (eNOS), smooth vascular myocyte migration and proliferation control disturbance, hemostasis disorders, etc.

Inflammation and more important circulating markers

Chronic or low-grade systemic inflammation or subclinical inflammation is considered as a key factor leading to VE impairment and dysfunction, beyond its direct action on atherogenesis progressing. Contemporary concept corroborates chronic inflammation as a sustained elevation of circulating cytokines released by a diversity group of cells, including adipocytes, which promotes an impact on remote endothelial cells. Classical canon underlines obesity, metabolic syndrome and insulin resistance as most important pathological entities leading to subclinical inflammation even in both relatively young people and apparently healthy adults [7-9].

Pathophysiological interface of inflammation induced VE disorders is very complex, multi hierarchical and interdependent [10, 11]. Among most important mechanisms should be revealed:

- Inflammatory cytokines induced endothelial cell apoptosis and pyroptosis.
- Activation of oxidative stress.
- Reduced expression and activity of eNOS.
- Diminution of tetrahydrobiopterin, a cofactor of NO synthesis by endothelial cells from L-arginine.
- Increased activity of arginase, which takes supplementary amounts of L-arginine from eNOS-citrulline cycle and converts this amino acid in ornithine cycle leading to urea formation [12]. Remarkably, arginase II expression in mitochondrial apparatus is augmented by inflammatory cytokines released by proinflammatory macrophages type 1 (M1). On the other hand, excess of polyamines released in ornithine cycle triggered by arginase are able to increase production of asymmetric dimethylarginine which inhibits activity of eNOS [13].

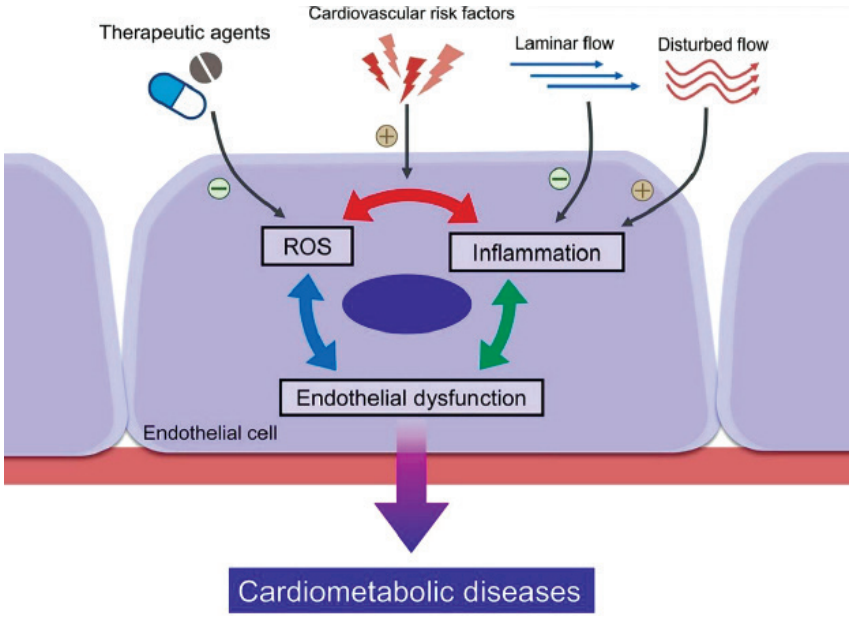

Fig 1. Mutual relation ROS-inflammation is sustained by disturbed blood flow [11]

Inflammation boosting resulting in endothelial dysfunction is higher in arterial vessels due to increased risk of disturbed blood flow. Contrarily, laminar blood flow like flow in veins is not a so strong factor capable to trigger inflammatory response, and as consequence it means a lowered hazard for excessive synthesis of ROS, reactive oxygen species (figure 1).

Finally, inflammation either directly or indirectly (thereby of NO deficiency) exacerbates the process of atherosclerosis a one of crucial supports responsible for VE damage and dysfunction. Inflammation also is a factor promoting vascular ageing either of arteries or veins which strongly correlates with VE dysfunction severity [14].

Accordingly, the assessment of inflammatory markers is an important diagnostical tool of VE dysfunction and respectively a prediction of inherent cardiovascular disorders. The most frequently used marker of inflammation and VE dysfunction is high-sensitive C-reactive protein (hsCRP), which compresses a large amount of evidence. Likewise, hsCRP is considered a key predictor of coronary and heart failure risk [15, 16]. To note in this regard that the serum level of hsCRP is taken as cardiovascular risk stratification such as the levels of <1 mg/L, 1–3 mg/L, and >3 mg/L should be interpreted respectively as low, moderate, and high vascular risk, respectively, based on a large number of population studies [17]. Must be mentioned that elevation of serum hsCRP level more than 15 mg/L should be interpreted as a presence of pathogen induced inflammatory processes. Furthermore, moderate increased hsCRP levels should be taken into consideration in cases when where are other cardiovascular risk factors like arterial hypertension, diabetes, obesity, smoking, dyslipidemia, stress, etc. [18].

The CRP role in endothelium damage and dysfunction is well documented in an enormous number of clinical and experimental studies and is based on certain mechanisms.

First, CRP is early and tightly involved in triggering and progressing of atherosclerosis in arteries and thrombosis in veins (figure 2).

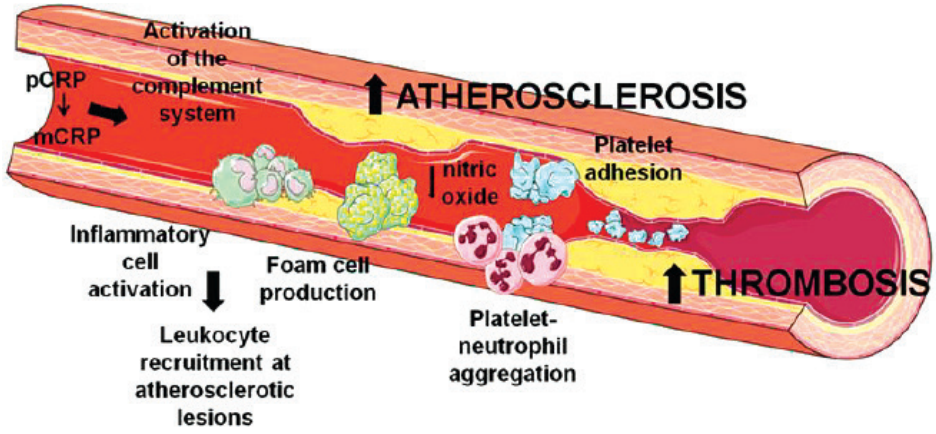

Fig 2. Synoptic mechanisms of pentameric CRP (pCRP) and monomeric CRP (mCRP) involvement in the processes of atherosclerosis in arteries and thrombosis in veins [19].

The main mechanisms of CRP induced inflammation driving VE dysfunction are linked to complement activation, stimulation of foam cell formation and production of ROS and cytokines, circulating leukocyte recruitment, stimulation of platelet adhesion and aggregation, inhibition of eNOS and NO diminution [19, 20]. More than that, the amount of CRP found in atherogenic plaque robustly correlates with intensity of inflammatory response, endothelial dysfunction degree and risk power regarding acute coronary syndrome. In regard to the ratio of monomeric/pentameric CRP forms in veins and arteries is to underline the role of mechanical and physical characteristics of the blood stream which determine the activity of pentamer conversion in monomer as well as the enzyme activity of the vascular locus. It is considered that in arteries the concentration of monomeric CRP is bigger than in veins, primarily due to a stronger blood stream, and a higher proteolytic enzyme concentration especially in the region of atherosclerotic injury [21]. It is still unknown the precise mechanisms explaining more aggressive pro-atherosclerotic action of CRP monomers in comparison to CRP pentamers although both structural patterns activate the same pathophysiological events, such as complement activation, platelet and leukocyte adhesion, endothelial cells damage [22]. Moreover, in the arterial endothelial zones of injury the concentration of white blood cells is higher compared to veins, a fact which should be taken into consideration, because according to some opinions, lymphocytes can synthesize CRP monomers [23]. Respectively, the CRP-linked and mediated inflammatory process is significantly more active in arteries than veins. Endothelial inflammation is manifested by increased expression of phospholipase A2 which facilitates the pCRP entering in the liposomes of cell membrane (e.g., endotheliocytes, macrophages, smooth muscle cells) where it is exposed to a process of dissociation in partly due to an acidic microenvironment. So, in a case of a certain suspected risk to endothelial dysfunction both pCRP and mCRP circulating levels are diagnostically more important for arteries.

Second, CRP increases expression of monocyte chemoattractant protein (MCP-1), as well as of selectins in endothelium [24]. These events facilitate the monocyte and neutrophil traffic through endothelial layer. The trapped white cells trigger the process of smooth myocytes migration and proliferation not only in arteries, but also in veins, defined as vein wall remodeling [25]. It is sustained by extracellular matrix (ECM) excessive degradation and synthesis under the action of fibroblasts and ECM metalloproteinases. Noteworthy that under statins action, the serum MCP-1 content decreased and it resulted in improvement varicose vein remodeling [26]. Likewise, it is a corroborated suggestion that susceptibility of the vein ECM to proinflammatory agents' action is linked to some proteomic and genetic disorders, which justifies the application of multi-marker panel in order to unravel the real predictors of chronic venous disease [27].

Third, CRP decreases activity of HDL capacity to take cholesterol from foam cells leading to atherogenic phenomenon boosting. On the other hand, HDL markedly reduces the proinflammatory activity of CRP, and therefore lipid profile improvement manifested by LDL decrease and HDL raise confines the pathogenic approach of CRP in inflammation induced VE damage and dysfunction [28]. This effect is especially suitable in arteries and represents a pillar of lipid-lowering therapy.

Another remarkable marker of inflammation and predictor of VE dysfunction is tumor necrosis factor alpha (TNF-α). TNF-α is considered as a pivotal proinflammatory marker whose expression is dependent on activity of 2 transcription factors: nuclear factor kappa B (NF-kB) and nuclear factor of activated T lymphocytes (NFAT) which provide the signals from membrane Toll-like receptors to nuclear DNA. The strong contribution of TNF-α in endothelial inflammation and dysfunction was confirmed in a lot of clinical and fundamental studies. Especially are the emphasized results showing that intra-arterial TNF-α infusion in healthy volunteers resulted in acute vascular inflammation associated with impaired endothelium-dependent vasorelaxation [29].

A few important detrimental effects of TNF-α are recognized as (figure 3):

- Increased activity of endothelial membrane NADPH-oxidase leading to excessive formation of superoxide anion which neutralizes the NO by accumulation of peroxynitrite ($ONOO^-$), a radical able to constrict arteries and exacerbate atherosclerotic process.
- Inhibition of cytochrome 450 resulting in lowering of epoxyeicosatrienoic acids (EETs) formation from arachidonic acid, which is able to relax arteries by hyperpolarization mechanism.
- Decreased expression of eNOS.

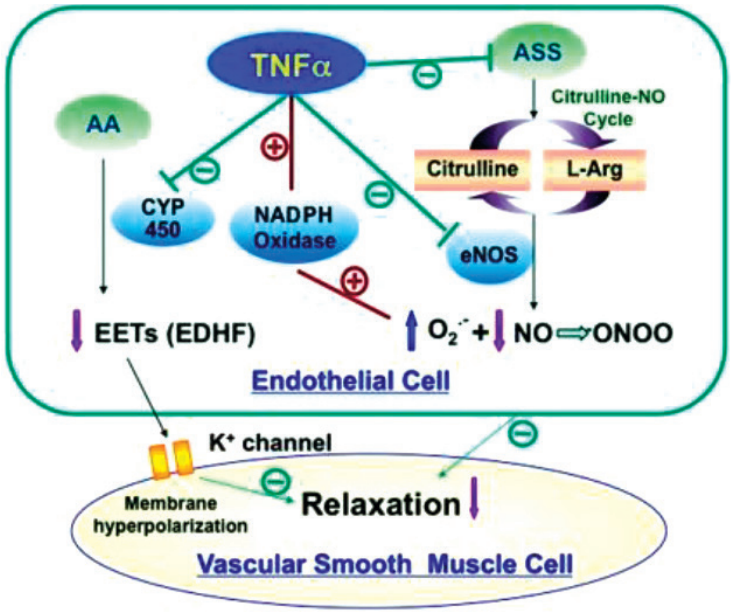

Fig 3. Mechanisms of TNF-α induced endothelial dysfunction [29].
AA – arachidonic acid; EDHF – endothelial derived hyperpolarizing factor; L-Arg -L arginine.

The circulating TNF-α levels directly correlate with severity of endothelial dysfunction in both arteries and veins. In some cases, overexpression of TNF-α is linked to genetic disorders found in autoimmune diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus) associated with VE dysfunction worsening [30]. TNF-α stimulates expression of endothelial receptor LOX-1 which boosts sequestration of oxiLDL molecules from blood in order to build the atherogenic plaque in arteries. LOX-1 activation also leads to increased expression of NF-kB and arginase2. The last reduces NO formation because consumes excessively L-arginase in the ornithine cycle.

The impact of low grade of inflammation or subclinical inflammation often found in diverse metabolic disorders (e.g., obesity, diabetes, insulin resistance syndrome) on VE even in young persons is provided via elevated circulating levels of TNF-α. Impairment of microcirculation (cerebral, cardiac), a system of vascular network which encompasses resistant vessels such as prearterioles, arterioles and capillaries, having a risk power for stroke and acute myocardial infarction, is strongly linked to TNF-α elevation [31]. Being directly and actively involved in the ROS formation, TNF-α leads to endothelial cell activation in order to release endothelin 1 (ET-1) which is considered as one of most potent natural vasoconstricting agents. So, collectively TNF-α, ROS and ET-1 are important pathogenic factors acting together and able to induce VE dysfunction and promote its consequences. More than that, TNF-α increases expression of ET-1 receptor in smooth arterial myocytes (i.e., types ETA and ETB), thus tightly contributing to artery remodeling, especially resistant arteries [32]. Stimulation of ET-1 generation by TNF-α is mediated by c-Jun N-terminal kinase pathway, which is also a mechanism of ROS release. Perivascular adipose tissue is influenced via inherent adipokines the rate of TNF-α expression. It has been proven that adiponectin decrease provides TNF-α expression, but in excess leptin and resistin, in contrary, stimulates expression. The substance P can decrease in natural conditions the TNF-α impact on VE thereby a mechanism linked to modulation of proteinkinase B (Akt) triggering eNOS activity [33].

Recent studies have shown that diverse families of micro-ARN (miR) can have a dichotomic manner of VE regulation. In this regard is important the action of mir-29a-3p manifested by decrease of TNF-α receptor expression, and as consequence the TNF-α induced activation of adhesive molecules, such as E-selectin and ICAM-1 (intercellular adhesion molecule) and VACM-1 (vascular adhesion molecule), hence playing a role of natural VE protector and a mechanism counteracting endothelial dysfunction worsening [34].

The role of TNF-α in the pathogenesis of inflammation induced and assisted endothelial injury is quite well documented [35]. However, the predictive power of TNF-α regarding early endothelial dysfunction is better studied and known for arterial bed.

Both main markers of VE dysfunction, CRP and TNF-α, are conceptually strongly linked to another proinflammatory marker, IL-6. Inflammation, oxidative stress, and Ang II stimulate the IL-6 expression in veins and arteries wall. Being a multifunctional cytokine, IL-6 acts on diverse cells (endotheliocytes, myocytes, adipocytes, monocytes, and cardiomyocytes) in both endocrine and paracrine pathways. Like CRP, IL-6 can be synthesized locally, in the atherosclerotic plaque, and its level positively correlates with expression of LOX-1 receptors and pathological pattern of vascular remodeling. IL-6 receptor is found in two arrangements [36]:

- As a membrane receptor without intrinsic kinase activity and with low affinity at the cell surface.
- As a gp130 transmembrane site having intrinsic tyrosine kinase activity able to bind the circulating complex acting as a ligand: IL-6+ soluble IL-6 receptor.

Activation of both markers leads to activation of STAT-1 and STAT-3 (signal transducer and activator of transcription) and consequently to nuclear DNA activation. The gp130 is expressed ubiquitously, but membrane IL-6 receptor – selectively, in dependence of the cell type.

In a comparative analysis of IL-6 role vis-à-vis of venous and arterial endothelial dysfunction it is important to underline 2 distinct traits. The first, vascular wall capacity to release IL-6 is higher in arteries. Second, the gp130 expression level in veins is lowered.

Among pleiotropic effects of IL-6 should be highlighted the following actions in regard to its role in VE dysfunction [37, 38]:

- augments the detrimental action of TNF-α and CRP on VE;
- increases production of ROS;
- stimulates expression of chemokines;
- elevates the level of plasmin activator inhibitor;
- stimulates synthesis of active-phase proteins by liver (e.g., fibrinogen, ceruloplasmin);
- increases expression of adhesive molecules and facilitates the trans-endothelial traffic of white blood cells;
- triggers the migration and proliferation of endothelial cells and smooth vascular muscle cells;
- activates the ECM fibroblasts and promotes the vascular remodeling.

According to IL-6 intake in the venous endothelial dysfunction the majority of evidence indicate that in diverse patterns of vein remodeling the circulating IL-6 level is significantly elevated and robustly correlates with the serum amount of IL-8, TNF-α and MCP-1 [39, 40]. Anyway, still the amplitude of diagnostic and pathophysiological relevance of the main proinflammatory markers such as CRP, TNF-α and IL-6 in veins remains less appreciated in comparison with arteries. First and foremost, deep venous thrombosis remains the principal venous pathology requesting monitoring of inflammation markers as predictors of endothelial dysfunction and inherent repercussions.

Markers of endothelial lesion and reendothelization

Endothelial lesion is a continuous process triggered by various pathologic factors such as: ROS, inflammation

mediators, shear-stress, hyperglycemia, dyslipidemia, hyperhomocisteinemia etc. Many comorbidities (e.g., arterial hypertension, diabetes, autoimmune diseases) worsen endothelial injury leading to a progressive decline of VE dysfunction. The most important markers of endothelial lesion are endothelial microparticles (EMP), endothelial exosomes which derive from special vesicles erupted from endothelial cell membrane and endothelial apoptotic bodies [41-43]. The circulating level of EMP positively correlates with the degree of endothelial injury especially in arterial bed, atherosclerotic process activity as well as risk of acute vascular accidents like acute myocardial infarction and stroke. Likewise, elevated EMP level is associated with higher serum amounts of CRP, TNF-α, IL-6, IL-8, MCP-1, and diabetes induced VE damage [44]. Hyperhomocysteinemia, smoking and hypodynamic lifestyle are often associated with raised EMP and free endotheliocyte circulation that indicates on endothelial layer injury and endothelial inflammation because the level of phospholipase A2 is also increased.

In the venous system, the pathophysiological significance of EMP is rather linked to procoagulant activity and risk of thrombosis, and this marker predicts endothelial cell senescence and functional weariness [45]. To note in this context that EMP increases directly and indirectly expression of selectins, VACM-1 and ICAM-1, activates platelets and monocytes, increases release of tissue factor from endotheliocytes, thrombocytes and mononuclear cells. These procoagulant events are also associated with elevated levels of thromboxane A2 (TxA2), ET-1, ROS and peroxynitrite, growth factors and plasminogen activator inhibitor. So, EMP is an early predictor of vein thrombosis, and obviously this marker should be assessed in association with other biomarkers referring to coagulant, anticoagulant and fibrinolysis systems feasibility.

The process of reendothelization is a crucial phenomenon aiming substitution of either damaged cells or senescence cells by new endotheliocytes. In regard to renewing of senescence cells population, should be emphasized that both endotheliocytes and smooth vascular myocytes demonstrate an advanced statement of senescence in the boosted atherosclerotic process [46]. Endothelial senescence and damage lead to diminution of tetrahydrobiopterin, a cofactor of NO synthesis, and respectively a predictor of VE dysfunction. Therefore, tetrahydrobiopterin became a reliable marker of early VE dysfunction and atherosclerosis progression [47].

Basically, reendothelization is realized and emphasized by following paramount markers: VEGF, angiopoietins, and endothelial progenitor cells (EPCs).

VEGF is considered a strong endotheliocyte mitogen and angiogenic factor. In vitro studies have demonstrated that VEGF stimulates the growth of arterial, venous, and lymphatic endotheliocytes which are forming a new capillary network. Hypoxia, ischemia, and oxidative stress are main factors triggering expression of VEGF, which is underlined also as vasoprotector factor due to its antiapoptotic effects derived from activation of Bcl-2 proteins. Mechanical stress of VE induced by diverse maneuvers of angioplasty damages endothelial cells and stimulates release of VEGF. Nitric oxide and eNOS play an important role in the VEGF induced angiogenesis [48]. Remarkably, VEGF induces vasodilation in a dose dependent manner, and this effect is fought to be mediated by prostacyclin. Two types of receptors mediate the large spectrum of VEFG effects: R1 and R2. The VEGF-R2 receptor is expressed on arterial and venous vasculature, being involved in control of vasodilation, atherosclerosis, cell migration and proliferation [49].

VEGF-R1 receptor is mostly found as a soluble receptor capable to bind the circulating VEGF and the affinity of receptor against specific ligand is 10-fold higher versus VEGF-R2. However, the kinase activity triggered by via VEGF-R1 is 10-fold weaker [50]. In contrast to VEGF-R2, VEGF-R1 does nor mediate angiogenesis in embryonal tissue. In adulthood it is expressed in both endothelial cells and macrophages and worsens the atherosclerotic process.

Accumulated data suggest that VEGF is a predictor of arterial wall remodeling, and its elevated level is well proven in patients with arterial hypertension and type 2 diabetes [51]. The authentic role of VEGF in reendothelization was confirmed in vivo by administration of an antibody neutralizing this growth factor after angioplasty induced vascular injury, which led to VE recovery annihilation. It has been deduced that released VEGF from damaged endothelial cells in the blood promotes the function of EPCs, which are sequestrated from bone marrow under action of this growth factor. Increased production of endogenous NO after an adequate reendothelization realized basically by EPCs, decrease expression and activity of VEGF. More than that, exogenous NO released from nitrite donors reduces the activity of reendothelization in vivo due to decrease of VEGF level as well as level of APCs. Interestingly, carbon monoxide, another endothelial derived factor, acts unlike NO, contributing to VEGF expression increase thereby hypoxia inducible factor. Finally, VEGF being appreciated as a growth factor expressed not only by endotheliocytes (e.g., by macrophages, platelets, keratinocytes, renal mesangial cells) plays a certain role in other functions besides reendothelization, such as: hematopoiesis, wound healing, bone tissue synthesis. To be noted in this context that tumor cells also are capable to express VEGF, and angiogenesis becomes a pathogenic factor facilitating tumor growing and metastasis [52]. Likewise, use of the blockers of VEGF receptors led to a suppressing effect on tumor growth and lowered risk of tumor metastasis.

Thus, EPCs are proven as an important tool triggering and sustaining reendothelization because they are capable to differentiate into endothelial cells and hence provide phenomenon of new blood vessels formation. Therefore, EPCs are often named as circulating angiogenic cells. Mobilization of EPCs from bone marrow is realized not only by VEGF, but other factors are also available, such as ECM metalloproteinase 9 and stromal cell-derived factor 1. Also, EPCs can secrete some paracrine factors, such as IL-8 and stromal cell-derived factor 1. Hypoxia and ischemia are factors that mobilize EPCs in blood flow resulting in their migration to-

ward the zone of endothelial injury where they proliferate, and differentiate into mature endothelium, thereby leading to reendothelialization and neovascularization [53, 54]. Being injected intravenously EPCs can reach the infarcted region within 48 hours.

Decreased levels of EPCs or their functional disability are strong predictors of endothelial dysfunction and cardiovascular disease as well [55]. Lower levels of EPCs are associated with a weaker process of reendothelization even when the circulating level of VEGF is quite high. It is a well proven fact, that low levels of EPCs are accompanied by decreased production of NO and vascular reactivity impairment. Weak sequestration of EPCs from bone marrow augments VE dysfunction and accelerates the progression of cardiovascular disorders. Many cardiovascular risk factors confine the activity of EPCs recruitment and their home in the zone of VE injury, such as hyperglycemia, hypercholesterolemia, hyperhomocysteinemia, low grade inflammation, leukocytosis, oxidative stress, etc. Therefore, for a better understanding of real pathogenic interface of VE dysfunction, EPCs should be assessed together and correlatively with a lot of other markers and risk factors of vascular, especially arterial damage. Noteworthy, the level of circulating EPCs is recognized as an independent predictor of atherosclerosis progression and suspected disorders of artery remodeling and reactivity [56-58].

Nowadays the serum concentration of EPCs is viewed as an important diagnostic marker as well as a therapeutic target of cardiovascular disorders associated with VE dysfunction. Intracoronary infusion of EPCs lead to reduced myocardial infarction zone and to improvement of myocardial and ECM remodeling in post-infarction period.

In this regard it is important the opinion of K. Lenk *et al.* (2021) who suggest that EPCs are a one of key tools for providing physical exercise benefits on atherosclerosis and coronary disorders [59]. Physical activity maintains the structural integrity of VE regardless of any risk factors action, and in case of endothelium alteration, the level of EPCs is higher and their involvement in vascular repair becomes more efficient.

Regarding some differences of EPCs role in arteries and veins, there is no conclusive data. Nevertheless, could be relevant hypothesis that the EPCs role in both arterial and venous endothelium repair is the same.

Nowadays, a new marker of reendothelization is angiopoietin which belongs to group of growth factors. Two most important families of angiopoietin are known: Ang-1 and Ang-2, which are natural ligands of the Tie-1 and, respectively, Tie-2 receptors tyrosine kinase, which are expressed primarily on endothelial cells and early hematopoietic cells [60, 61].

Ang-1 acts as a potent angiogenic growth factor, but Ang-2 plays an important role in various physiological processes and its impairment is inherent to a lot of homeostasis disorders, including for the lymphatic system.

Tie-2 receptor is abundantly expressed in endothelium, especially of arteries. Likewise, vascular fibroblasts can express Tie-2 receptors, whose attribution is considered to be tightly linked to vascular injury repair. Therefore, this receptor is considered as atheroprotective in arterial endothelium [62]. So, both angiopoietins fulfill a lot of suitable functions, such as: adhesion and survival of endothelial cells, augmentation of EPCs action vis-à-vis of endothelium repair, reendothelization and angiogenesis from new formed capillaries. However, the authentic equilibrium between Ang-1 and Ang-2 in postnatal vascular morphology and physiology control is still not well established. Most hypotheses state that Ang-1 acts in a paracrine agonistic manner inducing Tie-2 phosphorylation and subsequent vessel stabilization. In contrast, Ang-2 is produced by endothelial cells and acts as an autocrine antagonist of Ang-1-mediated Tie2 activation. Conceptually is important that Ang-2 blunts the action of proinflammatory cytokines on vascular endothelium and therefore prevents and mitigates the VE injury and remodeling. Lastly, Ang-2 and EPCs action on VE might be boosted by TGF-beta (transforming growth factor) whose signal is received by a special endothelial receptor, endoglin (membrane glycoprotein) whose activation promotes the neoformation of capillaries and integrity of vessel walls, either in the embryo or postnatal life [63].

Together with VEGF, angiopoietins could stimulate angiogenesis induced tumor metastasis, and respectively are depicted as targets of therapy, especially concerning the Ang-2. More data is needed in order to highlight physiological and pathophysiological entities of these growth factors regarding VE dysfunction.

Markers of VE dysfunction in connection to hemostasis disorders

Vascular endothelium dysfunction is associated with severe hemostasis disorders manifested finally by a prothrombotic state induction. Accordingly, VE dysfunction becomes a condition of prothrombotic risk, but at the same time the formed thrombi comprise a pathogenic interface for DE dysfunction exacerbation. Therefore, inherent markers of hemostasis disorders should be important and significant predictors of VE dysfunction severity and its prognostic outcomes.

Basically, prothrombotic state is a result of either overactivation of coagulation system or impairment of anticoagulant processes. Frequently these factors act together. Fibrinolytic activity of the blood also plays a notable role because can ensure in time the resolution of fibrin thrombus. Endothelium incompetency in the field of hemostasis control is emphasized as following main entities:

- Discovery of the integrins receptors expressed by subendothelial collagen fibers.
- Platelet activation and increase of its adhesive and aggregation receptors.
- Increased release of von Willebrand factor (vWF).
- Excessive accumulation of the tissue factor released in partly from damaged endotheliocytes.
- Decreased expression of endothelial receptors playing a crucial role in the anticoagulant protein C activation.

- Diminution of the anti-thrombin III level.
- Reduced NO and prostacyclin production.

The earliest consequences of VE lesion and dysfunction in regard to hemostasis control are elevated circulating level of vWF, antithrombin III and incompetence of anticoagulant tandem protein C-protein S. Von Willebrand factor, a pentameric glycoprotein, is mainly synthesized by endothelial cells (megakariocytes also express vWF) and is stored in Weibel-Palade bodies and a-granules respectively of endotheliocytes. Normally the circulating level of vWF is linked to ABO blood groups and other genetic arrangements. Non-genetic background of vWF plasma level changes is determined by age, gender, inflammation, oxidative stress, and surely by endothelial cell integrity in either arteries or veins. Most important hemostatic functions of vWF are driven by: (i) stabilization of the factor VIII in the circulation because vWF serves as its plasma carrier, and (ii) boosting of platelet adhesion to vascular wall and platelet aggregation, respectively.

Majority of pathologic states associated with vascular endothelium injury and dysfunction demonstrate elevated circulating levels of vWF [64-66]. Increased plasma vWF concentration means a risk for prothrombotic state activation leading to thrombi formation in arteries of veins (so, white and red thrombi). Most important conceptual and practice question remains as: does the vWF has a more decisive role in thrombus formation in arteries or in veins?

Solitary narrations indicate the importance of discovery of all active sites of vWF pentamer in the blood flow in order to achieve maximal hemostatic functions [67-69]. This is possible in an arterial rapid and intense blood flow comparable to the slow flow in. So, the predictive power of increased plasma level of vWF concerning risk of thrombus formation is obvious in arteries. In regard to vWF role in venous thrombus it is linked to lowered polymer degradation and consequently less fully discovery of active sites of glycoprotein needed for factor VIII binding due to weak blood flow in veins [70].

Another hemostatic factor linked to endothelial availability is antithrombin III (AT-III), recognized as an endogenous serine protease inhibitor (glycoprotein consisting of 432 amino acid residues), thus of thrombin. Endothelial cells injury leads to decreased release of AT-III resulting in a lowered capacity of thrombin inactivation of the blood. Likewise, AT-III also inhibits other factors of coagulation system, such as IX, X, XI and XII [71].

So, blood levels of AT-III predicts VE dysfunction and risk of serious cardiovascular diseases like acute myocardial infarction and stroke [72].

Finally, anticoagulant protein C is a key antithrombotic factor, but its functional feasibility is closely linked to endothelial receptor (type 1 transmembrane glycoprotein) needed for protein C activation. Therefore, even the normal or elevated circulating levels of protein C could be inefficient to prevent thrombus formation if due to endothelial damage its activation is compromised. However, there are interesting approaches to study the predictive value of soluble endothelial receptors to protein C. Remarkably, in the blood stream this receptor binds to circulating protein C, but this does not result in anticoagulant factor activation, but in contrary leads to its inhibition due to lost ability to inhibit factor Va [73].

Thus, increased level of soluble endothelial receptor of protein C could be a reliable marker of anticoagulant capacity fall due to protein C misfunctioning, and respectively a predictor of prothrombotic state activation in both arteries and veins.

Conclusions

1. The multi-marker panel of endothelial dysfunction is a key opportunity to identify the markers referring to main pathogenic mechanisms, such as inflammation, reendothelization and hemostasis disorders having a reliable early prediction for arterial and venous endothelial dysfunction.
2. Although majority of markers have a same predictive value of endothelial dysfunction in both arteries and veins, the circulating levels of CRP and vWF are more important in regard to arteries because here depolymerization activity of these pentamers is higher leading to pathogenic mission augmentation concerning vascular remodeling and prothrombosis.
3. The circulating level of protein C is not a reliable marker of endothelial dysfunction induced risk of thrombosis. However, the elevation of its soluble endothelial receptor could predict lower anticoagulant activity of protein C due to the loss of capacity to inactivate factor Va.

Competing interests

None declared.

Authors' contribution

Both authors contributed equally to the literature searching, conceptual highlighting of the material as well as writing of the manuscript. The authors read and approved the final version of the manuscript.

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REVIEW ARTICLE

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The biochemical approach to thromboembolism: the relevance of molecular aspects

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ABSTRACT

Introduction. Arterial and venous thromboembolism is a disease with a high impact on morbidity and mortality. Their pathological mechanisms of aggregation directed by the clotting factors along with the variations in clinical manifestation are regarded to a high moiety of genetic polymorphisms along with a wide diversity of comorbidities.

Material and methods. A comprehensive literature review was conducted, which included a total of 119 sources. Among these, 60 sources were systematically collected, while the remaining 59 sources were selected through non-systematic methods.

Results. We have identified different treatment options that regard both the venous or arterial thromboembolism in contrast with numerous pathogenetic outcomes, population groups along with biomarkers that significantly modify the clinical aspects of the therapeutical and post-clinical treatment aspect. At the moment its diagnosis is continuously improving worldwide, taking into consideration a high diversity of experts' opinions with a wide practical experience.

Conclusions. Arterial and venous thromboembolisms are serious medical conditions that can be prevented and effectively managed with modern diagnostic and therapeutic techniques.

Keywords: arterial/venous thromboembolism, biomarkers, blood, coagulation.

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Key messages

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

Currently, there are no biomarkers available that can guarantee a 100% accuracy in the differential diagnosis of arterial and venous thromboembolism, thereby increasing the complexity of the diagnostic process.

The research hypothesis

There are different biomarkers which in conjunction with each other can leave a trace of different conditions thus implying the necessity of using these molecular titers in a sensible and specific way. The biomarkers which are most efficient must be identified along with the auxiliary clinical methods for the management of thromboembolism.

The novelty added by manuscript to the already published scientific literature

Many studies consider the molecules either the surgical methods that are acceptable in daily clinical routine in an isolated manner but none of them have tried to see a broad picture of this memorable pathological condition.

Introduction

In-depth research reveals thromboembolism as a significant global issue that demands a timely diagnosis for swift assessment and appropriate treatment. The utilization of thrombosis markers in line with the diagnostic algorithm and disease stage remains an undiscovered aspect that warrants exploration [1].

Thromboembolism is a disease, which can affect various anatomical vessels, including arterial, venous and central portion [2-4]. Morphologically it is represented by thrombogenesis that will in turn lead to blood flow obstruction in that anatomical region [5].

Arterial thromboembolism was investigated with less detail. It can arise from the central region of the cardiovascular system or from atherosclerotic plaques in large-diameter blood vessels [4]. In certain animals, this condition can be extremely painful and debilitating, raising ethical concerns in veterinary medicine regarding euthanasia as a means to alleviate suffering [6].

The contemporary concept describes the mechanism of the coagulation cascade in which the factors are designated by Roman numerals, except for the first four factors which have specific names: Factor I (fibrinogen), Factor II (prothrombin), Factor III (tissue factor), and Factor IV (bivalent calcium ions). These factors can interact with each other to form a primary thrombus, which will undergo further fibrinolysis [7].

It has been observed that the main factors that are associated with the arterial thromboembolism are the anomalies in the coagulation cascade, cytokines, the soluble form of the P-selectin, the elevation of clotting factors, thrombocytosis and leukocytosis [4]. These factors are more marked in advanced age, females, and during exacerbation of comorbidities such as arterial hypertension, diabetes mellitus, myocardial infarction, heart failure, and stroke [8].

In the context of the emerging SARS-CoV-2 pandemic and COVID-19, there has been observed an increase in the incidence of thromboembolism and signs of ischemia following heart failure. The highest incidence of arterial thromboembolism has been identified in the lower limbs (71%), upper limbs (14%), mesenteric arteries (4%), and the arterial Willis' circle (10%). In certain cases, multiple sites may be affected, and there is a possibility of concomitant venous thromboembolism [9].

It has been demonstrated that in COVID-19, pulmonary venous thromboembolism is more prevalent than deep venous thromboembolism. This is attributed to the heart lesions that are induced in the cytokine storm that is stimulated by the virus antigens, without a primary focus on venous thromboembolism, contrary to initial reports from Wuhan, Hubei province, China, and surrounding regions, as indicated by a meta-analysis [10].

Malignant neoplasms are characterized by arterial vascularization and subsequent dissemination of thromboemboli in the arterial system. The highest incidence of arterial thromboembolism has been observed in cases of cerebral, pulmonary, colorectal, and pancreatic cancer [11].

The most prominent and up-to-date marker for thrombosis is D-dimer, which can be elevated in the elderly population, as well as in cases of cancer, infections, and chronic inflammations. This biomarker exhibits high sensitivity but limited specificity for venous thromboembolism [2]. Additionally, D-dimer levels can be influenced by various genetic polymorphisms that affect fibrinogen sequences, which may reduce its reliability in a clinical setting [12].

The Zaharin-Head regions have been shown to reflect somatosensory sensations, such as acute or chronic pain, and can serve as markers of organic lesions in the preterminal or terminal stages [13]. In cases of upper limb arterial occlusion, the pain may manifest as sensations of itching and numbness along the ulnar nerve pathway [14].

The objective of this study was to investigate the biochemical mechanisms underlying thromboembolism, with the aim of identifying effective therapeutic approaches and highlighting future research directions in the areas of prevention and treatment. We specifically focused on exploring the interactions between various biomarkers and their potential impact on avoiding quantitative biases in biochemical marker analysis.

Material and methods

A randomized literature study was conducted on 01.02.2023 in order to identify randomized clinical trials, meta-analyses and review articles. The search was conducted using numerous platforms including the PubMed, HINARI, EMBASE and Elsevier libraries.

After applying a set of criteria, a list of 60 random papers were selected. The including criteria were: relevance of the topic, had at least one of the key-words in the title or abstract – "thrombosis", "thromboembolism" in combination or with the "arterial", "venous" or "pulmonary" and were coincidant with our era and prevalent lifestyles on a cultural background with a relatively strict selection of the sources from the past 10 years. Some exceptions were admitted for the time period for the literary sources which are still standing as leading. The exclusion criteria were: outdated information, irrelevant geographical location and irrelevant time period of the results.

For an increased confidence of the research query were independently studied 59 sources. There were details that required additional clarification this in turn demanding this search. Overall, we have studied 119 literary pieces. All of them had to be regarded as being of high quality in respect to the journal in which they were published and its impact factor.

We did not follow any current guidelines, but we have used a protocol enlisted in the Essential Evidece-Based Medicine, 2nd edition by Dan Mayer [15].

Results and discussion

General aspects

Hypercoagulability can be defined as a condition characterized by an increased propensity for blood clot formation, influenced by both endogenous and exogenous factors. It is important to note that arterial hypercoagulability differs

significantly from venous and central hypercoagulability [16]. A notable physiological aspect of hypercoagulability is the Virchow's triad, which associates alterations in local blood circulation with blood vessel lesions [17].

Comorbidities associated with an increased risk of thrombosis are classified as prethrombotic states, while spontaneous thrombosis represents the primary pathology. Notable causes of thrombosis often involve deficiencies in protein S, protein C, antithrombin III, as well as various dysfibrinogenemias. Malignant conditions, pregnancy, oral contraceptives, myeloproliferative disorders, hyperlipidemia, diabetes mellitus, and vascular anomalies, along with alterations in blood rheology, are also considered prethrombotic states [18]. Hemophilic infections and viral infections are the primary contributors to both venous and arterial thromboembolism. These infections can be further complicated after splenectomies or during leukemia, potentially leading to confusion in laboratory data interpretation [19, 20].

It has been demonstrated that geomagnetic storms may have a potential correlation with increased incidence of cardiovascular diseases, including myocardial ischemia and cerebral stroke. The intensity of radiation emitted on the Earth's surface is higher during wintertime due to the planet's proximity to the sun, which contrasts with the equatorial region where there is a higher incidence of cardiovascular diseases throughout the year due to increased radiation exposure [21].

Comorbidities play a significant role in these mechanisms as they can affect both the quantity and quality of clotting factors, thereby modifying their functionality and impacting fibrinolysis. In kidney diseases, a tendency towards hypocoagulability is observed, while in chronic cardiac pathology, states of hypercoagulability are observed. The coagulation index can be measured using an integral approach that assesses the fibrinolytic potential and the overall hemostatic potential [22].

To understand the coagulation system in relation to other systems, it is important to consider a collection of intracellular operons that are based on microparticles. These granules can be observed in conditions such as atherothrombosis or type 2 diabetes mellitus, where they can be activated without a physiological reason and initiate the coagulation cascade involving tissue factors and factor VIIa. This provides an explanation for the occurrence of thromboembolic events in patients with these diseases [23-26].

Arterial thromboembolism

The factor which can determine the arterial thromboembolism is usually the fibrinogen that will in turn be transformed into fibrin (which can lead to the COVID-19 ground-glass pneumonia along with the vitamin K, coagulation factor XIII which is responsible for the fibrin formation from fibrinogen and von Leiden factor (V)) [27]. The main integrins that represent the subunits which constitute the membrane receptors that are implicated in the coagulation cascade are represented by the glycoprotein Ia, glycoprotein Ib, glycoprotein IIb, glycoprotein V and glycoprotein IX [28].

Thrombomodulin activates protein C and thus it represents a mediating factor for the thrombotic states. The platelet surface receptor which will lead to thrombogenesis are surface glycoprotein IIb/IIIa, Ia/IIa and Ib/IX/V [27].

It is well-known that oral contraceptives are a major risk factor for the arterial thrombosis but the mechanism is scarcely mentioned. A rising concentration of clotting factors in the blood circulation determined by these drugs will in turn lead to a primary arterial thromboembolic event [29].

Thrombocytes possess an intracellular microsomal system known as the secreatome, which is activated in a cascade manner upon stimulation of the aforementioned receptors. This system involves the secretion of β-thromboglobulins from α-granules located on the surface of monocytes, granulocytes, T-lymphocytes, and mastocytes. In the context of arterial thromboembolism in the cerebral vascular system, the direct involvement of matricial metalloproteinases MMP-2 and MMP-9 has been observed in the mechanisms associated with the secreatome, thus establishing their connection with the proteasome [30].

The platelet secreatome has been found to play a significant role in the pathogenesis of osteoarthritis, particularly through the amplified secretion of interleukin 17 and interleukin 17A. This leads to the activation of p38 and p65, which further enhance the expression of the NF-κB pathway. Consequently, matrix metalloproteinases MMP-1, MMP-3, MMP-9, and MMP-13 are activated, contributing to the regulation of anabolic hormone synthesis [31]. Additionally, a correlation was demonstrated between the tissue inhibitor of metalloproteinase-1 (TIMP-1), heat shock protein 70 (HSP-70), thymosin β4 (TB4), superoxide dismutase (SOD), and the generation of osteoblasts. These factors are released within the platelet secreatome, further highlighting their involvement in osteoblast development [32].

The disbalances in the ubiquitin-proteasome system that are determined by an increased concentration of immunoglobulins is one of the primordial factors that will determine an excess of procoagulant system components. The β1, β2 and β5 subunits are predominantly implied and will be conjugated into β1i/LMP2, β2i/MECL-1 and β5i/LMP7 [33]. Overall, they can form an immunoproteasome that is not reusable and may interact with the elements of the coagulation cascade. The immunocompromising factors like the human immunodeficiency virus 1 (HIV-1) will harshen the state of the patient because it will exhaust the anticoagulant system prior to a surgical intervention (which implies external stimuli) [34]. Compared with humans, the canines have a greater coagulation-anticoagulation system in terms of components. Although it is functionally inferior and has a slower interaction due to a less great interaction surface between the molecules making it less efficient [35].

In order to assess thrombosis in emergency states, the D-dimer biomarker was implemented. It is a reminiscence of the fibrin portion afore the thrombogenesis and less often they can be associated with dysfibrinogenemia [36]. The quantitative aspect of the D-dimers was associated with ter-

minal states like cancer or a chronic cardiovascular disease like a coronaropathy [37].

Fibrinogen was associated with an increased incidence of thrombosis along with additional elements besides the Virchow's triad because of the systemic inflammation and the renin-angiotensin system dysregulations [38]. In the context of COVID-19, fibrin was one of the primordial factors that determined the ground-glass pneumonia in the inferior lobes. Its consolidation was determined by the croupous inflammation that leads to respiratory arrest and death via cardiac failure [39].

The C-reactive protein (CRP) which is a global biomarker for the specific inflammatory states was proven to be associated with the arterial thromboembolism incidence. This in turn is regarded to the atherosclerosis in which the *tunica intima* is swollen and the secretion of inflammatory cytokines or other components takes place into the neointima because of the young myocytes in this layer [40].

A clinically applied biomarker that is widely used is the quantification of platelets count in the bloodstream. An increase in platelet count is often observed, especially in the presence of arterial comorbidities. This elevation is associated with an increased likelihood of platelets coming into contact with arterial walls, leading to microtrauma and potentially contributing to thrombus formation [41]. Conversely, in cases of antithrombin III deficiency, there is a depletion of procoagulant factors, which can increase the risk of arterial thrombosis [42]. The deficiency of protein C or protein S, primarily caused by genetic factors, is another significant factor contributing to the increased incidence of arterial thromboembolism, particularly in young individuals without underlying chronic cardiovascular diseases [43].

Complications associated with arterial thromboembolism are primarily caused by ischemia and its subsequent reactive hypoxia. These complications arise due to restricted blood flow in conditions such as myocardial infarction, ischemic cerebral strokes, and pulmonary embolism. They are common in the COVID-19 patients [44]. The complexity of a biochemical analysis in these patients is thus greater and has numerous inclusion and exclusion criteria.

Matrix metalloproteinases 1 and 8 (MMP-1 and MMP-8), as well as neutrophil gelatinase-associated lipocalin, are commonly used biomarkers for predicting the recurrence and chronicity of comorbidities associated with arterial thromboembolism [45].

Abciximab, a murine monoclonal antibody derived from immunoglobulin G, exerts its pharmacological effects independent of the active centers of platelet membrane receptors. Instead, it forms biochemical bonds with the stable portion of the protein, specifically the β_3 perimembranous chains. This mechanism explains its ability to inhibit secretions from endothelial cells, myocardial cells, and leukocytes through interactions with αβ or $\alpha_v\beta_3$ and $\alpha_m\beta_2$ integrins, respectively.

Eptifibatide, a glycoprotein IIb/IIIa inhibitor, competes with proaggregant molecules such as fibrinogen, von Willebrand factor, and other adhesive ligands for binding to the GP IIb/IIIa receptor and its associated $\alpha_v\beta_3$ integrin. By blocking these interactions, eptifibatide prevents platelet aggregation.

Tirofiban, on the other hand, is a selective antagonist for the GP IIb/IIIa receptor and does not exhibit specificity for other integrins. It can be used as a valuable criterion for considering vascular comorbidities when assessing the risk associated with administering the aforementioned pharmacological drugs [46].

Other receptors like $P2Y_{12}$ are used for the non-surgical treatment of the arterial thromboembolism; the most notorious being clopidogrel, which can be substituted with prasugrel and ticagrelor, though their superiority in clinical practice was not proven in comparison with clopidogrel. The first-mentioned drug is usually associated with acetylsalicylic acid (aspirin) and heparin [47].

In the past, the treatment was constituted from the administration of vasodilators, thrombolytics, anticoagulants, antibiotics and analgesics. Currently only anticoagulants and thrombolytics are used [48-50]. This pathophysiological particularity determined the topical usage of the anti-inflammatory drugs in prethrombotic cases like osteoarthritis, tendinitis, muscular strains, or muscular retractions [51]. A type II glycopeptide of natural origin, which is called ristocetin from the ristocetinic complex that has its active site, labelled „Spontin", was in past proven to be the cause of platelet aggregation in the von Willebrand disease of platelet type along with the acquired exhaustive thrombocytopenia. Now it is used as a diagnostic hallmark without being applied in the antibiotic-resistant bacterioses [52].

Venous thromboembolism

The venous thromboembolism has a sudden onset that is none determined by specific causes [2]. The additive factors are obvious, thus being correlated with the venous thromboembolism and are represented by the advanced age, tobacco consumption and increased adiposity [53]. The genetic factors can be primordial in determining the risk for the venous thromboembolism along with an enhancing of the clotting factors activity in the hormonal therapy with oral contraceptives besides other pharmacological drugs. They have genetic polymorphisms and will lead to the prior initiation of the coagulation cascade via the direct, alternative, or lectinic pathway [54]. Drugs that can be deterministic to the hypercoagulable states are systemic estrogen, tamoxifen, corticosteroids, the selective reuptake inhibitors of serotonin, cisplatin, talidomide and lenalidomide [7].

Pregnancy, sepsis (sometimes puerperal), long-time immobilization (trauma, paralysis, sedentary lifestyle) are the figuring main factors in venous thromboembolism development [54-56] along with inflammatory bowel disease and advanced age. The Zahn lines, which are characteristic for the venous thrombus, are due to the stratification of the consequent layers which are composed of erythrocytes and leukocytes, the red color comes from the hemoglobin and the white color due to empty platelets after degranulation in the primary hemostasis [57]. Due to the diverse approaches

employed across multiple disciplines in assessing patients at a population level, there is a potential for an overestimation of venous thromboembolism occurrences [58].Cancer, congestive cardiac failure, recent surgical interventions, and primary and secondary immunodeficiency are established risk factors for venous thromboembolism. The secondary thrombus formation involves not only platelets but also neutrophil granulocytes, monocytes/macrophages, and exfoliated endothelial cells from the *tunica intima* [53, 57]. Different types of cancer exhibit varying incidences of venous thromboembolism. Breast and urinary bladder cancers have a 3% incidence, while colon and prostate cancers have a higher range of 4-7% incidence. Stomach, lung, ovary, and brain cancers demonstrate a higher incidence range of 10-12%, and pancreatic cancer has the highest incidence at 15% [2]. The recurrence rate of venous thromboembolism over a 10-year period is approximately 30% [55].

The risk factors for venous thromboembolism can be classified into three categories: transient risk factors (synergistic), persistent risk factors (additive), and hereditary risk factors (genetic) [7].

The venous thromboembolism is clinically divided in the peripheral (profound venous) and central (pulmonary thromboembolism) that is frequently incriminated in all thromboembolism, being the leading cause of death [53]. Profound venous thrombosis (PVT) can be acute (<14 days) or chronic (>28 days). The subacute form is characterized by a duration of 14-28 days [57].

Topographically, it has been identified 40% of profound venous thromboembolism cases proximally and 25% distally [2]. Imagistic methods are used in 40% in the pulmonary thromboembolism or in 85% in the profound venous thrombosis [58]. The most frequent form is the profound thromboembolism thus of primary origin and the pulmonary embolism along with the superficial venous thromboembolism are usually complications and sometimes are regarded as primary pathological entities [55].

The most important symptoms in the profound venous thromboembolism are pain, edema, and distal proximal ulceration. In the last years, there is a tendency for increased estimable costs or for the hospitalization of patients with venous thromboembolism. Readmission in the state or private clinics costs more than the primary admission (with 48% more) [59]. The superficial venous thromboembolism is often confounded as a unique clinical entity but is usually regarded as a symptom of the profound venous thromboembolism. The symptoms of the pulmonary venous thromboembolism are apnea, pleuritic chest pain, hemoptysis, tachycardia, or hypoxemia but sometimes severe symptoms like sudden death, shock, hypotension, syncope and confusion may be observed [2]. Existing clinical descriptions do not adequately correspond to the severity states of venous thromboembolism, necessitating the imperative use of clinical intuition to ensure accurate diagnosis and suspicion. In order to facilitate the diagnostic process, there are clinical scores like the Wells score for pulmonary thromboembolism, the Wells score for profound venous thromboembolism and the Geneva score for the pulmonary thromboembolism [58].

There is a tight racial correlation that proves an increased susceptibility for the Afro-American race and obese individuals, though the Afro-Americans have a low obesity incidence. These population groups have increased risks due to genetic determinants and an increased viscosity of the blood related to a decreased permeability of the skin, which can be in turn thinner and less adapted for an interaction with the external factors. Obese individuals are proven to have an increased risk for atherosclerosis that is the main factor that determines thromboembolism. The dominant treatment during hospitalization is the administration of unfractioned heparin and low-molecular weight heparin along with the compression of the lower limbs using compression socks. Recently enoxaparin (a heparin with low-molecular weight) and betrixaban (a direct inhibitor of the Xa factor) have proven to be superior in the clinical trials compared to other drugs. The most important biomarker used in the clinical assessment of the venous thromboembolism are the D-dimers [60]. Other markers like C-reactive protein (CRP), P-selectin and the synthesis of thrombin may be applied [58].

The American Association of Hematology recommends an ambulatory approach in the venous thromboembolism prophylaxis with an international normalized ratio (INR) of 2.0-3.0. The recurrent states need to be treated with vitamin K antagonists, but the stable forms are treatable with direct oral anticoagulants [61]. The treatment length must be within the limits of 3-6 months [2]. Vitamin K along with heparins are preferable in patients with renal insufficiency, antiphospholipid syndrome, and cancer [62].

The monoclonal antibodies can serve as lytic adjuvants because of their amelioration in the intercellular signaling pathways between the circulating leucocytes. The neutralization of the C-reactive protein (CRP), interleukin 6 (IL-6), interleukin (IL-8), interferon-γ (IFN-γ), tumor necrosis factor α – Tumor necrosis factor α receptor rp55 (TNF-α/ TNF-α receptor rp55) and P-selectin by the monoclonal antibodies or the corresponding polyclonal antibodies will induce an ameliorated cellular answer compared to the low entropy of signaling during the venous thrombus lysis. The potentiation of the p53 pathway (during the quinacrine usage) is the pharmacologic alternative during the factor insufficiency [57].

Reperfusion techniques in the venous or pulmonary thromboembolism may be vital in order to prevent the exacerbation of the state thus to the development of a morbid complication. The filters for the vena cava are proven efficient in lowering of a profound venous thromboembolism but may be used only in reserved cases [62, 63]. Mechanical therapy with the compression of the legs is a significant adjuvant factor because it will contribute essentially to the physiological venous blood flow [62]. Bleeding is the most frequent adverse reaction [64].

Pregnant patients are tested using perfusion-ventilation tests in order to avoid the excessive irradiation. The lack of

adequate treatment in profound venous thromboembolism in turn will lead to the confrontation of more severe conditions like pulmonary thromboembolism with congestive heart failure. The symptoms will be hemoptysis, dyspnea, pleuritic chest pain, and hypoxic hypotension. Magnetic resonance imaging has a restriction due to use of gadolinium contrast because it is very time-consuming and can lead to cardiac arrhythmias due to the electromagnetic fields effect. Computed tomography is preferable. Additional biomarkers along with the D-dimers are the soluble form of P-selectin, the first and second clotting factors along with the factor VIII [65]. We observe an increased quantity of interleukin-6 (IL-6) and inter-cellular adhesion molecule 1 (ICAM-1) [57].

In the pediatric population the most important risk factor for the venous thromboembolism is the central venous catheter, but is less often found compared to the adult population, having now a steady increase in incidence [64, 66].

The deficiency of protein C is common, Protein S deficiency is a relatively rare condition, but it is more frequently observed in the Asian population, the mutations for the genes which codify the von Leiden factor (V) are characteristic for the Caucasian population. Hereditary thrombophilia was reported in approximatively 8.8% of the studied cohorts in Asia and the deficiency of the antithrombin III was not estimated along with the von Willebrand disease or acquired/hereditary hyperhomocysteinemia. The treatment is the same like in the adult population with a variation in the doses of the pharmacological drugs according to the body-weight [64].

The most notorious complication of the venous thromboembolism is the post-thrombotic syndrome which is chronicled by the venous thromboembolism symptoms (pain, edema and ulceration) with a toleration for the pain feelings and the difficulty to walk while there is a progression for the elephantiasis (like the cardiac insufficiency disabilities). The incidence of the post-thrombotic syndrome varies between 25-50% and is lower in the endovascular interventions favoringpharmacological thrombolysis [57]. This fact will determine the necessity of an individual approach in the diagnostic and treatment options for the venous thromboembolism [67].

Molecular biomarkers

The literature proves that there are no serum biomarkers that may confirm a diagnostic of thromboembolism in a specific manner, no matter of its genesis. Some markers have a high sensitivity with a low specificity thus making them unable to confirm thromboembolism. This context creates the conditions in which additional studies are necessary in order to prove other biochemical markers with diagnostic and prognostic potential. Following, will be described a short list of markers, some of which are experimental.

Protein C

It is one of the clotting factors that has a similar structure and function with the prothrombin, factors VII, IX and X. It has a light chain and a heavy chain that are interconnected via disulfide bonds formed between the variable cysteine residues [68]. It has a molecular mass of 52.071 Da and is codified by the PROC gene that is located on 2q14.3 [69]. Its structure is stabilized by the Ca^{2+} ions that have an increased affinity for the GLA domain [68]. This protein has nothing to do with the C-reactive protein and is not involved in the inflammatory reactions [70]. During the menstrual cycle, ovulatory phase notorious resistance to the activated protein C was noticed [71]. The lack of protein C or the resistance to the activated protein C is manifested as venous thromboembolism [72].

Protein S

It is the cofactor of the activated protein C and the tissue factor pathway inhibitor. From a structural overview, it is a glycoprotein that is rich in γ-carboxyglutamate [73]. It has a molecular mass of 75.123 Da and is codified by the PROS1 gene that is localized on the 3q11.1 chromosome band [74]. The anticoagulant effect is due to the fact that it may form glutamyl-heparin bounds in the exits of the factor IXa no matter of its concentration and the activity of the VIII factor [73]. Estrogen is influenced by the concentration of the serum protein S due to this fact we can explain its variation in the patients that administer oral contraceptives and its monthly fluctuation during the menstrual cycle [75]. Like bilirubin, the measurement of protein S is based on the free portion, the portion that is bound with the γ-globulins and is made along with the measurement of protein C. The normal values of these proteins are found in Table 1 [76]. Venous thromboembolism has a significant statistical correlation with protein S concentrations [72].

Table 1. Normal concentrations for protein C and protein S.

Protein C	**Values (UI/dl)**
1-5 years	40-92
6-10 years	45-93
11-16 years	55-110
Adult	64-128
Protein S (total fraction)	**Values (UI/dl)**
1-5 years	54-118
6-10 years	41-114
11-16 years	52-92
Adult	60-113
Protein S (free fraction)	**Values (UI/dl)**
1-5 years	21-69
6-10 years	22-62
11-16 years	26-55
Adult	27-61

D-dimers

D-dimers are the product of fibrin degradation due to its interaction with thrombin. They can become a valuable biomarker in the diagnostical process of arterial and venous thromboembolism [77]. There is no gene which may codify this compound but there are antibodies against D-dimers that are expressed by the corresponding genes, the most important of them being tumor necrosis factor α (TNF-α),

CD40 ligand and interleukin 10 (IL-10) [78]. There are no well-established intervals for the D-dimers concentration in blood thus their concentration is often subjective above the values of 0.5 μg/ml [76, 79]. A disadvantage is that D-dimer has a high sensibility but a low specificity thus a low prediction capacity with many false-positive cases [80, 82, 83]. Factors like age, pregnancy, inflammatory diseases, cardiovascular diseases and disseminated intravascular coagulation can make the result even more inconclusive. The measurement of D-dimer necessitates their association with other biomarkers in order to confirm a diagnostic [81-83].

Fibrinogen

It is a soluble protein that can form bonds with keratin, myosin, and epidermin. It's composed of three chains – Aα, Bβ and γ [84] that are codified by the corresponding genes – FGA and FGB localized in a cluster on 4q31.3 and FGG on 4q32.1 [85-87] with a 94.973 Da [85], 55.928 [86] and 51.512 Da molecular masses [87]. The final biochemical structure has α-helicoidally conformation and 3 domains – A (N-terminal), B and P (C-terminal). Fibrinogen has the capacity to bind Ca^{2+} ions. Interacting with thrombin it is cleaved into fibrin [84]. Fibrinogen has markedly increased serum concentration along with an erythrocyte number in venous thromboembolism [88, 89].

Selectin P, Selectin L and Selectin E

Selectins are peptides that are part of the type C lectin superfamily and are composed of a N-terminal domain that can bind Ca^{2+} ions along with an epidermal growth factor (EGF), peptidic tandems and a transmembranary domain [90]. P selectin has a molecular mass of 90.834 Da [91]. L selectin has a molecular mass of 42.187 Da [92] and E-selectin has a molecular mass of 66.655 Da [93]. These proteins are codified by a gene cluster located on the 1q24.2 chromosomal band [91-93]. Selectins are expressed on the surface of the platelets, endotheliocytes and leucocytes. The elevated blood concentrations of the soluble P-selectin forms along with the soluble E-selectin forms can be observed in association of an emerging venous thromboembolism in human study polls. We must mention that E-selectin can be genetically determined for unusual alleles that can predict an increased risk for thromboembolism and are dependent on the menstrual cycle (the luteal phase has its soluble form elevated in plasma) [94-95]. The soluble form of L-selectin was associated with thrombosis events [96].

Interleukins

They are represented by a series of proteins that are located in the regulatory leukocytes and are responsible for intercellular communication. They are codified and expressed in clusters and furthermore cleaved and stored. We identify 38 types of interleukins (IL 1-38) [97]. It was proven that interleukin 6, interleukin 8 and the monocytic chemoattractant protein 1 (MCP-1) are capable of inducing coagulation events. One of the major interleukins which secretion is low in the thromboembolism events realizes the differential diagnosis with the majority of unspecific inflammatory states is interleukin 10 (IL-10) [98] while leukocytosis is not mandatorily associated [99].

C-reactive protein (CRP)

It is represented by a pentameric protein synthetized in the liver and is activated by the interaction of interleukin 6 (IL-6) on the genomic structures in inflammatory states [100]. It has a molecular mass of 25.039 Da and is expressed by the CRP gene located on the 1q23.2 chromosome band [101]. For the adult population, concentrations that are lower than 0.3 mg/dl are normal, those that are located between 0.3-1.0 mg/dl are mildly elevated, 1.0-10.0 mg/dl moderately elevated, 10.0-50.0 mg/dl markedly elevated and >50.0 mg/dl is severe elevation [100]. We have proven that it has increased concentrations in venous thromboembolism [102-103] and arterial thrombosis [103].

Thrombin/Antithrombin (TAT)

Thrombin is represented by an enzyme that is responsible for the fibrin conversion from fibrinogen [104]. Antithrombin III is a glycoprotein composed from 432 aminoacids that inhibit thrombin and thus is part of the serin-dependent protease inhibitor from the serpine group. The α-antithrombin has a conformation that can bind all the 4 domains of the thrombin while β-antithrombin is able to glycosylate only 3 of them [105]. The balance between the thrombin-antithrombin complex is maintained on the principles exposed in the Michaelis-Menten equation and thus can express the intensity of the platelet aggregation processes and in turn the thrombin generation must be measured without getting an insight into their concentration [104].

Plasma proteins (Albumins, Globulins)

Albumins are the main protein fraction in the blood plasma, being synthetized in the liver [106]. Globulins are represented by the reminiscent portion that is made out of the α_1, α_2, β and γ portions that are represented by the immunoglobulins, complement and transport proteins like haptoglobulin, transferrin, ceruloplasmin and many more [107]. The albumin concentrations are variable between 3.5-5 g/dl [106] while globulins have variable values because they are dependent on the total protein fraction and albumin fraction: $C_{globulins} = C_{proteins} - C_{albumins}$ taking into fact that the total protein fraction has concentrations in the limits of 6-8 g/dl [108]. The low albumin concentrations along with the globulins represent and important indicator for the venous thromboembolism risk in the presence of emerging conditions like the nephrotic syndrome and some physiological states like the low-gravitational field in the cosmic space where the physiology is different [109, 110].

Prostaglandins, Leukotrienes and Nitric Oxide

Prostaglandins and leukotrienes are eicosanoids, derivatives of the arachidonic acid and polyunsaturated fatty acids. Their synthesis is assured by the cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) [111]. It is well known that the nitric oxide has a synergistic effect with the prostaglandins, with an unspecific action while the eicosanoids have their own receptors or can interact with analogue receptors [112]. The main effects of the prostaglandins in the thromboembolism context are the vasodilation (PTGE2), allergic response (PTGD2), musculature contraction and pulmo-

nary vessel contraction (PTGF2) and platelet aggregation inhibition (PTGI2). Leukotrienes like LTB4 are implied in the turnover if the endotheliocytes while LTC4, LTD4 and LTDE4 will determine bronchoconstriction, neutrophil extravasation, and vascular response. Nuclear polymorphisms will influence the variability of the functionality of these mediators and the pharmacological response to COX-1 and COX-2 inhibitors (non-steroidal antiinflammatory drugs) [113]. Thromboxanes are not proven to be implied in the thromboembolism in humans, this in turn being proven only for mice species [114, 115].

Matrix metalloproteinases (MMP)

This family of proteases is represented by Zn^{2+} dependent endopeptidases that will control the degradation of the matrix metalloproteinases. Structurally they have a hemopexic domain that is effective for proteolysis in the presence of Zn^{2+} ions [116]. They are part of disintegrin and metalloproteinase motif or disintegrin and metalloproteinase thrombospondin motifs and are inhibited by the tissue inhibitor of metalloproteinase 1-4 (TIMP 1-4) which can interact on the C domain of hemopexin [117]. It was proven that myeloperoxidase (MPO) is capable of inhibiting or activating matrix metalloproteinases that are dependent on their subtype and the structure of the C domain of hemopexin [118]. Matrix metalloproteinase 2 (MMP-2) will be activated by the myeloperoxidase (MPO) and will exacerbate the progression of the venous thromboembolism [118, 119] and matrix metalloproteinases 1 (MMP-1) will be regulated only by the tissue inhibitor of metalloproteinase 1-4 (TIMP 1-4) [117, 119]. In mice, the proaggregant action of MMP-9 and MMP-14 was proven without being regarded for the human species [120].

Our comprehension of thromboembolism has advanced, allowing for a more convenient interpretation of its relationship with systemic inflammation, metabolic changes, and other pathophysiological conditions. While these markers offer promising results, relying on any one of them alone may lead to false-positive or false-negative outcomes.

To ensure an accurate diagnostic or prognostic process, it is essential to apply these markers in combination. Further studies are necessary to meet the requirements of medical laboratories and enhance their utility.

Conclusions

(1) Although arterial and venous thromboembolism typically share common biomarkers, certain molecules may exhibit distinctive characteristics, enabling differential diagnosis. However, their practical applicability in clinical settings is limited.

(2) The majority of biochemical compounds utilized as biomarkers demonstrate strong interrelationships, allowing for the identification of cascade patterns of expression in both physiological and pathophysiological conditions.

(3) Understanding the structural aspects and mechanisms of action within the procoagulant cascade, in conjunction with biomarkers, is crucial for determining an appropriate treatment strategy.

Competing interests

None declared.

Authors' contribution

DC conceptualized and realized the study. EP revised, analyzed, and redacted critically the content of the study. All authors revised and approved the final version of the manuscript.

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REVIEW ARTICLE

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Current affairs in the use of medical ozone. Biological effects. Mechanisms of action

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ABSTRACT

Introduction. Oxygen-ozone therapy stands as a medically endorsed practice confirmed by numerous international clinical studies. Various authors have illustrated the beneficial clinical outcomes of ozone therapy in terms of its capacity to regulate redox balance, cellular inflammatory responses, and adaptation to ischemia/reperfusion processes. Ozone therapy extends to encompass a range of viral infections, inflammatory disorders, and degenerative ailments, used as both monotherapy and as an adjunct to unified conventional therapies.

Material and methods. Narrative literature review study. Bibliographic search was conducted using the *PubMed, Hinari,* and *SpringerLink* databases, as well as the *National Center of Biotechnology Information* and *Medline*. Articles published between 1990 and 2022 were selected using various combinations of keywords, including "ozone", "ozone therapy", "mechanisms of ozone action", "biological effects of ozone", "antioxidant effect", "anti-inflammatory effect" and "immunomodulatory effect." Information regarding ozone's mechanisms of action was identified and processed. Following the database information processing and search criteria, a total of 475 full-text articles were found. The final bibliography consists of 52 relevant sources that were deemed representative of the materials published on the topic of this synthesis article.

Results. The effects of ozone on oxygen metabolism are explained by changes in the rheological properties of blood, including inhibition of erythrocyte aggregation and stimulation of 2,3-diphosphoglycerate in erythrocytes, favoring the transport and delivery of oxygen to tissues while facilitating the substantial elimination of nitric oxide and increasing blood flow. Intracellular triatomic oxygen enhances the oxidative carboxylation of pyruvate, stimulating ATP production, which also contributes to reducing peripheral vascular resistance.

Conclusions. Ozone generates a moderate oxidative stress. Yet, it can set off several beneficial biochemical mechanisms that reactivate both the intra- and extracellular antioxidant systems and reverse chronic oxidative stress in various inflammatory and degenerative processes. Ozone induces a mild activation of the immune system by triggering neutrophil activation and stimulating the synthesis of certain cytokines (IL-2, TNF-α, IL-6, and IFN-γ), thereby initiating a complete cascade of immune responses. Ozone therapy yields the following biological reactions: optimization of blood circulation and oxygen delivery to ischemic tissue, regulation of cellular antioxidant enzymes, initiation of a slight immune system activation, and enhancing the release of growth factors.

Keywords: ozone, ozone therapy, mechanisms of ozone action, biological effects of ozone, antioxidant effect, anti-inflammatory effect, immunomodulatory effect.

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Key messages

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

The article summarizes the latest foreign publications on the mechanisms of the antioxidant, anti-inflammatory, regenerative,

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The research hypothesis
Systematization and critical analysis of published data on most recent findings regarding ozone therapy's mechanisms of action, including its methodology and optimal timing of application.
The novelty added by manuscript to the already published scientific literature
This comprehensive review reassesses the reported mechanisms of medical ozone, clinical potency and the latest medical approaches towards the effects of medical ozone.

Introduction

Ozone (O3), a gas discovered in the mid-19th century and composed of three oxygen atoms, represents a highly reactive allotropic form of oxygen. It exhibits high solubility in plasma, extracellular fluids, and water (approximately 10 times more soluble in water than conventional oxygen). At room temperature, it is unstable, causing rapid decomposition into ordinary diatomic oxygen. Notably, its half-life measures 25 minutes at 30°C, 40 minutes at 20°C, and 140 minutes at 0°C [1-10].

Medical ozone is a blend of oxygen and ozone derived from medical-grade oxygen through the utilization of a medical ozone generator. This medical ozone contains a concentration of 1-5% ozone and 90-95% pure medical oxygen, or 10-80 μg/mL (0.21-1.68 μmol/ml) of ozone per milliliter of blood. Ozone therapy stands as a current and significant avenue of research in contemporary medicine [1, 3-5, 7, 9, 10-15].

Oxygen-ozone therapy is a medically validated practice supported by numerous international clinical studies. Nowadays, many clinical trials have shown its beneficial effects on the modulation of the oxidoreduction balance, cellular inflammation state, and adaptation to ischemia/reperfusion processes. Ozonotherapy is an effective, safe, feasible, and easy-to-perform technique, which finds applications in various inflammatory, infectious, degenerative diseases, as well as in rehabilitation following acute cardiac and cerebral ischemic events. It demonstrates good efficacy both as an independent treatment and, notably, as an adjunct to conventional therapies [3-5, 7, 11, 16-22]. By incorporating this medical practice, patients can attain significant clinical benefits. When combined with standard therapies, it often leads to reduced medication dosages, complication rates, treatment duration, medication toxicity, and medical expenses. It also addresses the issue of bacterial resistance to antibiotics [2, 4, 18, 19, 21, 23].

In the context of the aforementioned, the purpose of this article is to present a synthesis of the most recent findings regarding ozone's mechanisms of action.

Material and methods

To achieve the outlined purpose, an initial search of specialized scientific publications was conducted. These were identified through the Google Search engine, namely, *PubMed, Hinari, SpringerLink, the National Center of Biotechnology Information,* and *Medline*. The article selection criteria encompassed contemporary data regarding the mechanisms of action of ozone therapy, utilizing the following keywords: "ozone", "ozone therapy", "ozone mechanisms of action", "biological effects of ozone", "antioxidant effect", "anti-inflammatory effect", and "immunomodulatory effect." 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, articles published between 1990 and 2022. After a preliminary analysis of the titles, original articles, editorials, articles of narrative synthesis, taxonomy, and meta-analysis were selected, which contained up-to-date information and contemporary concepts regarding the mechanisms of ozone therapy. Furthermore, a search was conducted within the reference lists of the identified sources to highlight additional relevant publications that were not found during the initial database searches.

The information from the publications included in the bibliography was gathered, organized, evaluated, and synthesized, showcasing the key aspects of the contemporary understanding of ozone's mechanisms of action, namely, its antioxidant capacity, vascular and hematological modulation, immune system activation, as well as its anti-inflammatory, bactericidal, virucidal, and fungicidal effects.

To minimize the potential systematic errors (bias) in the study, a meticulous search was conducted within databases to identify a maximum number of relevant publications for the study's purpose. Only studies that satisfy validity criteria were evaluated, rigorous exclusion criteria for articles under consideration were applied, and a comprehensive review was conducted of both positive outcome studies and those that did not highlight the treatment's benefits.

If necessary, additional sources of information were consulted to clarify some concepts. Duplicate publications and articles that did not meet the purpose of the article and were not available for full viewing were excluded from the list of publications generated by the search engine.

Results

Following the data processing, as identified by the Google Search engine and from databases such as *PubMed, Hinari,*

SpringerLink, National Center of Biotechnology Information, and *Medline*, in accordance with the search criteria, a total 475 articles on the topic of ozone therapy were found. After a primary analysis of the titles, 59 articles were eventually deemed relevant for the given synthesis. Upon repeated review of these sources, a final selection of 52 relevant publications was ultimately made in alignment with the intended purpose. The final bibliography of this work comprises 52 articles that have been considered representative of the materials published on the subject of this synthesis article.

Publications, the content of which did not reflect the relevant topic, despite being selected by the search program, as well as articles that were not accessible for open viewing through the *HINARI (Health Internet Work Access to Research Initiative)* database or available in the scientific medical library of the *Nicolae Testemițanu* State University of Medicine and Pharmacy, were subsequently removed from the list.

Although ozone is the most potent natural oxidant, capable of oxidizing a wide range of organic and inorganic substances, and has the potential for cytotoxicity, researchers believe that under controlled conditions, it possesses numerous therapeutic effects. Moreover, the reactivity to ozone can be effectively mitigated by the blood and cellular antioxidant system [1, 2, 15, 24-28]. Initially used as an empirical approach, oxygen-ozone therapy has now evolved to a stage where the majority of ozone's biological mechanisms of action have been extensively studied and elucidated, these findings being found within the fields of biochemistry, physiology, and pharmacology [18, 22, 25, 27-29].

Ozone is not a pharmaceutical medicine but rather a regulatory molecule capable of generating bioactive mediators. The effects of ozone have been demonstrated to be consistent, safe, and associated with minimal preventable side effects [2, 30]. Chronic oxidative stress, chronic inflammatory processes, and immune overactivation are present and highly detrimental in a wide variety of diseases. The effectiveness of ozone therapy is determined by moderate oxidative stress, resulting from the interaction of ozone with the biological components of the body, triggering an endogenous cascade of biochemical reactions [31].

Ozone can function as an oxidant either directly, when it dissolves in plasma and other biological fluids, immediately reacting with polyunsaturated fatty acids, antioxidants, cysteine-rich proteins, and carbohydrates; or indirectly, by generating reactive oxygen species (ROS) and lipid oxidation products (LOP) [25, 28, 32-37]. At the onset of ozone therapy, an endogenous cascade is triggered, releasing bioactive substances in response to transient and moderately induced oxidative stress by ozone ('oxidative eustress'). Ozone can easily induce this oxidative stress due to its plasma solubility. Reacting with polyunsaturated fatty acids and water, ozone forms ROS in human fluids and tissues. The main molecule among ROS is hydrogen peroxide (H_2O_2) – a non-radical oxidant. Concurrently, ozone also gives rise to LOP – the lipoperoxide radical, hydroperoxides, malondialdehyde, isoprostanes, ozoneides, alkenes, and predominantly, 4-hydroxynonenal. ROS and LOP are the effector molecules responsible for modulating several biological and therapeutic effects in the body following ozone therapy [3, 8, 13, 27, 34, 37-39].

Having reacted with a number of biomolecules, ozone disappears, and hydrogen peroxide, the main molecule of ROS, and other mediators rapidly diffuse into cells, activating various metabolic pathways with numerous biological and therapeutic effects [3, 5, 27, 28, 35, 40]. Therefore, ROS and LOP are "biological messengers of ozone" and are responsible for the biological and therapeutic effects of ozone. ROSs are short-acting early messengers and are responsible for immediate biological effects, while LOPs are important late and long-term messengers [3, 5, 10, 13, 14, 17, 28, 36].

The formation of ROS in plasma occurs extremely quickly (less than a minute), accompanied by a moderate and transient decrease in the antioxidant capacity of the blood (from 5% to 25%). However, this antioxidant capacity returns to normal within 15-20 minutes [3, 9, 17, 28, 31, 40, 41].

Discussion

Although not fully comprehended, the present article will delve into the mechanisms underlying the antioxidant, anti-inflammatory, immunomodulatory, antimicrobial, antiviral, and analgesic effects of ozone.

The ***antioxidative capacity*** is considered one of the key impacts of ozone therapy. Moderate oxidative stress induced by ozone within the therapeutic range (10-80 μg/mL), most commonly 30-45 μg/mL (the 'physiological' dose of ozone), physiologically effective and recommended levels for systemic application, elicits controlled low doses of ROS acting primarily as signaling molecules, thereby stimulating the formation of LOP. ROS triggers the activation of nuclear erythroid factor 2 (Nrf2), well known as a pivotal regulator of manifold cytoprotective responses, responsible for upregulating antioxidant enzyme activity. In response to transient, moderate oxidative stress, the levels of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase, glutathione S-transferase, catalase, and heme oxygenase-1 increase. Thus, moderate, transient, and repetitive oxidative stress causes an intense modulation of antioxidants in the body. A multitude of cells across various organs upregulate the synthesis of antioxidants, which are capable of significantly countering excessive ROS, thereby alleviating chronic oxidative stress, which is present and extremely harmful in a variety of diseases Consequently, ozone, either through oxidative preconditioning or adaptation to chronic oxidative stress, safeguards tissue integrity against ROS-induced damage, fostering a balance between antioxidant and pro-oxidant factors while preserving cellular redox balance [27, 30, 41-45].

Nrf2 and nuclear factor kappa B (NF-κB) represent the primary signaling pathways through which ozone exerts its effects. Nrf2 activation regulates cell defense and maintains cellular homeostasis [36]. Furthermore, ozone therapy fosters adaptation to oxidative stress by gently triggering the

immune system, releasing growth factors, and/or activating metabolic pathways that contribute to maintaining redox balance [38].

By activating Nrf2, LOP induces oxidative stress proteins, including heme-oxygenase-1 (HO-1), another inhibitor of the NF-κB pathway and one of the most crucial antioxidant defense enzymes. Through inhibiting the high expression level of hypoxia-inducible factor-1α (HIF-1α), it contributes to reducing the production of proinflammatory cytokines, directly activating anti-inflammatory cytokines, enhancing antioxidant protection, and consequently, safeguarding cellular integrity [8, 28, 30, 44-46].

Thus, ozone mimics acute oxidative stress which, when properly balanced, is not harmful, but can trigger several beneficial biochemical mechanisms. It can reactivate the intra- and extracellular antioxidant system, thereby reversing chronic oxidative stress in various inflammatory, degenerative processes, etc. During ozone treatment, cells throughout the body receive gradual and subtle impulses of LOP, significant long-term messengers that play a crucial role in up-regulating antioxidant enzymes in multiple cell types while rebalancing the oxidant/antioxidant system [46, 47].

Vascular and Hematological Modulation. Ozone serves as a catalyst for transmembrane oxygen flow. The increase in cellular oxygen levels resulting from ozone therapy enhances the efficiency of the mitochondrial respiratory chain. Moreover, ozone amplifies the production of prostacyclin, a widely acknowledged vasodilator [2, 6, 8, 26].

The effects of ozone on oxygen metabolism can be explained by promoting (1) changes in the rheological properties of blood (reversal of erythrocyte aggregation, increased flexibility and elasticity of red blood cells, favoring the transport and delivery of tissue oxygen), leading to enhanced blood flow in microcirculation; (2) increasing the speed of glycolysis in erythrocytes; and (3) the release of substances (adenosine triphosphate, nitric oxide, and prostaglandins) that may contribute to reducing peripheral vascular resistance and increasing oxygen supply to tissues [18, 25, 35, 37, 40, 48-50].

Hydrogen peroxide (H_2O_2) diffuses from the plasma into the cellular cytoplasm and serves as the triggering stimulus. Depending on the cell type, various biochemical pathways can be simultaneously activated in red blood cells, white blood cells, and platelets, leading to a multitude of biological effects [10, 28, 32].

The Impact of Ozone on Erythrocytes. Erythrocytes are the focus of ROS. During erythropoiesis, submicromolar concentrations of LOP positively regulate the synthesis of antioxidant enzymes. Consequently, ozone therapy increases the glycolytic rate by enhancing intracellular adenosine triphosphate production. This approach intensifies erythrocyte generation, yielding metabolically enhanced erythrocytes (super-endowed erythrocytes) capable of more effectively transporting and delivering oxygen to tissues, including ischemic tissues, thereby correcting hypoxia in vascular diseases [7, 12, 25, 27, 28, 39, 48]. Coupled with increased nitric oxide synthase activity, there is a significant increase in nitric oxide, an essential element in maintaining optimal levels of vasodilation and blood perfusion [1, 6, 8, 40].

Ozone therapy, through careful regulation of ozone dosage, stimulates the production of antioxidant enzymes within the system (catalase, glutathione peroxidase, and superoxide dismutase) while mitigating excessive formation of ROS, thereby reducing chronic oxidative stress [1, 6, 12, 14, 39, 43, 49].

The impact of ozone on leukocytes. Ozone acts as a mild cytokine and serves as a cytokine inducer by lymphocytes and monocytes, thereby enhancing the immune system's activity. This stimulation fosters intercellular matrix synthesis and contributes to the healing process [1, 12, 32, 35, 37, 39].

The Impact of ozone on platelets. Hydrogen Peroxide (H_2O_2) and other ROS generated through blood ozonation initiate a cascade of enzymatic reactions. These reactions gradually elevate intracellular Ca levels and trigger the release of prostaglandins (F2a and E2), leading to irreversible platelet aggregation. Increased levels of growth factors released from platelets, mobilization of endogenous stem cells, and stimulation of neoangiogenesis promote tissue regeneration, as well as healing of injuries and wounds [27, 49, 51].

Thus, the impact of ozone on oxygen metabolism is explained by how it alters the blood's rheological properties (reversing red blood cell clumping, enhancing the flexibility and elasticity of red blood cells, promoting the transport and delivery of oxygen to tissues). This, in turn, facilitates blood flow in the microcirculation, increases glycolysis in red blood cells, and triggers the production of substances (such as adenosine triphosphate, nitric oxide, and prostaglandins) that help reduce peripheral vascular resistance.

Activation of the immune system. Ozone promotes an increase in the production of interferon-γ (IFN-γ) and some cytokines, with interleukin-2 (IL-2) being the primary one, subsequently triggering a whole cascade of immunological reactions [1, 2]. It has been shown that ROS, including H_2O_2 and LOP generated by ozone therapy, can easily diffuse into plasma cells and activate NF-κB, inducing the production of immunoactive cytokines in normal cells (IL-2, tumor necrosis factor alpha - TNF-a, IL-6 and IFN-γ), thereby enhancing the immune response [9, 13, 14, 26, 32, 40, 44].

Ozone indirectly activates the innate (non-specific) immune system by enhancing phagocytosis and promoting the synthesis of cytokines and interleukins in neutrophils and leukocytes. It also triggers the components of both cellular and humoral immunity [8, 26, 33, 39]. Within mononuclear cells, ozone stimulates immune responses by modulating the NF-kB transcription factor, thereby reactivating the suppressed immune system [27, 28].

Furthermore, ROSs trigger the activation of the immune system, which acts through monocytes and lymphocytes, promoting the production of a variety of cytokines (IL-1, IL-2, IL-6, IFN-β, IFN-γ, TNFα) [6, 49].

Thus, ozone induces mild immune system activation by stimulating neutrophils and initiating the synthesis of cer-

tain cytokines that trigger a whole cascade of immunological responses.

Bactericidal, virucidal and fungicidal action of ozone. Ozone used in vitro acts directly on the membrane of bacterial cells (direct oxidative effect), disrupting and damaging the integrity of bacterial cell membranes, oxidizing phospholipids and lipoproteins, thereby impeding their enzymatic function. Additionally, ozone damages the viral capsids, disturbing their structure and interfering with the virus-cell interaction, leading to disruption in the reproductive cycle. When it comes to fungi, ozone inhibits cell growth by perturbing intracellular homeostasis, resulting from the compromised barrier properties of the plasma membrane [2, 6, 12, 16, 26, 44, 48, 50].

Although ozone is one of the most potent disinfectants, used in various ways, it cannot deactivate any pathogens (bacteria, viruses, and fungi) in vivo. This is because pathogens are well protected, especially within cells, by the cell's powerful antioxidant system. Consequently, ozone acts as a gentle enhancer of the immune system by activating neutrophils and stimulating the synthesis of certain cytokines [1, 10, 19, 22, 28, 39, 46].

The ***anti-inflammatory effect*** is revealed in ozone's ability to influence the inflammatory cascade by oxidizing biologically active substances (arachidonic acid and its derivatives - prostaglandins), which participate in the development and sustenance of the inflammatory process. Additionally, ozone significantly reduces the levels of pro-inflammatory cytokines (IL-1β, IL-6, IL-8, and TNF-α) without any signs of toxicity or recorded side effects [8, 26, 30, 31]. These cytokines induce the prostaglandin E_2 pathway, which causes pain or increases the sensitivity of nerve roots to other algogenic substances (such as bradykinin) [31].

Severe oxidative stress, triggered by high concentrations of ozone, along with proinflammatory cytokines (IL-1β, IL-6, IL-8, TNF-α), activate NF-κB, a key regulator of the inflammatory response and muscle atrophy. This contributes to an increased inflammatory response and tissue damage, including the release of other inflammatory factors that enhance the migration of eosinophils and neutrophils [9, 13, 17, 47, 49].

On the contrary, mild oxidative stress induced by precise and small doses of ozone activates Nrf2. The latter indirectly inhibits the pro-inflammatory mechanism driven by the NF-kB pathway. As a result, there is a reduction in NF-κB activity along with a modification in the expression of inflammatory cytokines associated with NF-kB activity. This triggers an anti-inflammatory effect, leading to a decrease in IL-1, IL-2, IL-6, IL-7, and TNFα, as well as an increase in interleukins such as IL-4, IL-10, IL-13, and the transforming growth factor beta – TGF-β [11, 13, 19, 38, 43, 49, 50].

Nrf2 also plays an important role in intracellular inflammatory signaling pathways. Triggering the Nrf2-antioxidant signal can dampen NF-kB activity, leading to the downregulation of the inflammatory response by suppressing essential inflammatory mediators and cytokines (IL-6, IL-8, and TNF-a) [31, 38, 42, 50].

Moreover, a small amount of H_2O_2 stimulates the NF-kB pathway, which is typically balanced out by the Nrf2's blocking action, resulting in an immunomodulatory effect [11].

The ***analgesic effect*** of ozone is ensured by the oxidation of the byproducts of albuminolysis, known as algopeptides, which act on the nerve endings in the damaged tissue and determine the intensity of the pain response. Additionally, the analgesic effect is attributed to the restoration of the antioxidant system and, subsequently, the reduction of harmful molecular byproducts from lipid peroxidation [26]. Recent preclinical studies have elucidated the role of ROS in hyperalgesia by activating N-methyl-D-aspartate receptors [11].

Following ozone therapy, there has been a demonstrated increase in antioxidant molecules (serotonin and endogenous opioids), which induce pain relief by stimulating antinociceptive pathways [31, 39].

Data from scientific research acknowledge that the mechanisms of action of ozone are due to: (1) a decrease in the production of inflammatory mediators; (2) oxidation (inactivation) of metabolic mediators of pain; (3) improvement of local blood microcirculation leading to improved oxygen delivery to tissues; (4) elimination of toxins and resolution of physiological disorders that generate pain [42, 52].

Therefore, ozone exhibits pleiotropic properties, extending beyond its exclusive role as an antioxidant, anti-inflammatory, or immunomodulatory one. It also encompasses the capacity to employ ROS as a signaling molecule rather than merely as intracellular toxic substances. In existing experimental models and clinical studies, the anti-inflammatory, antioxidant, regenerative and immunomodulatory effects of ozone therapy have been associated with several molecular mechanisms, the main ones being the NF-kB/Nrf2 balance and IL-6 and IL-1β expression. NF-kB and Nrf2 are the most studied and important transcription factors and regulatory proteins that control the expression of a wide range of genes, encoding proteins involved in a multitude of vital biological functions, including those associated with redox status, immunity, and inflammatory responses. Additionally, indirectly through these pathways, LOP initiates the HIF-1α, HO-1, and NO/iNOS pathways.

The main pharmacological effects of medical ozone through ozone-produced peroxides are as follows: (1) increased oxygen release by erythrocytes due to activated metabolism; (2) immunomodulation due to leukocyte activation; and (3) regulation of cellular antioxidants via Nrf2 signaling. Ozone therapy can elicit the following biological reactions: (a) improved blood circulation and oxygen delivery to ischemic tissue; (b) optimization of overall metabolism by improving oxygen delivery; (c) regulation of cellular antioxidant enzymes and induction of HO-1; (d) triggering a mild immune system activation and intensified release of growth factors; (e) providing a state of well-being in most patients, probably due to stimulation of the neuroendocrine system.

Conclusions

1. Ozone induces both mild and moderate oxidative stress. When appropriately balanced, this stress poses no harm; instead, it can initiate several beneficial biochemical mechanisms. These mechanisms, in turn, reactivate the intracellular and extracellular antioxidant systems, effectively countering long-term oxidative stress in various inflammatory and degenerative processes, etc. Cells throughout the body receive small and gradual bursts of lipid oxidation products, important late and long-term messengers that are responsible for activating antioxidant enzymes in many cell types to rebalance the oxidant/antioxidant system.
2. The impact of ozone on oxygen metabolism is explained by changes in the blood's rheological properties. This involves reversing red blood cell aggregation, enhancing the flexibility and elasticity of hemoglobin, and promoting the efficient transport and delivery of oxygen to tissues. This process also facilitates blood flow within microcirculation, speeds up glycolysis within red blood cells, and triggers the release of substances like adenosine triphosphate, nitric oxide, and prostaglandins, which help to reduce peripheral vascular resistance.
3. Ozone triggers a slight activation of the immune system by up-regulating and activating neutrophils and promoting the synthesis of cytokines (IL-2, TNF-a, IL-6, and IFN-γ), setting off a chain reaction of immune responses.
4. Ozone therapy induces the following biological responses: enhanced blood circulation and oxygen delivery to ischemic tissue, regulation of cellular antioxidant enzymes, mild immune system activation, and intensified release of growth factors.
5. Ozone is an inherently toxic gas that should never be inhaled, cannot be stored, and must be handled with caution. Generally, no toxic effects were reported, and only the respiratory tract was found to be highly sensitive to inhaled ozone since the respiratory mucosal cells contain a minimal amount of antioxidants and are extremely susceptible to oxidation.
6. Although ozone ranks among the most potent disinfectants, being employed in various ways, it cannot neutralize any pathogens (bacteria, viruses, and fungi) in vivo, since pathogens are effectively shielded by the strong blood and cellular antioxidant system. Furthermore, elevated ozone concentrations induce severe oxidative stress, prompting increased inflammatory responses and tissue damage.

Competing interests

None declared.

Authors' contribution

NC and RB conceived the study, participated in the study design and assisted in drafting the manuscript. SŞ and IC performed the analysis and data interpretation. IG drafted the manuscript. SC conceived the significant revision of the manuscript and provided significant intellectual involvement. The authors have read and approved the final version of the manuscript.

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REVIEW ARTICLE

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The role of the lateral pterygoid muscle in temporomandibular disorders

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ABSTRACT

Introduction. The clinical concept that would argue that the activity of the lateral pterygoid muscle, being disturbed, would play an important role as an etiological factor in temporomandibular joint dysfunctions is still widely accepted, being also a decisive factor in the correct choice of the treatment plan. However, because of the fact that very few research and clear evidence were conducted and presented to support completely that concept, it continues to remain a very controversial one.

Material and methods. For this literature review were considered and studied scientific articles published between 2000 and 2023, in the following electronic databases: *PubMed, MEDLINE, Google Scholar, BIR Publications, ScienceDirect.* Research methods – analysis, synthesis, systematization, and description.

Results. Patients presenting temporomandibular joint dysfunction complain about pain in the temporomandibular joint or/and in masticatory muscles, limitation and sounds during mandibular activity. Temporomandibular dysfunction is a non-specific collective term, used to describe a heterogeneous group of pathological conditions located in the territory of the stomatognathic system. These are considered musculoskeletal conditions that cause pain while performing the function (mastication, speech, swallowing), with increased sensitivity in the masticatory muscles and/or the temporomandibular joint, with possible limitations of the range of motion, the appearance of joint noises and otological symptoms. One of the theories claims that in temporomandibular dysfunction, the lateral pterygoid muscle becomes hyperactive, hypoactive or that there is a lack of coordination between the superior and inferior branches of the muscle, or that there is a disturbance during the performance of the role of the muscle to control and stabilize the temporomandibular joint. However, the in-depth study of the specialized literature indicates that no scientific evidence is yet available that the function of the lateral pterygoid muscles in temporomandibular dysfunction is somehow disturbed. Moreover, the muscle's role during the execution of its normal function has also been questioned and remains a matter of controversy.

Conclusions. The lateral pterygoid muscle obviously plays an important role in the development of temporomandibular dysfunction through the prism of its anatomical and functional particularities, referring to the superior fascicle responsible for the correct anatomical maintenance of the articular disc during function.

Keywords: lateral pterygoid muscle, temporomandibular joint, temporomandibular dysfunction.

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Key messages

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

The literature study shows that there is still no clear scientific evidence that the function of the lateral pterygoid muscles in temporomandibular dysfunction is somehow disturbed. Additionally, their involvement in the development and evolution of temporomandibular dysfunction is uncertain.

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The research hypothesis

Examining the lateral pterygoid muscle requires a deep knowledge of its anatomy and function, as both palpation and improperly or superficially performed functional manipulation can provide us with erroneous data for the diagnosis and treatment of temporomandibular dysfunction.

The novelty added by manuscript to the already published scientific literature

A systematic study of the specialized literature was conducted regarding the role of the lateral pterygoid muscle in the development of temporomandibular joint dysfunctions, the evaluation of the anatomical aspects of the lateral pterygoid muscle and its interrelationship with the occurrence of temporomandibular joint dysfunction, as well as the elucidation of methods for examining the lateral pterygoid muscle and the importance of its role in the evolution of temporomandibular dysfunction.

Introduction

The clinical concept that argues that the activity of the lateral pterygoid muscle, being disturbed, plays an important role as an etiological factor in temporomandibular joint (TMJ) dysfunctions is still widely accepted. It also remains a decisive factor in the correct choice of the treatment plan. However, due to the fact that few rigorous studies and clear evidence have been conducted and presented to fully support this concept, it continues to be a very controversial one [1, 2]. Patients with temporomandibular disorders (TMD) complain of pain in the TMJ and/or masticatory muscles, limitations and sounds during mandibular movements. Temporomandibular disorders are a non-specific collective term used to describe a heterogeneous group of pathological conditions located in the territory of the stomatognathic/masticatory system. These are considered musculoskeletal disorders, which cause pain during the performance of the function (mastication, speech, swallowing), with increased sensitivity at the level of masticatory muscles and/or the TMJ, along with possible limitations of the range of motion and the occurrence of joint noises and otological symptoms [1, 3-6]. Current data demonstrate that TMD is one of the most commonly diagnosed forms of musculoskeletal pain in all age groups. Ethnicity, age, geographic location, and the time of assessment influence the level of prevalence, causes, factors, and spectrum of clinical manifestation of the pathology [7, 8]. In adolescents, the prevalence is a controversial subject, considering that adults are more frequently affected by TMD, but at the same time, a significantly increased incidence is noted in subjects with mixed dentition (worldwide incidence – 25%, and in the developed countries – between 2% and 6%) [1, 3-5, 9]. One theory claims that in TMD, the lateral pterygoid muscle becomes hyperactive, hypoactive, or that there is a miscoordination between the superior and inferior branches of the muscle, or that there is a disturbance during the execution of the role of the muscle to control and stabilize the TMJ [1, 10]. However, a rigorous review of the literature indicates that no clear scientific evidence is yet available to suggest that the function of the lateral pterygoid muscles is somehow disrupted in TMD. Moreover, the muscle's role during the execution of its normal function has also been questioned and remains a matter of controversy.

Material and methods

For this literature review, scientific articles published between 2000 and 2023 were considered and studied using the following electronic databases: PubMed, MEDLINE, Google Scholar, BIR Publications, ScienceDirect. Keywords such as „lateral pterygoid muscle", "temporomandibular joint", and "TMJ dysfunction" were used. A total of 137 articles were studied, out of which 12 were duplicate articles, 45 articles presented studies performed on cadavers, 21 articles did not provide sufficient data, and 19 articles were conducted before the year 2000. The exclusion criteria included studies performed on cadavers, studies conducted before the year 2000, and studies that did not present trustworthy information on the topic under study.

Results and discussion

Anatomical variations of the lateral pterygoid muscle. In recent years, several studies have been carried out with the purpose of proving a theory that there may be a third fascicle of the lateral pterygoid muscle or that the two fascicles may be anatomically inserted differently at the level of the disc and condyle among different individuals. To study the anatomical variations of the lateral pterygoid muscle and its insertion variations, analyzes were performed using magnetic resonance imaging (MRI). In a 2013 study by Valenzuela et al., 698 patients underwent MRI analysis of the TMJ. Three types of muscle insertion of the superior fascicle of the muscle have been described (Table 1). The first type of muscle insertion involved muscle fibers inserting onto the articular disc. The second type of muscle insertion was at the level of the articular disc and condyle, and the third

type was at the level between the articular disc and the articular capsule (Figure 1) [11, 12]. In another similar study conducted in 2016 by Eberhard et al., in which 382 patients with articular disc dislocation were evaluated by MRI, the results showed that the prevailing type of muscle insertion of the superior fascicle among subjects was type 2, at 67%, and the one with the lowest prevalence was type 1, at 7.6%. MRI images were taken in the position of maximum intercuspation (MI) and full opening of the oral cavity [13].

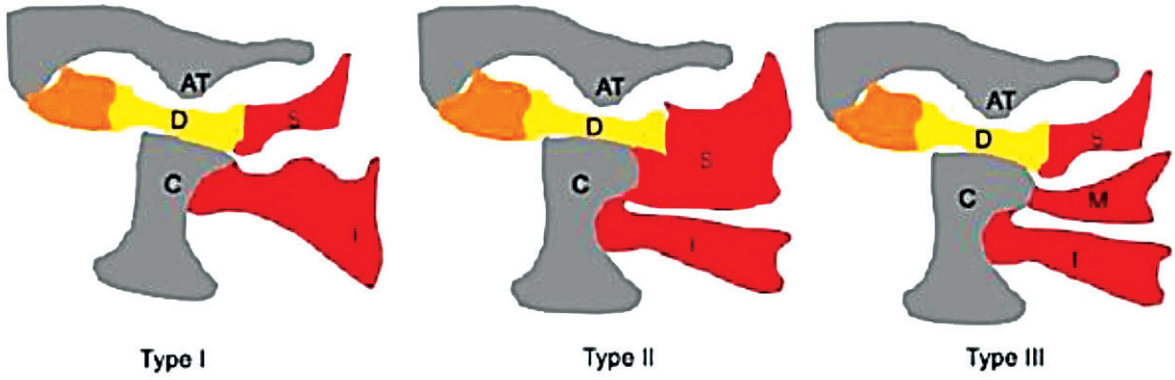

Fig. 1 Schematic representation of the three types of muscle insertion and the third fascicle of the LPM.
Note: (AT) - articular tubercle, (C) - condyle, (D) - disc, (S) - superior bundle of LPM, (I) - inferior bundle of LPM, (M) - medial bundle of LPM [12]

Table 1. Types of muscle insertion of the lateral pterygoid muscle. Litko classification [13]

Type of insertion	*Bundle*	*Insertion*
Type 1	Superior Inferior	Disc Condyle
Type 2*	Superior Inferior	Disc and condyle Condyle
Type 3	Superior Medial Inferior	Disc Condyle Condyle

Note: *(*)* – the highest prevalence with 76% of subjects according to Eberhard et al. studies.

Function of the lateral pterygoid muscles. The international theory about the anatomy of the lateral pterygoid muscle says that it is a masticatory muscle, which is part of the dento-maxillary system. It is considered to be an important masticatory component, in some sources even the main one, either structurally or functionally, due to its direct insertion at the level of the components of the temporomandibular joint. In some temporomandibular pathologies or dysfunctions, the muscle would be described as being directly or indirectly involved. Directly, it is described as the muscle that specifically causes TMD. The dysfunction involves the alteration of the movement of the joint and causes pain in neighboring structures, which are nervous, vascular or bone [11, 14, 15]. Pathologically, it is described that at the level of the joint there would be an impediment or an inconsistency between the elements, but the most affected component would be the articular disc, which is directly associated with the lateral pterygoid muscle that causes the anterior dislocation of the disc [11]. Mechanically, this joint dysfunction produces an anterior disc dislocation, which, in turn, compresses with the bony tissues, causing a bony stop for the articular condyle, reducing joint space and range of motion at the joint, producing combined clinical and mechanical symptoms [11, 12].

The functions of the lateral pterygoid muscle were defined as follows: the superior bundle is responsible for closing, retropulsion, and ipsilateral movements of the mandible, while the inferior bundle is active in opening, propulsion, and contralateral movements of the mandible. However, recent studies suggest that some fibers of the superior bundle may also be involved in opening, propulsion, and contralateral mandibular movements, and that it may consist of three mediolaterally arranged functional areas. This indicates that the concept that the occurrence of clicking, crackles in the TMJ is due to uncoordinated movements between both bundles of the lateral pterygoid muscle needs to be re-evaluated [10, 16]. Both fascicles of the lateral pterygoid muscle, in some recent reports, have been shown to be inactive during electromyography in the mandibular posture position. An important role of the lateral pterygoid muscle demonstrated electromyographically is that it generates laterality and propulsive movements of the mandible. This was observed when the activity of the inferior fasciculus changed when the direction of the horizontal force of the mandible oscillated from side to side [1, 10]. Murray et al. claim that the cause that would lead to different conclusions and results regarding the function of the lateral pterygoid muscle would be the incorrect placement of the electromyographic electrodes at the level of the muscle [17]. The results obtained could belong to other adjacent muscles such as the temporalis muscle or medial pterygoid muscle, or they could belong to the lateral pterygoid muscle (LPM) but be incorrectly assigned to a specific fascicle. With the help of computed tomography, they analyzed the correct placement of the electrodes at the level of the lateral pterygoid muscle and confirmed the classical notion

that the inferior LPM would be involved in opening, protrusion, and contralateral mandibular movements. Studying the functional unit of the muscle, they concluded that the supero-medial part of the inferior LPM has an important role in initiating contralateral movements of the mandible, while the infero-medial part has a role in controlling fine movements [17]. The activity of the superior lateral pterygoid muscle was also confirmed by the correct placement of the electrodes, and it turned out to be much more complex than originally thought [17]. In contrast to the idea that the superior LPM is active only in closure, retrusion, and ipsilateral movements of the mandible, most muscle fibers of the superior LPM are at least active in opening, protrusion, and contralateral movements. Moreover, the pattern of activity varies depending on the location of the muscle fibers in the muscle and has been classified into three functional areas (Figure 2) [17]. The medial area produces patterns of activity similar to those produced by the inferior LPM. The lateral area may be equally active in closure, ipsilateral movements, and retrusion, while the central area generates different patterns of activity. These obtained functional data play an important role in understanding the role of the lateral pterygoid muscle in TMD [17].

The relationship between the lateral pterygoid muscle and the elements of the temporomandibular joint. J. Okeson described the functional importance of the superior lateral pterygoid muscle, namely: it becomes evident when the unilateral masticatory function is exercised. When an individual unilaterally bites a hard substance, both temporomandibular joints are functionally loaded differently. This occurs because the force is not applied to the joint but to the hard substance. Intra-articular pressure increases contralaterally to the biting area, and decreases at the ipsilateral articular level. This fact can lead to a separation between the articular surfaces, resulting in a dislocation of the articular disc on the same side. To prevent this, the superior lateral pterygoid muscle becomes active during biting, rotating the disc forward on the condylar surface so that the thicker edge of the disc remains in permanent contact with the articular surface [5].

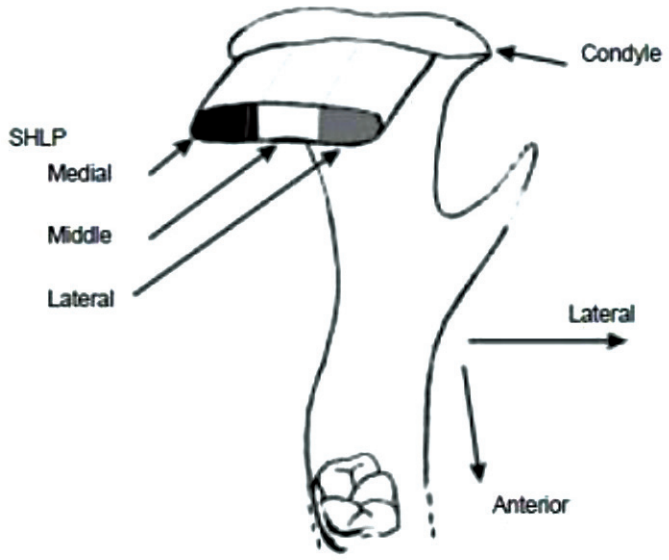

Fig. 2 Functional areas of the upper Lateral Pterygoid Muscle.
Note: (Medial) - medial area, on left; (Middle) - middle area, on center; (Lateral) - lateral area, on right; Directions - Anterior, Lateral [17]

The articular disc is composed of several layers of collagen fibers, each oriented in different directions to resist the shearing phenomenon that could occur during the sliding of the condyle. The disc is attached to the medial and lateral surfaces of the condyle, which allows it to move in unison with the articular condyle. The positioning of the disc is controlled by a combination of elastic fibers attached to the posterior surface of the disc, thereby holding the disc in tension against the action of the superior fasciculus of the lateral pterygoid muscle, which is attached to the anterior surface of the disc. When the disc ligaments pull the disc along with the movement of the condyle, the rotation of the disc on the condyle occurs due to the force of contraction or relaxation of the superior fascicle of the muscle (Figure 3) [1, 4, 18].

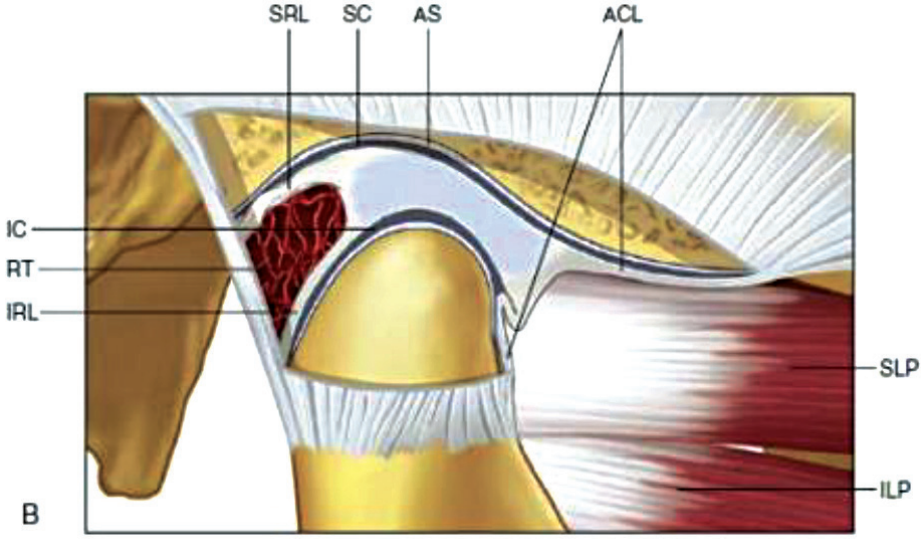

Fig. 3 Diagram of the anatomical components of the TMJ.
Note: (ACL) - anterior capsular ligament; (AS) - articular surface; (IRL) - inferior retrodiscal lamina; (RT) - retrodiscal tissues; (SC and IC) - superior and inferior articular cavity; (SLP and ILP) - superior and inferior lateral pterygoid muscle; (SRL) - superior retrodiscal lamina [5]

All the tissues attached to the disc have the role of preventing its anterior dislocation. However, the question arises as to how its anterior dislocation occurs, which is very common in patients with temporomandibular dysfunction. The only force that is directed anteriorly and can produce articular disc dislocation is the muscle that is attached to the anterior surface of the disc, namely the superior fascicle of the lateral pterygoid muscle. This muscle, together with the elastic collagen fibers attached posteriorly to the disc, controls the position of the disc on the surface of the condyle and is always aligned with the direction of the force when the condyle descends the slope of the tubercle downward [4]. If the articular condyle is located in centric relation, the articular disc is located in the most anterior position that the posterior ligaments allow. In this position, the condylar forces are directed upward toward the medial third of the disc and forward toward the anterior surface of the condyle. When the inferior fascicle of the lateral pterygoid muscle (+) begins to propel the condyle anteriorly, the superior fascicle of the muscle (-) relaxes its contraction to allow the elastic fibers to pull the disc over the condyle (Figure 4) [4].

At the maximum opening of the oral cavity, when the condyle reaches the most inclined portion of the articular tubercle, the disc should be directly located above the articular condyle, as the forces are directed superiorly. At this point, the elastic fibers rotate the disc posteriorly because the superior fasciculus of the lateral pterygoid muscle is in a controlled relaxation (Figure 5) [4].

Upon closing the oral cavity, the condyle moves posteriorly and superiorly on the slope of the articular tubercle, so that the disc is again positioned in front of the condyle. For this, the upper fascicle (+) begins to contract, while the lower fascicle (-) relaxes and allows the condyle to return to its original position (Figure 6) [4].

When the condyle returns to the centric relation position, the disc is located as anteriorly as possible, as far as the posterior ligament allows. If the ligament is intact and has not been stretched or torn, the disc is located in perfect

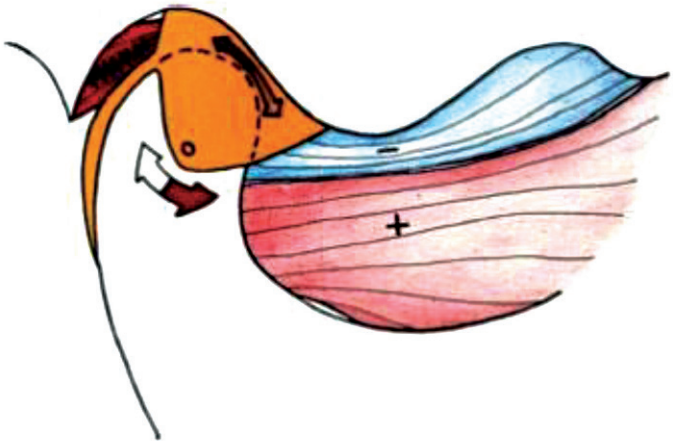

Fig. 4 Schematic structure of lateral pterygoid muscle in centric relation.

Note: (-) – the upper bundle of the lateral pterygoid muscle; (+) – the inferior bundle of the lateral pterygoid muscle [4].

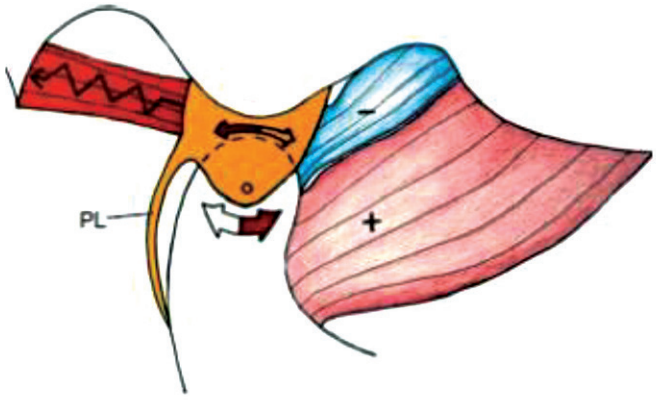

Fig. 5 Schematic structure of lateral pterygoid muscle at the maximum opening.

Note: (-) - the upper bundle of the lateral pterygoid muscle; (+) - the lower bundle of the lateral pterygoid muscle; (PL) - posterior elastic fibers [4]

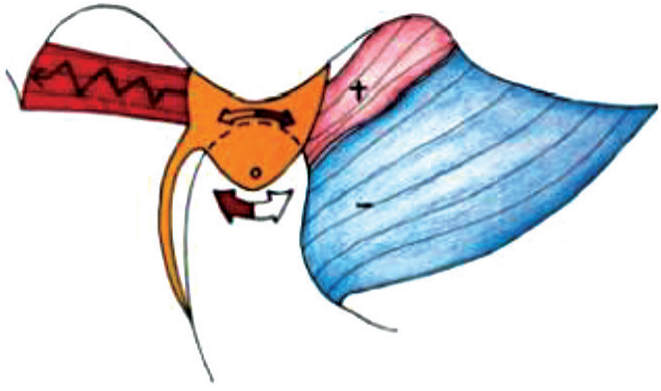

Fig. 6 Schematic structure of the lateral pterygoid muscle upon closing.

Note: (-) – the lower bundle of the lateral pterygoid muscle; (+) – the superior bundle of the lateral pterygoid muscle [4].

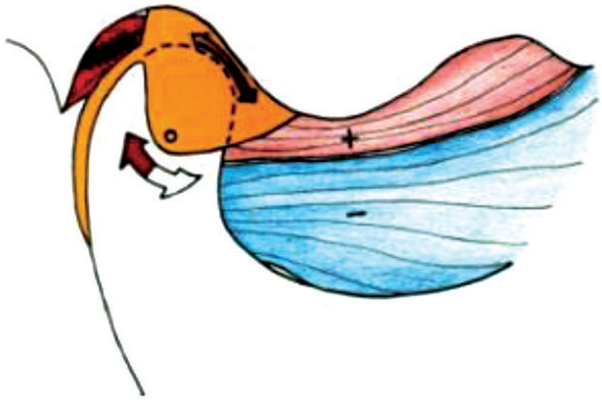

Fig. 7 Schematic structure of the lateral pterygoid muscle when the condyle returns to the centric relation position.

Note: (+) – the upper bundle of the lateral pterygoid muscle; (-) – the inferior bundle of the lateral pterygoid muscle [4].

alignment with the direction and position of the condyle. In the absence of occlusal interference, the inferior fascicle of the lateral pterygoid muscle is not active, even in the position of maximum intercuspation (MI). The superior fascicle remains contracted to keep the disc in the proper position (Figure 7) [4].

Correlation between anatomical variations of the lateral pterygoid muscle and temporomandibular dysfunctions. The correlation between the types of muscle insertions of the LPM and its anatomical variations is directly proven in studies of temporomandibular joint dysfunctions. Only two research studies from 2009 and 2012 (Dergin et al.; Matsunaga et al.) did not show any link between anatomical variations of the lateral pterygoid muscle and temporomandibular joint dysfunctions. This is because they represent studies carried out on corpses where only the morphological structure was studied and only the variations were observed [19, 20, 21]. The anatomical variation of muscle insertion type 2 according to the Litko classification [13] was the one that showed the closest connection with TMD, especially due to the insertion of the superior fasciculus of the LPM at the level of the articular disc and the articular condyle (Table 1). This variation can produce joint alterations or dysfunctions, characterized by local or loco-regional pain during closing or opening movements. However, studies have not shown any relationship between the type of insertion of the inferior fascicle of the LPM and TMD, both primary and secondary. The middle or accessory fascicle, which was described in 19% of cases in the analyzed studies, showed no connection between its presence and any TMD, primary or secondary, acute or chronic [21, 22-26]. Murray et al. state that observations made in recent years support the theory that the lack of coordination of movements between the upper and lower LPM could generate clicks, cracks, or articular blockages during the horizontal positioning of the disc in relation to the condyle. However, recent evidence indicates the possibility that fibers of the superior lateral pterygoid muscle that insert at the level of the articular disc could activate independently of other fibers of the same muscle, the superior LPM, and generate forces at the level of the disc that are not simultaneously generated with those inserted at the level of the condyle [17]. J. Okeson states that the exact percentage of insertion of the superior lateral pterygoid muscle at the level of the disc and at the level of the articular condyle is still under debate and is variable. However, it would be reasonable to assume that if the insertion of the muscle is predominant at the level of the neck of the articular condyle, and less at the level of the disc, the function of the superior muscle will have correspondingly less influence on the position of the articular disc. Moreover, vice versa, if the insertion of the superior muscle is predominant at the level of the articular disc, and less at the level of the condyle, the function of the muscle will greatly influence the position of the articular disc. This anatomical variation explains why, in some patients, the disc dislocates very quickly, without any antecedents or previous clinical features [5].

Conclusions

1. The lateral pterygoid muscle plays an obviously important role in the development of temporomandibular dysfunctions through the prism of its anatomical and functional particularities, specifically referring to the superior fascicle responsible for maintaining the correct anatomical position of the articular disc during function.

2. The anatomical variations of the muscle insertion according to various classifications are directly related to the development of TMJ dysfunctions, especially due to the insertion of the superior fascicle of the LPM at the level of the articular disc and the articular condyle. This variation can produce alterations at the level of the joint complex, characterized by the respective dysfunctional symptomatology. However, studies have not shown a clear link between the type of insertion of the inferior fascicle of the LPM and both primary and secondary TMJ dysfunction.

Competing interests

None declared.

Authors' contribution

All the authors have contributed equally at the results presentation in the paper, approved the „ready for print" version of the manuscript.

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CASE STUDY

OPEN ACCESS

Laser ureteroscopic endopyelotomy efficacy in pyeloureteral junction stenosis

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ABSTRACT

Introduction. Pyeloureteral junction stenosis (PUJS) is a condition that affects urinary drainage at level of the renal pelvis and upper ureter. It is found in approximately 1 in 500 newborns, with a higher prevalence in males (2:1 ratio). PUJS is the main cause of congenital hydronephrosis and can also be caused by other specific pathologies. Endoscopic management is the primary treatment for PUJS, particularly in cases of aperistaltic and <2cm intrinsic ureteral stenosis without aberrant vessels.

Aim of the study. Efficacy assessment of endoscopic retrograde incision of PUJS for urinary drainage recovery and duration of postoperatory effect.

Material and methods. 5 patients were operated, from November 2022 to February 2023. Each patient has been operated by using retrograde LASER endopyelotomy method. There were excluded patients with extrinsic ureteral obstruction, defected segment more than 2 cm, massive hydronephrosis, split renal function <20%, tumor in the obstruction area, high ureteral insertion, patients <18 years of age. Mean follow-up time of patients is 8 weeks.

Results. One month after intervention patients were recalled for investigations. There were observed way more better results in the patients with grade 1 hydronephrosis than those with grade 2 (p = 0.002). All patients at 3-month postoperative follow-up reported resolution of symptoms.

Conclusions. Efficacy of LASER endopyelotomy is 99.9% in first months of the follow-up, after double J stent extraction. More follow-up time and patients are required to present more statistically significant results.

Keywords: pyeloureteral junction, endopyelotomy, hydronephrosis, renal function.

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Key messages

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

There is no specific data about the PUJ patency years after the intervention. Our research is a prospective study of patients, and relapses that may occur after laser endopyelotomy.

The research hypothesis

In a defined patient subgroup with PUJ defects <2 cm, hydronephrosis grade 1 or 2, and renal function >20%, our study suggests that laser endopyelotomy significantly reduces relapse rates, highlighting its potential efficacy for sustaining PUJ patency.

The novelty added by manuscript to the already published scientific literature

This study uniquely investigates the prolonged efficacy of laser endopyelotomy in a specific subgroup with PUJ defects <2 cm, hydronephrosis grade 1 or 2, and renal function >20%. The manuscript fills a research gap in this context, enhancing our understanding of laser endopyelotomy's potential in maintaining PUJ patency.

Introduction

Stenosis of the pyeloureteral junction (PUJ) is a condition that disrupts the urinary passage at the level of the renal pelvis and the upper third of the ureter. It occurs in 1 in 500 newborns, with a higher prevalence in males than females, with a ratio of 2:1 [1, 2]. Stenosis of the pyeloureteral junction is considered the most common cause of congenital hydronephrosis, and it is determined by anomalies in the anatomical structure of the renal-urinary tract, such as ureteral hypoplasia, high insertion of the ureter, aberrant vessels, and adhesions that compress and irritate the ureter, leading to urodynamic disorders and subsequent morphological changes. However, PUJ stenosis can also occur later in life as a secondary condition, with etiologies including: (a) extrinsic factors such as compression of the PUJ due to retroperitoneal fibrosis, retroperitoneal lymphadenopathy, retroperitoneal tumors; (b) intrinsic factors such as fibrosis of the PUJ caused by stones, chronic inflammation, exposure to radiation, radiotherapy, transitional cell and urothelial tumors of the ureter, and iatrogenic causes [3-5].

There is a variety of minimally invasive interventions available for the treatment of stenosis and strictures of the pyeloureteral junction (PUJ): balloon dilation, laparoscopic pyeloplasty, antegrade (percutaneous) endopyelotomy, and retrograde endopyelotomy. Although laparoscopic pyeloplasty is considered the gold standard for all causes of PUJ stenosis, endoscopic management can be considered as a first-line treatment for aperistaltic ureteral segment and intrinsic ureteral stenosis (<2 cm) in the absence of aberrant renal vessels [6]. Methods with maximal precision and direct ureteroscopic visualization of the lesion are preferred over blind incisions with Acucise, as they carry a higher risk of intraoperative bleeding [7, 8].

Ureteroscopic endopyelotomy with LASER was first described by Inglis and Tolley in 1986 [9]. Retrograde incision of PUJ offers several advantages: shorter procedure and recovery duration, reduced hospitalization time, minimal use of postoperative analgesics, and avoidance of external urinary drainage. Although it does not show more significant results compared to open surgeries, studies on the success rate of LASER endopyelotomy in patients with primary or secondary PUJ continue to this day. Literature results show a success rate of retrograde LASER endopyelotomy ranging from 39% to 100% in primary or secondary PUJ [10, 11]. Other studies present ureteroscopic LASER endopyelotomy as a treatment method for PUJ stenosis with a success rate starting at 89% [12, 13]. The primary objective of any intervention, whether open or minimally invasive, is to preserve renal function.

The purpose of presenting these cases is to evaluate the effectiveness and morbidity of retrograde endoscopic incision for restoring urinary passage in PUJ, as well as the duration of maintaining the postoperative result.

Case presentation

Inclusion and exclusion criteria, patients' description, case history, investigation results.

Patient selection was based on specific indications for retrograde endopyelotomy, aiming to achieve a higher success rate in cases without significant hydronephrosis, with a ureteral segment affected by stenosis less than 2 cm in length, and lacking aberrant vasculature. The study data was collected by recording the clinical and paraclinical data of the enrolled subjects. Patients with extrinsic ureteral obstruction, stenotic segments exceeding 2 cm, severe hydronephrosis, renal function less than 20% of the affected kidney by PUJ stenosis, tumor involvement at the site of obstruction, high ureteral insertion, pediatric patients, and individuals whose treatment modality did not involve the employed method were excluded from the study.

From November 2022 to February 2023, a cohort of 5 female patients of 35 to 45 years of age WITH URETEROPELVIC JUNCTION STENOSIS WERE ADMITTED FOR RETROGRADE LASER PYELOTOMY. All participants presented with ureteral stenosis and secondary PUJ obstruction resulting from chronic inflammation due to renal calculi that periodically lodges in PUJ. The primary symptoms reported by patients included lumbago, recurrent urinary tract infections, and, in one case, hematuria. Among the five patients, three exhibited grade 2 hydronephrosis, while the remaining two showed grade 1 hydronephrosis, as confirmed by ultrasonography. The renal function of the affected kidney was evaluated using scintigraphy, revealing a function greater than 30% in all candidates. The diagnosis was established based on computed tomography and intravenous pyelography, with the identification of contrast medium restriction at the junction without visualization of the ureter or encountering delayed passage (image of the distal portion of the ureter appear after 30 minutes). The length of the defect and the degree of hydronephrosis were determined using the aforementioned imaging methods. Renal function was assessed through dynamic scintigraphy. CT angiography was performed to exclude the presence of aberrant vessels.

The patients were prepared for the intervention by treating urinary tract infection, if present, performing ureteral stenting to facilitate drainage of infected urine, and resolving the renal calculi.

Treatment plan, surgical technique, and follow-ups

In all cases, a semirigid ureteroscope was employed for the procedure. In instances where the ureter presented excessive narrowness, impeding access to the upper third, a 5-Fr JJ stent was inserted for a duration of 2 weeks to facilitate ureteral dilation and establish an adequate working space. Subsequently, the patients were scheduled for stent extraction and the continuation of the previously determined treatment protocol. All patients underwent spinal anesthesia. Initially, intraoperatively, retrograde ureteropyelography was performed by injecting contrast medium into the ureter via the ureteroscope, allowing for repeated assessment of the anatomy of the PUJ stenosis. A semirigid ureteroscope, specifically the OES Pro 6.4/7.8 Fr x 430 mm model with a 4.2 Fr working channel, was utilized. The safety guide was used to reach the level of the pelviureteric junction stenosis as we can see in Fig. 1. A 365-μm laser fiber was employed during the procedure. Using real-time imaging guidance, the incision was made in a postero-lateral, caudo-cranial direction until visualization of the normal

urothelium was achieved (Fig. 2), utilizing Ho:YAG LASER with an energy range of 0.5-3.5J and a frequency of 5-20. A control retrograde ureteropyelography was performed to determine the estimated length and level of the stenosis (Fig. 3). Post-incisional retrograde ureteropyelography was performed to assess the outcome (Fig. 4). Following the procedure, patients received the placement of 7-8 Fr JJ stents, which remained in place for a period of 4 weeks. After the completion of the procedure, patients were catheterized with a 16 Fr Foley catheter for 24 hours, following which they were discharged for outpatient follow-up and continuation of medical treatment at home. After a period of 3-4 weeks, patients were called back for reevaluation and analysis of postoperative results. During the first month, ultrasound, urine culture, and intravenous urography (IVU) were performed to assess urinary passage. If the positive result was maintained, the patient would be scheduled for a follow-up examination after 6 months. Successful treatment was defined by the resolution of symptoms, restoration of urinary passage, shortened visualization time of contrast in the ureter as observed through imaging studies, absence of obstructive patterns in dynamic scintigraphy, and preserved renal function. The half-life (T ½) of the radiopharmaceutical preparation (RFP) was less than 20 minutes. If these criteria were not met, the treatment was considered unsuccessful. Postoperative complications were classified according to the Clavien-Dindo classification system [14]. All patients have been informed about the surgical technique, purpose, and complications of the procedure. Consent for the surgical intervention and the use of personal data for the study has been obtained from each patient.

Results

Subjects involved in the present study were all detected with grade 2 (3 patients) and grade 1 (2 patients). When assessing pre-procedural (PUJ stenosis (Fig. 3)) and post-procedural results in retrograde ureteropyelography, we observe the restoration of urinary passage in all cases (Fig. 4). The average duration of the surgical intervention was 42.8 ± 2,58 minutes, and the mean duration of hospitalization was 4 ± 1.73 days. The patients were followed up for an average period of 8 weeks. One patient experienced a postoperative complication in the form of a urinary tract infection on the second day, which prolonged the hospital stay by an additional 4 days. During the one-month follow-up, a statistically significant difference was observed between patients with grade 1 hydronephrosis (complete restoration of urinary flow) and those with grade 2 hydronephrosis (faster contrast visualization in UIV) ($p < 0.005$). Furthermore, all patients reported the resolution of symptoms within three months of postoperative clinical monitoring.

Discussion

Open pyeloplasty represents the gold standard in the treatment of ureteropelvic junction (UPJ) stenosis, with a success rate ranging from 80% to 97% [15, 16]. However, due to its drawbacks, such as the need for a large surgical approach, lengthy recovery period, and high cost, alternative minimally invasive methods have been explored. Despite the lower postoperative success rate, these alternative approaches offer advantages in terms of cost, intraoperative complications, recovery time, and length of hospital stay.

Thorough preoperative evaluation and investigation of the patient allow for the selection of suitable candidates for minimally invasive interventions, which offer a high postoperative success rate. The European Association of Urology guidelines recommend laser endopyelotomy as the first-line treatment with a grade C recommendation. It is primarily applied in cases of intrinsic stenosis with a defect length of less than 2 cm, absence of a dilated pelvis, high ureteral insertion, renal split function less than 20%, and ipsilateral renal calculi. The level of evidence for this recommendation is 4 [17]. Some studies have reported a higher efficacy of laser endopyelotomy in cases of primary UPJ obstruction. Other authors did not observe a difference, while some reported a higher success rate in patients with secondary etiology of UPJ obstruction [11, 18]. Gupta and colleagues have reported on the success rate of retrograde endopyelotomy based on the degree of hydronephrosis and the length of the stenotic segment. In both cases, as the degree of hydronephrosis and the length of the stenotic segment increase, the success rate decreases. However, the difference in results between the two cases is not statistically significant [19]. Similarly, Rassweiler and colleagues have also addressed the correlation between multiple factors that influence the success rate, with one of the factors being hydronephrosis. They found that as the severity of hydronephrosis increases, the postoperative outcomes become less favorable [20], which is consistent with the findings of the current study. The complication rate is estimated to be between 5% and 35%, with the most common complication being urinary tract infection (UTI) [21]. In our case, the complication rate was 20%, with a UTI being identified in a patient on the second day postoperatively during a urine analysis. The duration of stent placement for ureteral integrity restoration post-endopyelotomy varies among different authors. Anil Mandhani and colleagues conducted an animal study that showed positive results with complete recovery after 2 weeks [22]. Geavlete reported a necessary period of 6 weeks, as the urothelium regenerates in 5 days and the muscular tissue in 6 weeks [23]. In the current study, the stent was left in place for 4 weeks. The success rate in our study, at an 8-week patient follow-up, is currently 99.9%. However, patients require ongoing monitoring to assess the maintenance of JPU patency. Nevertheless, although the effectiveness of the intervention is continuously studied, JPU patency can decrease over time, as mentioned by Shalhav Al and colleagues in 1989 [24].

Conclusions

Compared to other minimally invasive methods such as antegrade endopyelotomy, retrograde incision with balloon (Acucise), laparoscopic, and robot-assisted procedures, the retrograde technique has several advantages, with cost-effectiveness being the most significant one: comparatively affordable equipment, shorter recovery period, and hospital stay. To achieve a clear success rate, certain factors need to be considered: defect length <2 cm, insignificant hydronephrosis, absence of a large pelvis, and high ureteral insertion, with preserved ipsilateral

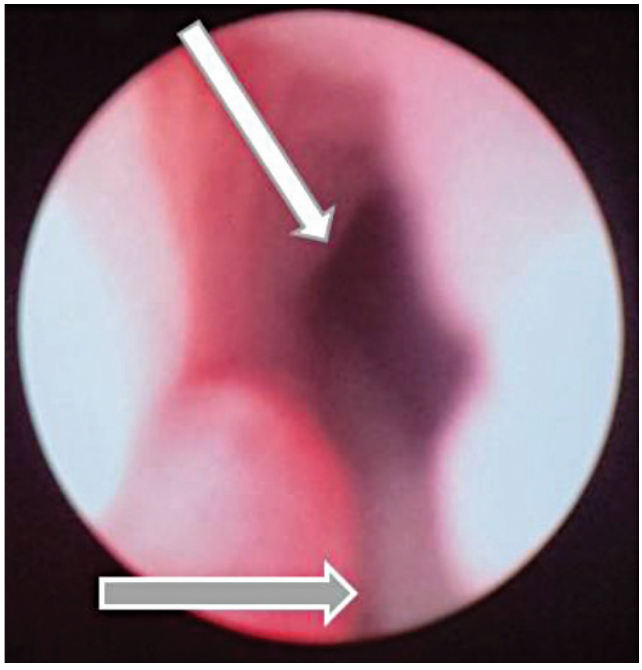

Fig. 1. Affected PUJ guided with safety wire.

Grey arrow shows safety wire; White arrow shows PUJ stenosis. Images are from our own collection

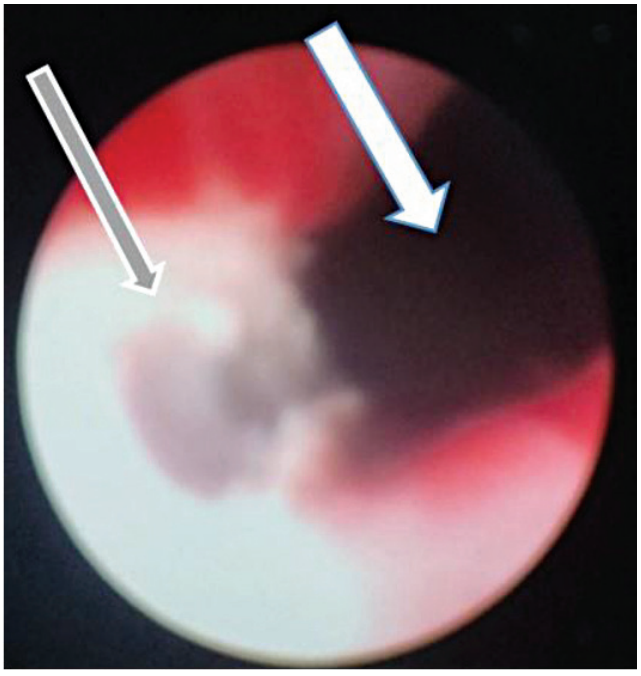

Fig. 2. PUJ after incision with Holmium:yttrium-aluminum-garnet LASER (Ho:YAG)

Grey arrow shows incision made with Laser; White arrow shows widened PUJ. Images are from our own collection.

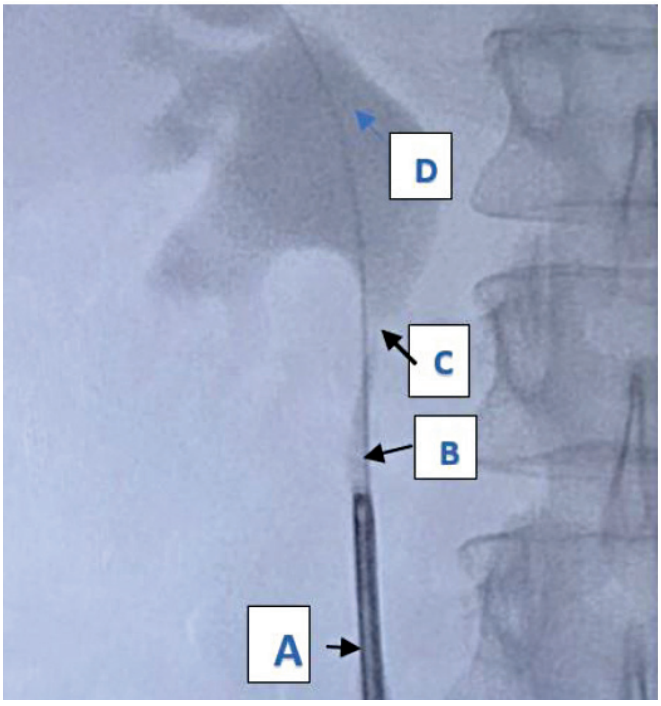

Fig. 3 Radioscopic visualization of PUJ stenosis in retrograde ureteropyelography.

(a) Ureteroscope, (b) Safety wire, (c) PUJ stenosis, (d) Dilated renal pelvis and calyces. Images are from our own collection.

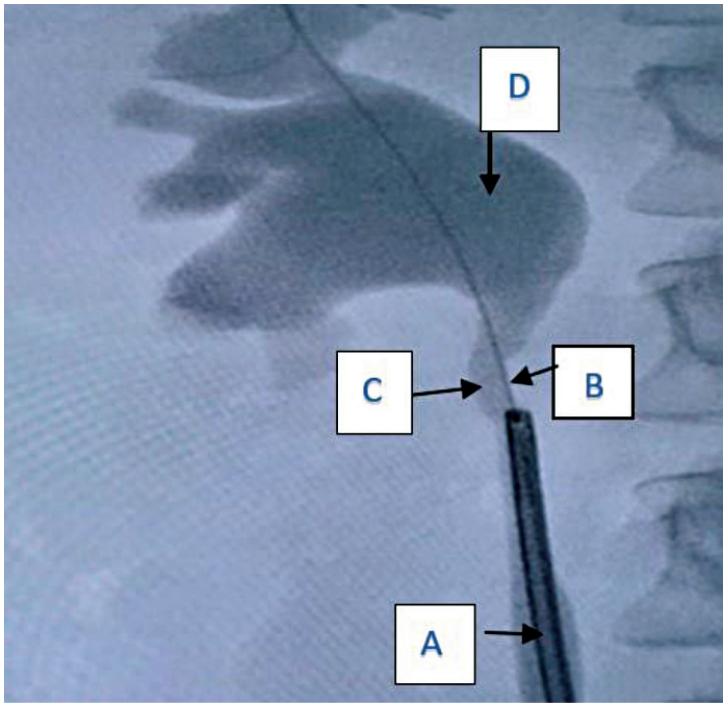

Fig. 4 Postincisional retrograde ureteropyelography with visualization of restoration of the urinary passage.

(a) Ureteroscope, (b) Safety wire, (c) PUJ after incision, (d) Dilated renal pelvis and calyces.Images are from our own collection.

renal function. The current study results demonstrate an efficacy rate of 99.9% in the early months of monitoring. However, longer monitoring with a larger patient sample is necessary to provide more conclusive data in the future.

Competing interests

The authors report no conflicts of interest in this work.

Patient consent

Obtained.

Funding Statement

The authors report no financial support.

Authors' contributions

VC conceived and designed the study, performed surgical interventions, collected and analyzed data, and significantly contributed to manuscript writing and revision. EP assisted in study design, participated as an assistant in surgical interventions, contributed to data analysis, and played a substantial role in manuscript drafting and critical review. AM assisted in study design, participated as an assistant in surgical interventions, provided clinical insights, and contributed to manuscript revision. CM assisted with data collection, participated as an assistant in surgical interventions, and participated in manuscript revision. Final manuscript was read and approved by all authors.

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CASE STUDY

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Treatment of deep carious lesions with mineral trioxide aggregate: clinical case report

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ABSTRACT

Introduction. Deep carious lesions are a dental disease widely spread among population of all ages. From clinical point of view, they have little symptoms and go unnoticed by the patients a long time, until they provoke dental pulp inflammations. If diagnosed and treated properly, the tooth can be treated conservatively with certain techniques of pulp vitality preservation. An important role in this process plays the innate capacity of regeneration of the pulp-dentine complex and the enhanced stimulating properties of new biomaterials used in dentistry. The aim of this clinical case report is to describe the clinical manifestations and the diagnostic algorithm used in deep caries and to establish a clinical guideline of treatment of deep carious lesion with a calcium silicate hydraulic cement.

Material and methods. Description of clinical case of a permanent tooth with a deep carious lesion, treated by indirect pulp capping with mineral trioxide aggregate cement. Clinical and paraclinical methods of investigations were used, the patient was evaluated after 6 and 12 months after the received treatment.

Results. The sensitivity to cold stimuli presented by the patient before the treatment attenuated shortly after he received dental care. After 6 and 12 months, the tooth is asymptomatic, the clinical findings and paraclinical parameters show no evidence of pulp inflammation.

Conclusions. Mineral trioxide aggregate showed long term successful results when used as a biomaterial for vital pulp therapy.

Keywords: deep carious lesion, vital pulp therapy, direct pulp capping, mineral trioxide aggregate.

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Key messages

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

Vital pulp therapy in carious teeth is still a controversial topic regarding in conservative dentistry. It has shown very high rates of success in non-carious dental pulp exposures. However, it remains to be established what the long-term success rates are for cases of deep carious lesions.

The research hypothesis

The cariously exposed dental pulp requires an enhanced treatment protocol in order to ensure the preservation of pulp vitality.

The novelty added by manuscript to the already published scientific literature

A case of a deep carious lesion in a permanent tooth is presented, along with its clinical and paraclinical manifestations, as well as the enhanced treatment algorithm with mineral trioxide aggregate.

Introduction

Vital pulp therapy (VPT) is defined as an ultra-conservative treatment that aims to preserve and maintain pulp tissue in a healthy state that has been compromised but not destroyed by caries, trauma, or restorative procedures [1]. Based on the level of pulp preservation, VPT includes stepwise excavation, indirect pulp capping, direct pulp capping, miniature pulpotomy, partial pulpotomy (Cvek) and complete coronal pulpotomy [2]. In our case report, we used the technique of direct pulp capping, a method that involves placing a bioactive dental material directly on an exposed pulp with the aim of stimulating the reparative function of the pulp-dentine complex.

In deep carious lesions, the bacteria present in the infected dentine initiate a reversible inflammatory process in the pulp. However, if the aggression is removed and proper conditions are established, the vital pulp-dentine complex has a high potential for self-repair [3]. This is due to the complex mechanisms that are initiated once the removal of the infected dentine occurs, a process known as tertiary dentine formation. Tertiary dentine is divided into two different categories based on the events that precede its formation and the mechanism of formation. Reactionary dentine is a form of tertiary dentine secreted by odontoblast cells that have survived a mild stimulus. Reparative dentine, on the other hand, is tertiary dentine secreted by a new generation of odontoblast-like cells in response to a strong stimulus (traumatic or carious pulp exposure), after the death of the original odontoblasts responsible for the primary and physiological secondary dentine secretion [4].

According to scientific research, hydraulic calcium silicate cements have a substantial positive influence on pulp-dentine complex regeneration [5]. The mineral trioxide aggregate (MTA) used in our case report (ProRoot MTA, Denstply) is categorized as a type 1 hydraulic calcium silicate cement [6]. MTA has the following positive properties: radiopacity, low solubility, and long-term stability; high biocompatibility; forms an excellent seal to prevent the ingress of bacteria; and promotes the formation of tertiary dentine by stimulating the production of growth factors and mediators in the injured pulp. However, MTA also has some drawbacks, such as a difficult handling technique, prolonged setting time, high cost, and tooth discoloration over time [7].

To achieve long-term success in the treatment of deep caries, the clinician needs to correctly assess the pulpal status at the beginning of the treatment and employ an enhanced treatment protocol. The proper diagnostic of the pulpal health status is crucial for the treatment's outcome [8].

Clinically, there are two available methods of assessing pulp vitality, based on the presence or absence of sensitivity to cold or electrical stimuli, with their combination leading to better precision. Parameters indicating pulp health or reversible inflammation are mild to strong sensitivity or pain to cold stimuli that disappears once the stimulus is removed. Additionally, values of electrical tests are as follows: for the incisors and canines group - from 2 to 9 μA, for the premolars group - from 6 to 15 μA, and for the molars group - from 8 to 18 μA. In the case of deep carious lesions in the molar group, values of electrical tests can increase to as much as 25-27 μA [9].

For paraclinical assessment of the dental caries, X-ray diagnostic methods are most commonly used, especially periapical, bitewing, and orthopantomography [10]. The radiological signs for deep carious lesions are:

- the presence of extensive radiolucency in the tooth of interest that has no communication with the pulp chamber;
- a preexisting faulty filling with underlying radiolucency, indicating the presence of secondary caries;
- no pathological periapical findings.

Once the diagnosis of a deep carious lesion with a vital pulp is established, the treatment requires an enhanced protocol because severe microbial contamination is expected when the necrotic tissue is removed. The enhanced protocol includes: selective carious tissue removal guided by optical magnification, disinfection of the resulted cavity with 5.25% sodium hypochlorite, and application of a hydraulic calcium silicate cement to the bottom of the cavity [11].

Materials and methods

This article reports a clinical case study of a 30-year-old male who sought dental care with the chief complaint of mild sensitivity to cold and sweet stimuli in the upper right quadrant. The patient was evaluated from clinical and paraclinical perspectives. The following tests were conducted: dental probing, axial percussion, cold and electrical sensitivity tests to assess pulp vitality, and orthopantomography.

Results

Clinical examination revealed a deep carious cavity in tooth 17 with a displaced preexisting filling. Dental probing did not elicit any pain; the dentine had a soft consistency, and axial percussion was negative. For cold testing, a cotton pellet soaked in Endo-Frost spray was used, and the response indicated a vital pulp. Additionally, electrical pulp testing was performed with a DigiTest device, recording a value of 18 μA. On the orthopantomography, an extended radiolucency without communication with the pulp chamber was detected in tooth 17, along with a displaced radiopaque filling. No pathological periapical findings were observed. The diagnosis of a deep carious lesion, classified as class I cavity by Black, was established.

Following the principles of minimal invasive dentistry, a direct pulp capping procedure was performed on tooth 17 in a single visit, which involved the following steps:

1. Local anesthesia with Septanest 1:100.00;
2. Isolation of the working field using a rubber dam;
3. Selective carious cavity preparation, resulting in a small pulp exposure;
4. Cavity disinfection and hemostasis achieved with 5.25% sodium hypochlorite 5.25% for 10 sec.;
5. Application of a thin layer of mineral trioxide aggregate on the bottom of the cavity;

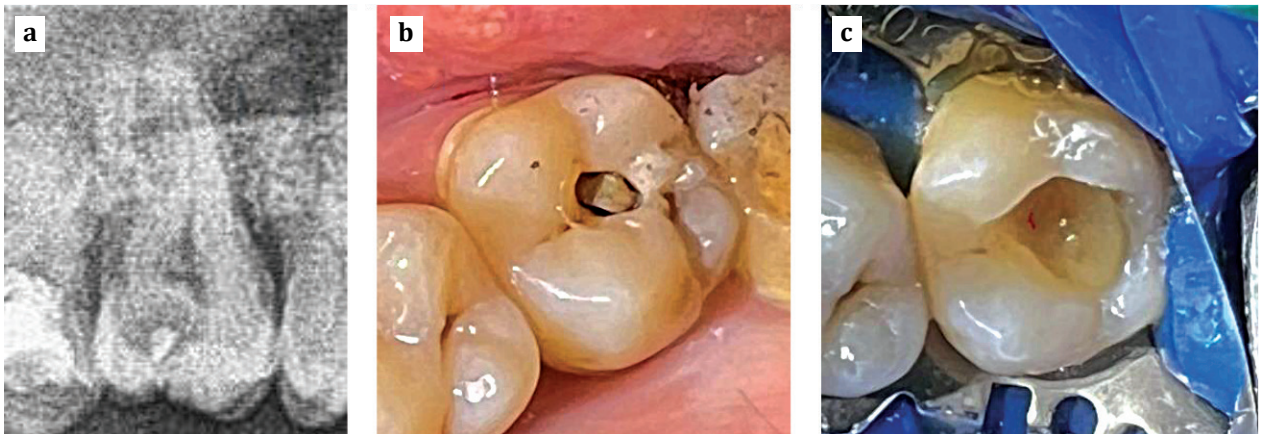

Fig. 1 Deep carious lesion in tooth 17, classified as a class I cavity by Black.
(a) - radiological image of the tooth; (b) - intraoral initial status; (c) - final preparation of the cavity.

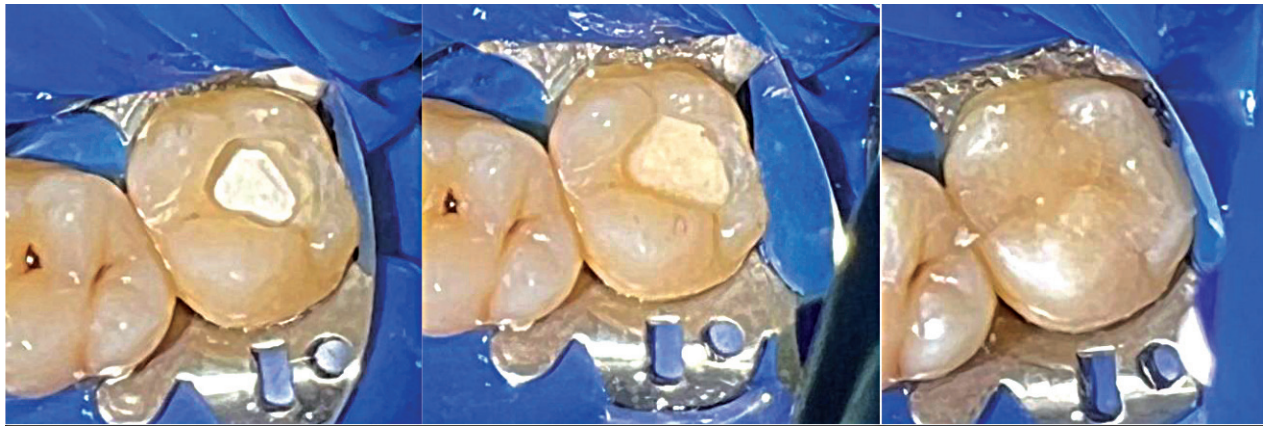

Fig. 2 Direct pulp capping of tooth 17 with MTA.
(a) - a thin layer of MTA covering the bottom of the cavity; (b) - the isolating layer made from glass ionomer cement; (c) - the final permanent restoration using light-curing composite.

6. Application of an isolating layer of glass ionomer cement;
7. Utilization of the total etch technique and V-th generation bonding;
8. Application of a permanent restoration using light-curing composite.

Discussions

This clinical case study describes the procedure of direct pulp capping, which involves the application of a thin layer of bioactive dental material over an exposed pulp. The exposure can occur due to traumatic injury or the removal of carious dentine. The procedure aims to maintain pulp vitality and stimulate the apposition of tertiary dentine. Studies have shown high success rates (92-97%) for MTA when used as a direct pulp capping agent [5]. This success is attributed to MTA's physical and bioactive properties, which result in the formation of a tertiary dentine bridge: high sealing capacity, low solubility, alkaline pH, slow release of calcium ions, and stimulation of TGF-α and BMP growth factors.

Direct pulp capping in cariously exposed dental pulps remains one of the most controversial areas in dentistry because the exposure is considered to occur through an infected layer of dentine, and in some cases, the pulp can show initial signs of inflammation. According to Bjørndal, these cases require an enhanced protocol that includes the removal of only the infected carious tissue, the use of optical magnification, disinfection and hemostasis with 5.25% sodium hypochlorite, as well as the use of a hydraulic calcium silicate cement as a pulp capping agent [11]. This is precisely the protocol we employed in our clinical case study.

The patient was recalled at 6 and 12 months after the applied treatment in order to perform vitality tests and radiographically assess potential changes. Electrical and cold tests confirmed the vitality of the pulp, and on the radiograph, a uniform and continuous layer of newly created tertiary dentine was observed, with no periapical pathologies depicted. Our findings correlate with the study results of Smith et al. [4]. Direct pulp capping can be done if the patient is of the proper age (under 35 years) and good general

health; in this case, the dental pulp still has a good ability to regenerate. After an enhanced protocol treatment, it can maintain its vitality and function, extending the shelf life of the tooth.

Conclusions

The direct pulp capping with MTA was considered successful as the chief complaint, which was sensitivity to cold and sweet stimuli, disappeared after the received treatment. Also, at the follow-up visits at 6 and 12 months, the cold tests indicated a vital pulp, the electrical pulp testing recorded values of 15 μA and 12 μA, respectively, indicating a positive dynamic, and no pathological periapical radiological modifications were depicted. In conclusion, we can say that MTA is an excellent pulp capping agent with a wide range of clinical uses, especially in VPT.

Competing interests

None declared.

Patient consent

Obtained.

Funding statement

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Authors' contributions

Both authors have equally contributed to the results presented in the paper and have approved the „ready for print" version of the manuscript.

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[Revised May, 2023]

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	Experimental Cohort (n=100)	Control Cohort (n=100)	p
Dysrhythmia	6.0%	30%	0.49
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

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