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The Moldovan Medical Journal is an international scientific double-blind peer reviewed periodical edition, 4 per year, of the Scientific Medical Association of the Republic of Moldova designed for specialists in the areas of medicine, dentistry, pharmacy, social medicine and public health. From its debut the journal has striven to support the interests of Moldovan medicine concerning the new concepts of its development.

The Editorial Board warmly welcomes both the readers of and the authors for the journal, all those who are enthusiastic in searching new and more effective ways of solving numerous medicine problems. We hope that those who want to make their contribution to the science of medicine will find our journal helpful and encouraging.

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ORIGINAL RESEARCHES

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Post-cardiac surgery bacterial contamination

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Author's ORCID iD, academic degrees and contribution are available at the end of the article

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Abstract

Background: Septic purulent nosocomial infections (SPNI) are one of the most significant healthcare challenges of post-surgical procedures. SPNI are associated with increased morbidity, mortality and admission costs. It is a priority to determine the level of nosocomial infections (NI). This study aims to evaluate the bacterial contaminations after cardiac surgery within the Department of Acquired Heart Defects (DAHD).
Material and methods: A cross-sectional study was designed and the medical records of 1189 patients who underwent cardiac surgery within the DAHD of a multiprofile hospital were retrospectively analyzed. The data were collected and stored in a Microsoft Excel spreadsheet.
Results: The incidence rate of SPNI following cardiac surgery was 317.57‰ compared to 15.02‰ officially reported (p <0.001). Of the most common infections among the total of 418 cases of SPNI studied, 32.06% were surgical site infections, 23.18% were associations of infections, 19.14% – respiratory tract infections. A patient with SPNI has an average of 22.25 days/bed spent in hospital, compared with the average for a patient without SPNI of 12.27 days/bed. The etiological structure includes 28 species of microorganisms including gram-positive (61.92%) and gram-negative (38.08%).
Conclusions: Given the relatively high incidence of the SPNI and its impact, it is imperative to take more serious measures to prevent and control these infections.
Key words: cardiac surgery, nosocomial healthcare-associated infections, microorganisms.

Cite this article
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Introduction

Nosocomial infections (NI) represent a serious problem in the medical facilities. They are associated with substantial morbidity, prolonged hospitalizations and possible higher mortality [1]. Several studies have examined the incidence and risk factors of NI after open heart surgery. Postoperative infection has been reported to occur in 5-21% of cardiac surgery patients in various institutions [1, 2].

The number of reported nosocomial infections in the Republic of Moldova contradicts the findings of this study. As compared to other European countries, the actual number of cases of NI is much higher in Moldova, indicating that infections are not detected or are detected but not reported.

The actual number of patients with septic purulent nosocomial infections (SPNI) in the Institute of Neurology and Neurosurgery was 32.2 per 1000 operated patients, this is 7.32 times higher than the officially reported number of 4.4‰ [3]. The actual incidence of 156.42 per 1000 operated was detected at the Traumatology and Orthopedics Hospital but only five cases (1.06%) of SPNI were officially declared. This shows the actual number to be 14.75 times higher than the officially reported number [4].

Healthcare-associated infections (HCAI) caused additional 6.1 days of hospitalization. The incidence of patients with HCAI in Spanish hospitals was relevant and similar to those found in studies in Canada and New Zealand [5].

The study which looked at critically ill patients in the hospital, found that morbidity attributed to nosocomial bacteremia increased the length of stay in a hospital by 14 days [6].

Of particular interest is the study of the etiological structure and peculiarities in septic-purulent nosocomial infections in cardiac surgery departments.

From an etiological point of view, NI are characterized by their constant evolution over time. The analysis demonstrates a high variation in the etiology of NI in the surgery departments, but also in terms of the location of the infection. Therefore, in cardiac surgery the predominant pathogens isolated were *S. epidermidis* (32.1%), *S. aureus* (28.4%), the gram-negative isolates were *Enterobacter* species (10.1%): *E. coli* (6.4%), *Seratia* species (4.6%), *P. aeruginosa* (4.6%), *K.*

pneumonia (4.6%) [1].

Although gram-positive microorganisms are the main bacteria involved, gram-negative bacilli, especially *Enterobacteriaceae*, are frequently involved in cardiac surgery, according to Jolivet S. et al., the main *Enterobacteriaceae* (36.5%) were *E. coli* (29%) and *E. cloacae* (15%) [7].

It is necessary to minimize infections, this in addition to reducing mortality, reduces the duration of hospital stay, and reduces the cost of treatment [8].

Hypothesis of the study: Considering the importance of timely diagnosis and treatment of infections, it is extremely important to concentrate on prevention by investigating and evaluating bacterial infections after cardiac surgery at the Department of Acquired Heart Defects (DAHD).

Material and methods

In this cross-sectional study, the research population included all patients undergoing cardiac surgery in the DAHD. Data was collected and stored in a Microsoft Excel spreadsheet.

An infection was classified as nosocomial when developed within the hospital and became clinically apparent while the patient was still hospitalized, according to the Patient Safety Component Manual [9].

Sample size. At the time of the study, 423 cases of infection were registered, indicating a sufficient sample size to determine connections.

Site pathogens and medical records. In order to determine the etiological structure of SPNI, the results of microbiological investigations within the medical records of post-cardiac surgery patients performed in the bacteriological laboratory of the multiprofile hospital were tabulated and analyzed. This resulted in detection of 281 strains of microorganisms.

Results

Following the retrospective analysis of 1332 patients treated in the DAHD, it was found that 423 of them developed SPNI, the frequency index being 317.57 per 1000 hospitalized patients.

Out of 423 cases registered in the DAHD, only 20 cases (4.73%) were reported to the Chisinau Public Health Center. The incidences of SPNI following cardiac surgery were 317.57 per 1000 inpatients compared to 15.02 per 1000 inpatients reported to the Chisinau Public Health Center or being 21.15 times lower than the actual number, t=5.03, p <0.001, the statistical link is highly significant (confidence 99.9%) (fig. 1).

Out of the total 418 cases of post-cardiac surgery SPNI, surgical site infection (SSI) represents the majority and constitutes 134 cases (32.06%), associations of infection (AI) are 97 cases (23.18%), respiratory tract infections (RTI) – 80 cases (19.14%), cardiovascular system infections (CV) – 74 cases (17.71%), bone and joint infections (BJI) – 16 cases (3.83%), bloodstream infections (BSI) – 6 cases (1.44%), eye, ear, nose, throat, or mouth infections (EENT) – 4 cases

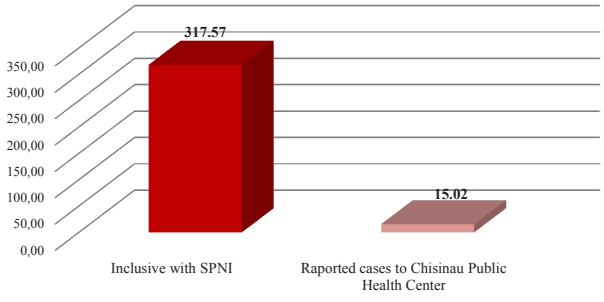

Fig. 1. General incidence due to cardiosurgical SPNI

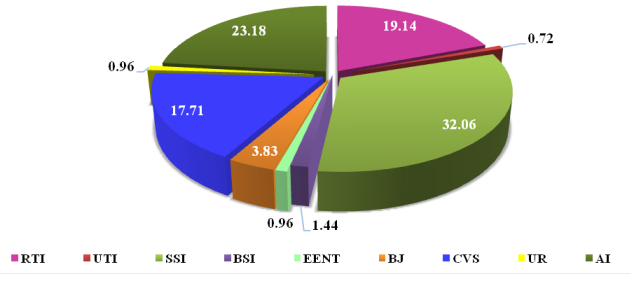

Fig. 2. The structure of post-cardiac SPNI according to the group of infections (%)

(0.96%), upper respiratory tract infections (UR) – 4 cases (0.96%) and urinary tract infections (UTI) – 3 cases (0.72%) (fig. 2).

During the studied period, 1189 patients were in the hospital 32920 days/bed, of which 418 patients with SPNI spent 14402 days in the hospital or 44.26% of all days spent in the hospital by all patients. On average, length of stay (LOS) beginning with hospitalization, for each patient with SPNI was 34.44 days/bed compared to 23.97 days/bed for patients without SPNI or 10.47 days/bed less (fig. 3).

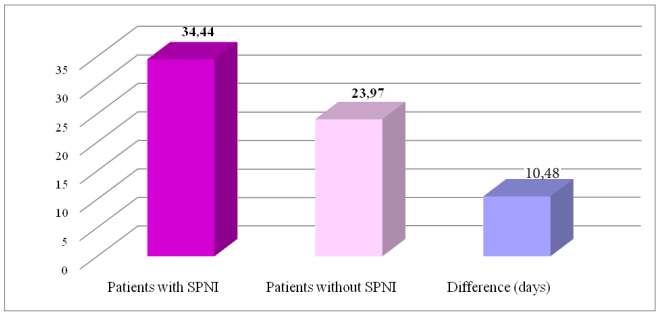

Fig. 3. The length of stay (beginning with hospitalization), (days)

For 1189 patients operated during the studied period, a total of 18691 days/bed were used. Therefore, 418 patients with SPNI used 9270 days/bed (49.93%) of the total days/bed. The length of stay (LOS) starting with surgery on average for a patient with SPNI was 22.25 days/bed, with 9.98 days/bed more than the average for a patient without SPNI which was 12.27 days/bed (fig. 4).

In the etiological structure of cardiosurgical ISPN, gram-positive microorganisms with 174 strains prevail (61.92%), with a wide spectrum of bacterial species: *S. epi-*

dermidis (27.40%), *S. viridans* (6.76%), *S. aureus* (6.05%), *S. saprophyticus* (5.34%), *E. faecalis* (7.47%), *E. faecium* (4.60%), *Enterococcus* spp. (4.02%), *S. haemolyticus* (1.72%), *Corynebacterium* spp. (1.72%), *Bacillus* spp. (1.72%), *Sarcina* spp. (1.72%), other gram-positive microorganisms (1.72%), *S. pyogenes* (0.57%), *S. agalactiae* (0.57%), *S. pneumoniae* (0.57%) (fig. 5).

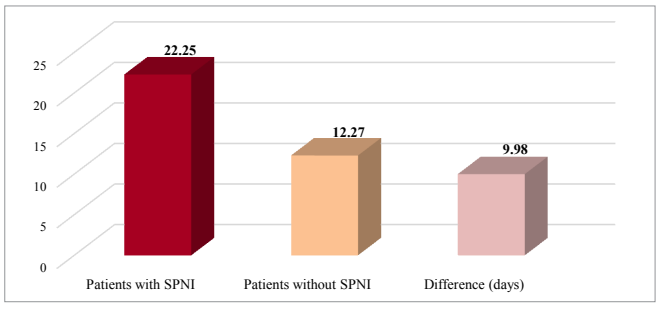

Fig. 4. The length of stay (starting with surgery), (days)

At the same time, 107 strains (38.08%) of gram-negative microorganisms were isolated, including *P. aeruginosa* (30.84%), *E. aerogenes* (28.97%), *A. baumannii* (11.21%), *E. coli* (10.28%), *K. pneumoniae* (6.54%), *K. oxytoca* (2.80%), *C. freundii* (1.87%), *P. mirabilis* (1.87%), other gram-negative microorganisms (1.87%), *E. cloacae* (0.93%), *P. vulgaris* (0.93%), *Neisseria* spp. (0.93%), *Branhamella* spp. (0.93%) (fig. 5).

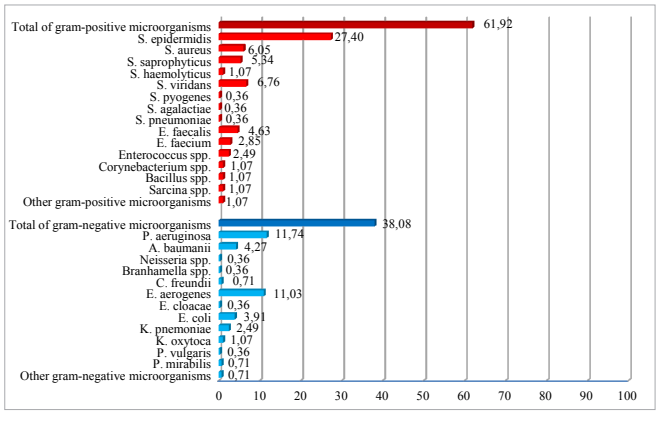

Fig. 5. The etiological distribution of cardiosurgical SPNI

Of the 281 strains isolated from patients with cardiac surgery for the study period, 112 were staphylococcal strains (*S. aureus*, *S. epidermidis*, *S. saprophyticus* and *S. haemolyticus*), which accounted for 64.36% of the total strains, 31 strains of streptococci (*S. agalactiae*, *S. pyogenes*, *S. pneumoniae*) and enterococci (*E. faecalis* and *E. faecium*) representing 17.81%, 13 strains (12.14%) of gram-negative cocci from the family Moraxellaceae (*Branhamella* spp. and *A. baumannii*), 58 strains of gram-negative microorganisms from the family Enterobacteriaceae – *E. coli*, *C. freundii*, *K. pneumoniae*, *K. oxytoca*, *P. vulgaris*, *P. mirabilis*, *E. aerogenes* and *E cloacae*, which constituted 54.20%, 33 strains of *P. aeruginosa* which constituted 30.84% of the total isolated strains (fig. 5).

The structure of microorganisms detected in cardiac surgery patients according to the investigated bio substrate shows that gram-positive bacteria predominate in samples taken from the content of the wound (41.38%), tracheal catheter (25.86%), valve vegetation (9.20%), while the share of microorganism's gram-negative amounts to 18.69%, 42.06%, 2.80% (fig. 6).

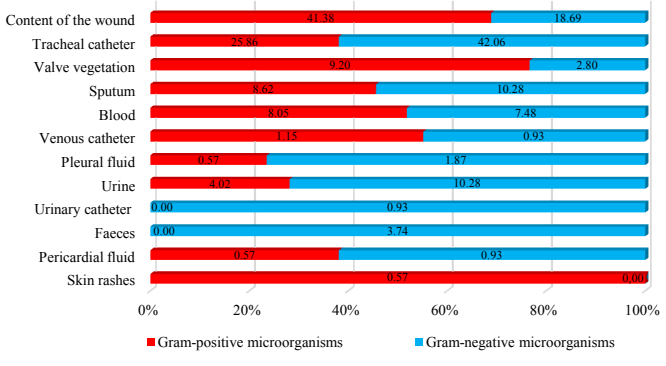

Fig. 6. Structure of microorganisms detected in cardiac surgery patients with SPNI according to the investigated bio substrate

Gram-negative microorganisms are more frequently isolated from tracheal catheter (42.06%), sputum (10.28%), urine (10.28%), while the share of gram-positive microorganisms is 25.86%, 8.62%, 4.02% (fig. 6).

An approximately equal rate of isolation of gram-positive and gram-negative bacteria was observed from blood (8.05%/7.48%), venous catheter (1.15%/0.93%), pericardial fluid (0.57%/0.93%) (fig. 6).

Only gram-negative bacteria were isolated from faeces (3.74%) and urinary catheter (0.93%), and only gram-positive microorganisms (0.57%) were identified in the skin rashes samples (fig. 6).

Discussion

According to the World Health Organization (WHO), healthcare-associated infections are the most frequent adverse event in healthcare delivery worldwide [10]. According to the WHO, seven of every 100 hospitalized patients acquire HCAI, with the infections causing approximately 99000 deaths per year in the United States [11, 12].

In various national and multicenter studies, including dozens of countries, it was determined that 3.5 to 12% of the patients were affected with at least one nosological form of SPNI [11].

Postoperative infections have been reported to occur in 5-21% of cardiac surgery patients in various institutions [1].

Different studies have shown that HCAI rates can range from 6% to 24% [8]. Therefore, Damavandi D. et al., reported being between 17% and 23% [8], as well as Ferreira G. et al., reported a similar range of 22.6% [13]. In the present study, the incidence of post-cardiac surgery SPNI is 31.76%, which is higher than normal limits.

Also, cardiac surgery involves superficial and deep sternal infections (SSI) [8]. Currently, it is considered that most SSI originate from the bacteria that enter the wound during

surgery [8].

In actual research, SSI represent the majority and constitute 32.06%, AI – 23.18%, RTI – 19.14% were the most common infections. Although in the other studies pneumonia (51.2%), SSI (22.0%), UTI (17.9%) and sepsis (14.6%) were the most common types of infection [8]. At the same time, Michalopoulos A. et al., established that the majority of NI were RTI (45.7%), CLABSI (25.2%) and wound infection (17.7%) [1]. Stanisławska M. indicated that the most frequent site of NI were pneumonia (44.4%), sepsis (42.0%) and SSI (33.3%), however every infected patient had 1-4 clinical forms [14], concurring with the present research.

One way to analyze the impact that the occurrence of infections has on LOS is to evaluate patients who remained more than nine days, which is considered as the ideal LOS for heart disease stays [13]. In the present study, a patient with SPNI has an average of stay in the hospital of 22.25 days/bed, with 9.98 days/bed more than the average for a patient without SPNI which was 12.27 days/bed.

Ferreira G. et al., demonstrated that patients who developed HCAI remained 1.5 times longer than those without infection, a 14-day increase in hospitalization after cardiac surgery [13].

Among hospital survivors, cardiac surgery patients acquiring NI had longer hospital LOS compared to patients without NI (20.1±13.0 days vs 9.7±4.5 days) [2].

From an etiological point of view, NI are characterized by their constant evolution over time. The analysis demonstrates a high variation in the etiology of NI in the surgery departments, but also in terms of the location of the infection.

In the actual research, gram-positive microorganisms are 61.92% and gram-negative microorganisms are 38.08%. Highlighted from the group of gram-positive microorganisms were *S. epidermidis* (27.40%), *S. viridans* (6.76%), *S. aureus* (6.05%), *S. saprophyticus* (5.34%), *E. faecalis* (7.47%), *E. faecium* (4.60%), *Enterococcus* spp. (4.02%), *S. haemolyticus* (1.72%), *Corynebacterium* spp. (1.72%), *Bacillus* spp. (1.72%), *Sarcina* spp. (1.72%), other gram-positive microorganisms (1.72%), *S. pyogenes* (0.57%), *S. agalactiae* (0.57%), *S. pneumoniae* (0.57%).

Of the group of gram-negative microorganisms, the largest share is manifested by *P. aeruginosa* (30.84%) followed by *E. aerogenes* (28.97%), *A. baumannii* (11.21%), *E. coli* (10.28%), *K. pneumoniae* (6.54%), *K. oxytoca* (2.80%), *C. freundii* (1.87%), *P. mirabilis* (1.87%), other gram-negative microorganisms (1.87%), *E. cloacae* (0.93%), *P. vulgaris* (0.93%), *Neisseria* spp. (0.93%), *Branhamella* spp. (0.93%).

In other studies, gram-positive cocci bacteria and gram-negative bacilli were the most common pathogens with a prevalence of 30.8% and 28.4% respectively [8]. *Acinetobacter* (48.6%), *Enterobacteriaceae* (37.1%), *P. aeruginosa* (17.1%) [8], the results of a Mazzeffi M. et al., study were similar [15]. Whereas Stanisławska M. has shown that the most common etiological factors of infection in cardiac surgery were gram-negative microorganisms such as *E. cloacae* and *P. aeruginosa* [14].

Although gram-positive microorganisms are the main bacteria involved, gram-negative bacilli, especially Enterobacteriaceae (36.5%), are frequently involved in cardiac surgery according to the results of Jolivet S. et al. [7]. In another research, the scientists found that gram-positive cocci were 67.9% and 30.7% were gram-negative bacteria [1].

In actual research, the structure of microorganisms detected in cardiac surgery patients according to the investigated bio substrate shows that gram-positive bacteria predominate in samples taken from the wound (41.38%), tracheal catheter (25.86%), valve vegetation (9.20%), while the share of microorganism's gram-negative amounts to 18.69%, 42.06%, 2.80%.

Gram-negative microorganisms are more frequently isolated from tracheal catheter (42.06%), sputum (10.28%), urine (10.28%), while the share of gram-positive microorganisms is 25.86%, 8.62%, 4.02%.

An approximately equal rate of isolation of gram-positive and gram-negative bacteria was observed from blood (8.05%/7.48%), venous catheter (1.15%/0.93%), pericardial fluid (0.57%/0.93%).

Only gram-negative bacteria were isolated from faeces (3.74%) and urinary catheter (0.93%), and only gram-positive microorganisms (0.57%) were identified in the rash samples.

Similar results obtained Liu Z. et al., (2021) recording the results of microbiological investigations of the sputum, blood, faeces and catheters [16]. Therefore, 73.13% out of all microorganisms were isolated from sputum (73.13%), blood (23.18%) and only 0.75% from urine [16].

This data suggests the urgency to take action in preventing SPNI following cardiac surgery that could decrease the incidence of the infections and allowing the patient a shorter possible stay in the hospital which will reduce healthcare costs for both the patient and the hospital.

Conclusions

1. According to the results of this study, SSI (32.06%) and AI (23.18%) represent the majority of recorded microbial infections.

2. A patient with SPNI spends an average of 22.25 days/bed in the hospital, which is 9.98 days/bed more than the 12.27 days/bed for a patient without SPNI.

3. The etiological structure of cardiac surgery SPNI is varied and includes 28 species of microorganisms. Gram-positive microorganisms are 61.92% and gram-negative microorganisms are 38.08%.

References

1. Michalopoulos A, Geroulanos S, Rosmarakis ES, Falagas ME. Frequency, characteristics, and predictors of microbiologically documented nosocomial infections after cardiac surgery. Eur J Cardiothorac Surg. 2006;29(4):456-60. doi: 10.1016/j.ejcts.2005.12.035.
2. Kollef MH, Sharpless L, Vlasnik J, Pasque C, Murphy D, Fraser VJ. The impact of nosocomial infections on patient outcomes following cardiac surgery. Chest. 1997;112(3):666-75. doi: 10.1378/chest.112.3.666.
3. Leu E. Epidemiologia şi prevenirea infecţiilor nosocomiale în staţionarele neurochirurgicale [Epidemiology and prevention of nosocomial infections in neurosurgical unit] [dissertation]. Chisinau: *Nicolae Testemit-*

anu State University of Medicine and Pharmacy; 2009. 141 p. Romanian.
4. Baranețchi I. Optimizarea supravegherii epidemiologice a infecțiilor nosocomiale septico-purulente în staționarele de profil Traumatologie și Ortopedie [Optimizing the epidemiological surveillance of septic-purulent nosocomial infections in the Traumatology and Orthopedics outpatient clinics] [dissertation]. Chisinau: *Nicolae Testemitanu* State University of Medicine and Pharmacy; 2016. 112 p. Romanian.
5. Aranaz-Andrés JM, Aibar-Remón C, Vitaller-Murillo J, Ruiz-López P, Limón-Ramírez R, Terol-García E. Incidence of adverse events related to health care in Spain: results of the Spanish National Study of Adverse Events. J Epidemiol Community Health. 2008;62(12):1022-9. doi: 10.1136/jech.2007.065227.
6. Pittet D, Tarara D, Wenzel RP. Nosocomial bloodstream infection in critically ill patients. Excess length of stay, extra costs, and attributable mortality. JAMA. 1994;271(20):1598-601. doi: 10.1001/jama.271.20.1598.
7. Jolivet S, Lescure FX, Armand-Lefevre L, Raffoul R, Dilly MP, Ghodbane W, Nataf P, Lucet JC. Surgical site infection with extended-spectrum β-lactamase-producing Enterobacteriaceae after cardiac surgery: incidence and risk factors. Clin Microbiol Infect. 2018;24(3):283-8. doi: 10.1016/j.cmi.2017.07.004.
8. Damavandi DS, Javan M, Moshashaei H, Forootan M, Darvishi M. Microbial contamination after cardiac surgery in a hospital cardiac surgery ward. J Med Life. 2020;13(3):342-348. doi: 10.25122/jml-2019-0071.
9. Centers for Disease Control and Prevention, National Healthcare Safety Network (NHSN). Patient Safety Component Manual [Internet]. Washington: CDC; 2021 [cited 2021 Jul 16]. Available from: https://www.cdc.gov/nhsn/pdfs/pscmanual/pcsmanual_current.pdf
10. World Health Organization; Ducel G, Fabry J, Nicolle L, editors. Prevention of hospital-acquired infections: a practical guide. 2nd ed. [Internet] Geneva: WHO; 2002 [cited 2021 Jul 16]. Available from: http://www.who.int/emc
11. World Health Organization. Healthcare-associated infections: Fact sheet [Internet]. Geneva: WHO [cited 2021 Jul 19]. Available from: https://www.who.int/gpsc/country_work/gpsc_ccisc_fact_sheet_en.pdf
12. Haque M, McKimm J, Sartelli M, Dhingra S, Labricciosa FM, Islam S, et al. Strategies to prevent healthcare-associated infections: a narrative overview. Risk Manag Healthcare Policy. 2020;13:1765-1780. doi: 10.2147/RMHP.S269315.
13. Ferreira GB, Sava Donadello JC, Mulinari LA. Healthcare-associated infections in a cardiac surgery service in Brazil. Brazilian J Cardiovasc Surg. 2020;35(5):614-8. doi: 10.21470/1678-9741-2019-0284.
14. Stanisławska M. [Monitoring of hospital infections in cardiac surgery ward]. Ann Acad Med Stetin. 2013;59(1):37-48. Polish.
15. Mazzeffi M, Gammie J, Taylor B, Cardillo S, Haldane-Lutterodt N, Amoroso A, Harris A, Thom K. Healthcare-associated infections in cardiac surgery patients with prolonged intensive care unit stay. Ann Thorac Surg. 2017;103(4):1165-70. doi: 10.1016/j.athoracsur.2016.12.041.
16. Liu Z, Zhang X, Zhai Q. Clinical investigation of nosocomial infections in adult patients after cardiac surgery. Medicine (Baltimore). 2021;100(4):e24162. doi: 10.1097/MD.0000000000024162.

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Author's contribution
AN conceptualized the idea, conducted literature review, collected the data, interpreted the data, and wrote the manuscript.

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Ethics approval and consent to participate
The study was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 47 of 17.04.2013). An informed consent from all participants in the study was obtained.

Conflict of Interests
There is no known conflict of interests to declare.

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Validation of the spectrophotometric method for the dosing of some combined capsules

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Abstract

Background: UV-Vis spectrophotometry remains the most accessible spectral method with a high degree of sensitivity and information. The advantage of the method consists in its universality, the ability to combine with other methods, the minimum error, as well as its economic efficiency. The objective of this study was the determination of some validation parameters for the spectrophotometric method of dosing piracetam and nicergoline in combined capsules.
Material and methods: Agilent 8453 UV-Vis spectrophotometer, reference standards of piracetam and nicergoline, 0.1 M HCl methanolic solution. Validation of the spectrophotometric method according to the requirements of the ICH guide "Q2R1: For analytical procedures and validation".
Results: Linearity was investigated on concentration ranges 5-40 μg / mL. The regression (R^2) values were 0.9998 for nicergoline and 0.998 for piracetam, respectively. The limit of detection was 1.737 μg / mL for nicergoline and 0.369 μg / mL for piracetam. Quantification limit values were also calculated as 5.265 and 1.118 μg / mL for nicergoline and piracetam, accordingly. The results obtained showed that the developed spectrophotometric method is accurate, precise and robust, because the value of the relative standard deviation was less than 1.0%.
Conclusions: The developed spectrophotometric method showed specificity, linearity, accuracy, precision and robustness, and can be applied on the concentration range between 80-120% of the nominal value of the content of nicergoline and piracetam in the preparation.
Key words: dosing, UV-Vis spectrophotometry, validation.

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Introduction

The combination of the medicinal substances in a single dosage form is a globally recognized practice. Due to the advantages of the fixed-dose combinations (FDCs) which have been shown over time, FDCs are widely applied in the treatment of a large number of pathologies, such as cardiovascular [1, 2] and respiratory [3, 4], tuberculosis [5, 6], HIV [7], diabetes [8], pain management [9], infectious diseases [10]. Among the most important advantages are: (a) the improvement of the response rate compared to monotherapy, based on different mechanisms of drug action in the combination; (b) reduced doses and toxicity; (c) achievement of the faster effect by FDC's than monocomponent; (d) better compliance by reducing the pill burden and adherence improvement; (e) low cost and saving of resources for the patients and the health system [11-13]. Most FDCs come in solid dosage forms: tablets and capsules, containing two active ingredients, rarely three and more.

Hard capsules are used as a delivery system for FDCs due to the fact that they are inert and do not interact with drug's components; they can have different sizes, including customized sizes and they are ideal for packaging of hygroscopic substances; the production technology is not complicated; they are convenient for administration [14].

The development of combination drugs includes several specific steps, being a process with many challenges, related to the nature and properties of the active ingredients which can cause incompatibilities, different doses, different pharmacokinetic properties and bioavailability etc. [15]. A major challenge in the development of FDCs is the development of analytical methods for the simultaneous determination of each component of the mixture. Researchers usually opt for physico-chemical methods, which offer the possibility of separating the components, are accurate and sensitive. Numerous factors, such as chemical structure, molecular weight, pKa values, UV absorption, concentration of active principle in the analyzed sample, solubility of compounds, are taken into account when selecting the method. Chromatographic methods, especially high pressure liquid chromatography (HPLC), are most often used for the analysis of combined products, the working techniques being complex and difficult to perform.

A very important step in the FDCs standardization is the development of the simple, accessible, fast and accurate methods of analysis. UV-Vis spectrophotometry is one of the

alternatives that can be proposed and meets all the nominated requirements. It is based on the absorption of electromagnetic radiation by molecules of the substances. Therefore, this method can be used successfully for the analysis of compounds with chromophore groups.

Hearing is one of the most important senses of human being, which is the source of sound, being an essential means of communication. Any difficulty in percepting the sound is defined as a hearing loss, pathology with various etiology and pathophysiological mechanisms. In neurosensory hypoacuzia, the difficulty of sound reset is determined by dysfunction in the inner ear [16]. Treatment of neurosensory hypoacuzia remains a serious problem. Corticosteroids are indicated as first-line medicines. At the same time, certain emphasis is placed on the advancement of the hemodynamics in the region of the innear ear, the improvement of the metabolism, the blood rheology and the stimulation of the auditory analyzer. The medical treatment includes several types of medicines: vasodilators, nutrients, anti-inflammatories, antioxidants. Medicines, in particular, vinpocetine, pentoxifylline, cerebrolysin, piracetam are given in the first days parenterally, then orally [17, 18]. The concomitant use of several medicines in treatment regimens for hearing loss justifies efforts to develop combined medicines, which would solve several pharmacotherapeutic problems of this disease. Thus, research was carried out at the Scientific Center for Drug Research (SCDR) in order to develop an original composition containing nicergoline, piracetam and Hawthorn dry extract in the one dosage form – capsules.

Nicergoline (fig. 1) is an analogue of ergot alkaloids, which associates in the molecule a nucleus of hydrogenated lysergic acid and nicotinic acid, belongs to the alpha-adrenolytics group, possesses vasodilating action. Due to the presence of nicotinic acid in its structure, it manifests direct spasmolytic myotropic action on the vessels muscles, increases their permeability for glucose. Nicergoline acts predominantly on the cerebral vessels and lower and upper limbs, improves cerebral, pulmonary and renal blood circulation [19].

Piracetam (fig. 1) is a cyclized derivative of gamma-aminobutyric acid, with nootropic action, intensifies energy and plastic processes in the brain, has the ability to restore the structure of cell membranes, the three-dimensional structure of membrane and transmembrane proteins, with the recovery of their functionality [20].

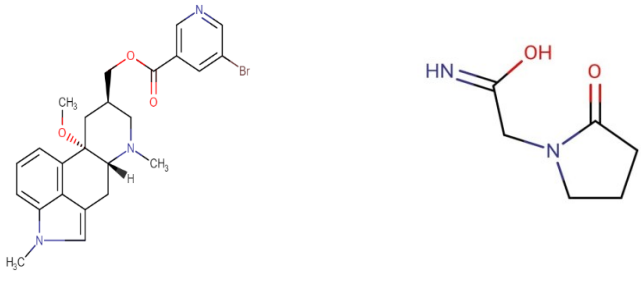

Fig. 1. The chemical structures of nicergoline (left) [21] and piracetam (right) [22]

Data from the literature provide information on several methods applied in bulk nicergoline analysis, in one-component forms or various combinations: HPLC [23], spectrofluorimetry [24, 25], spectrophotometry [26]. Chromatographic [27-29] and spectral [30, 31] methods are also reported for piracetam. It should be noted that the simultaneous determination of these two substances has not been reported, since they are associated for the first time in the same pharmaceutical form.

The purpose of this study was to develop and validate a simple, accurate and reproducible UV-Vis spectrophotometric technique to analyze nicergoline in combination with piracetam in capsules.

Material and methods

The experimental researches were carried out at the Laboratory of elaboration, analysis, standardization and control of medicines (LEASCM) of the SCDR within *Nicolae Testemitanu* State University of Medicine and Pharmacy.

The elaboration of the assay method of combined capsules was performed based on the requirements of the ICH guideline "Q2R1: For analytical and validation procedures" [32].

Three experimental series of laboratory-prepared operculated capsules containing nicergoline, piracetam, Hawthorn dry extract and excipients were used in the studies.

Apparatus

The Agilent Technologies 8453 Spectrophotometer equipped with 10 mm matched quartz cells was used.

Substances, solvents and reagents

Reference standards of nicergoline and piracetam (Sigma Aldrich), methanol (MeOH, Sigma Aldrich), hydrochloric acid (HCl) 0,1 M were used.

Preparation of the standard solutions

0.05 g of standard piracetam was transferred into a 25 mL volumetric flask, was dissolved in 0.1 M HCl methanol solution and then adjusted up to the mark with the same diluent. 1.2 mL of the obtained solution was transferred into a 25 mL volumetric flask and adjusted up to the mark with the same diluent (dil.1). After that, 2.5 ml of obtained solution was diluted to 10 mL with the same diluent (dil.2).

0.005 g of standard nicergoline was transferred into a 25 mL volumetric flask, dissolved in 0.1 M HCl methanol solution and adjusted up to the mark with the same diluent. Then, 5.0 mL of obtained solution was transferred into a 25 mL volumetric flask and volume was adjusted up to the mark with the same diluent (dil.1). After that, 2.5 ml of obtained solution was diluted to 10 mL with the same diluent (dil.2).

Preparation of the sample solutions

A content weight of one capsule was taken into a 50 mL volumetric flask, then 40 mL of 0.1 M HCl methanol solution was added and shaken well until it dissolved. After that, the obtained solution was filtered and made up to the mark with the same diluent *(stock solution)*. For the analysis of piracetam, 0.6 mL of *stock solution* was withdrawn and taken into a 50 mL volumetric flask. The volume was adjusted up to mark with the same diluent (dil.1). Then, 5 mL of obtained

solution was diluted to 10 mL with the same diluent (dil.2). For the analysis of nicergoline, 10.0 mL of *stock solution* was withdrawn and taken into a 25 mL volumetric flask. The volume was adjusted with the same diluent up to mark (dil.1). Then, 5 mL of obtained solution was diluted to 10 mL with the same diluent (dil.2).

Preparation of placebo solutions

Accurately weighed Hawthorn dry extract and excipients were calculated for one capsule and were taken into a 50 mL volumetric flask. Then, 40 mL of 0.1 M HCl methanol solution was added and shaken well until it dissolved. After that, the obtained solution was filtered and made up to the mark with the same diluent *(placebo solution).* For the analysis of piracetam, 0.6 mL of *placebo solution* was withdrawn and taken into a 50 mL volumetric flask. The volume was adjusted up to mark with the same diluent (dil.1). Then, 5 mL of obtained solution was diluted to 10 mL with the same diluent (dil.2). For the analysis of nicergoline, 10.0 mL of *placebo solution* was withdrawn and taken into a 25 mL volumetric flask. The volume was adjusted up to mark with the same diluent (dil.1). Then, 5 mL of obtained solution was diluted to 10 mL with the same diluent (dil.2).

Quantitative determination

The absorbance of standard and sample solutions was measured using 10 mm matched quartz cells and 0.1 M HCl methanol solution as reference solution.

The quantitative content of nicergoline and piracetam in capsules was determined by using Eq. (1):

$$X, g = \frac{A_{an} * m_{st} * W_{an} * P}{A_{st} * m_{an} * W_{st}}, \text{ in which:} \quad (1)$$

A_{an} – absorbance of the sample solutions;
A_{st} – absorbance of the standard solutions;
M_{an} – mass of the sample substance, g;
m_{st} – mass of the standard substance, g;
P – average content mass of one capsule;
W_{st} and W_{an} – the volumes of dilution for standard and sample solutions respectively.

Validation of the method

According to the ICH guide the method was validated for parameters, such as linearity, accuracy, precision, sensitivity (LOQ and LOD) and robustness, selectivity, solution stability [32].

The *linearity* of the spectrophotometric method of assay of piracetam and nicergoline was investigated in the concentration range from 5 – 40 μg / mL. Thus, standard stock solutions of substances were prepared: 5 samples with different concentrations of piracetam and nicergoline of 5, 10, 24, 30, 40 μg / mL and 5, 10, 15, 20, 30 μg / mL, respectively. The determinations were performed in triplicate, being constructed the calibration curve (fig. 2). Linear regression analysis was used to evaluate the linearity of the calibration curve using the least squares method.

The *selectivity* of the spectrophotometric method of assay of piracetam and nicergoline was investigated by measuring the absorbance of the placebo solutions at the spectrophotometer using the 10 mm matched quartz cells in the wavelength range 200 – 350 nm. 0.1 M HCl methanol solution was used as the reference solution.

Determination of the *accuracy* of the method was prepared using three samples consisting of nicergoline, piracetam and a mixture of excipients in 0.1 M HCl methanol solution at concentration levels of 80 – 120% of the stated amount, with 3 replicates for each concentration. The percentage recovery of the amount of substance and % RSD were calculated for each of the replicate samples (tab. 1).

The nicergoline and piracetam concentrations were determined using the calibration curve, and the percentage of regression has been established by using Eq. (2).

$$R = [(C_t - C_p)/C_a] * 100\%, \text{where:} \quad (2)$$

R – recovery, %;
C_t – concentration of the sample with the addition, μg/mL;
C_p – sample concentration without addition, μg/mL;
C_a – concentration of the standard with the addition, μg/mL.

Determination of *precision* of the method was performed by evaluating repeatability and intermediate precision [33]. Repeatability was determined using six samples of capsules, which were analyzed on the same day and under the same conditions. Intermediate precision was determined using six samples of capsules, which were analyzed in different days over the period of a week by different analysts (tab. 2). The concentration of nicergoline and piracetam from capsules was determined.

The *sensitivity* of the method was investigated by determining the limit of detection (LOD) and limit of quantification (LOQ) by analyzing the substance solutions and measuring the signal-to-noise ratio. LOD is the concentration, which is due to the signal/noise ratio of about 3:1, while LOQ is the concentration that gives a signal/noise ratio of about 10:1 with RSD values (n = 3) less than 10%.

The *robustness* of the method was investigated by varying the maximum absorption wavelength of nicergoline and piracetam with ±2 nm [32]. The determinations were repeated 3 times at each wavelength (tab. 3).

The *stability of standard and sample solutions* was determined by analyzing them immediately after preparation and after 24 hours at room temperature (25°C). Three determinations were performed, the absorbance was evaluated, the concentrations of analyte in the samples (relative to a freshly prepared reference solution) and the % RSD were calculated (tab. 4).

Statistical analysis

Statistical analysis was carried out by using the Statistical Package for the Social Sciences (IBM SPSS Statistics) 10.5 software.

Results and discussion

The piracetam and nicergoline standard solutions showed absorption maxima at 205 nm and 287 nm, respectively. The same maxima were detected on the spectrum of the sample solution. The spectrum of the placebo solution showed a very low absorption at 272 nm (fig. 2).

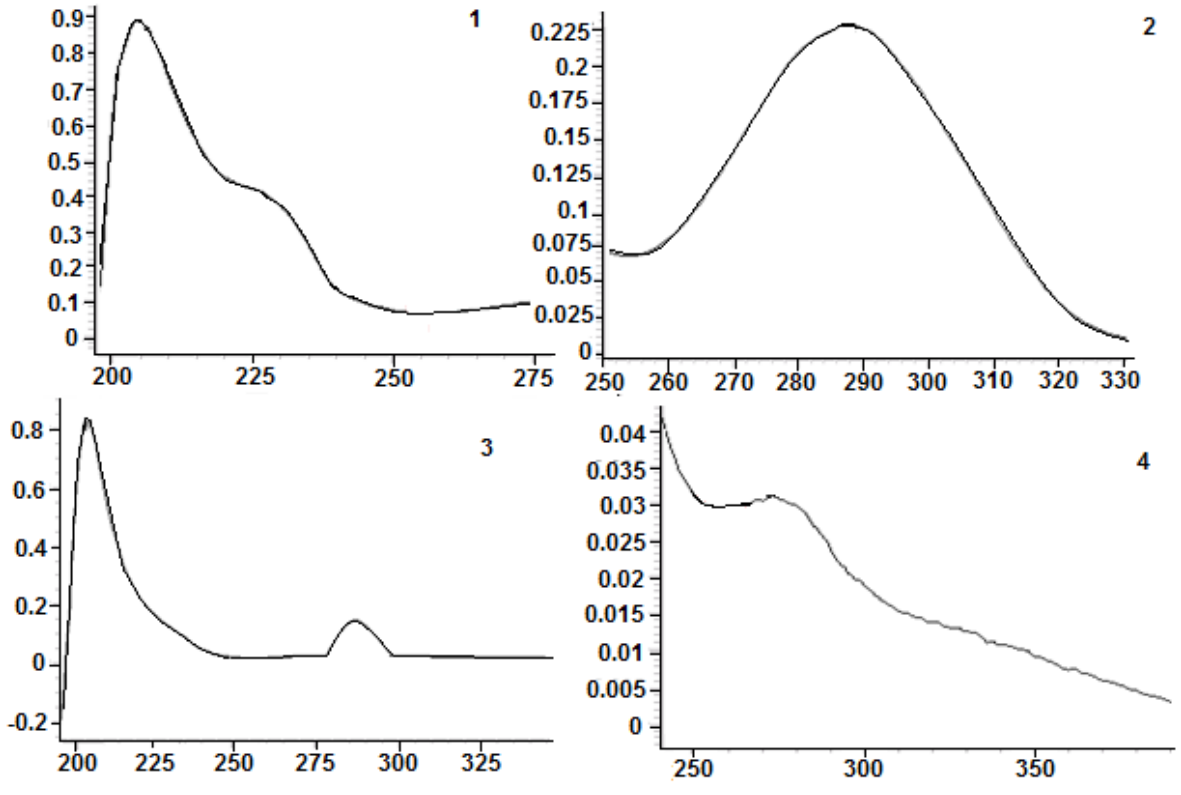

Fig. 2. Absorption spectra of standard piracetam (1) and nicergoline (2), sample (3) and placebo (4) solutions in 0.1 M HCl methanol solution

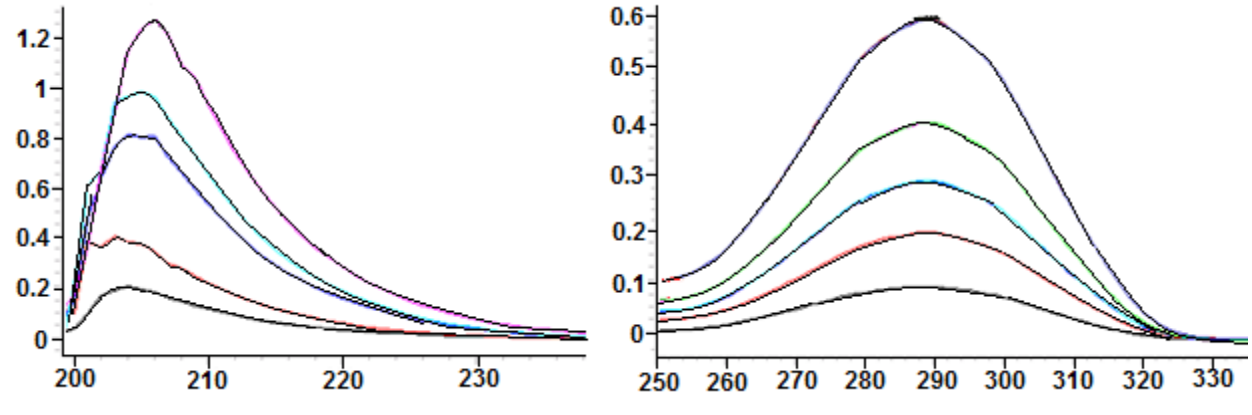

Fig. 3. Absorption spectra of piracetam (left) and nicergoline (right) standard solutions for linearity

The validation of the method was carried out in accordance with the ICH Guide "Q2R1: For Analytical Procedures and Validation" [32].

The *linearity* of the UV-Vis spectrophotometric method was determined in the concentration range of 5 – 40 μg/mL of piracetam and 5 – 30 μg/mL of nicergoline. Based on the obtained results, the calibration curve was drawn and the equation of linear regression was obtained (fig. 3 and 4).

The obtained results showed a linear regression of the method in the concentration range between 5 – 40 μg/mL of piracetam and 5 – 30 μg/mL of nicergoline.

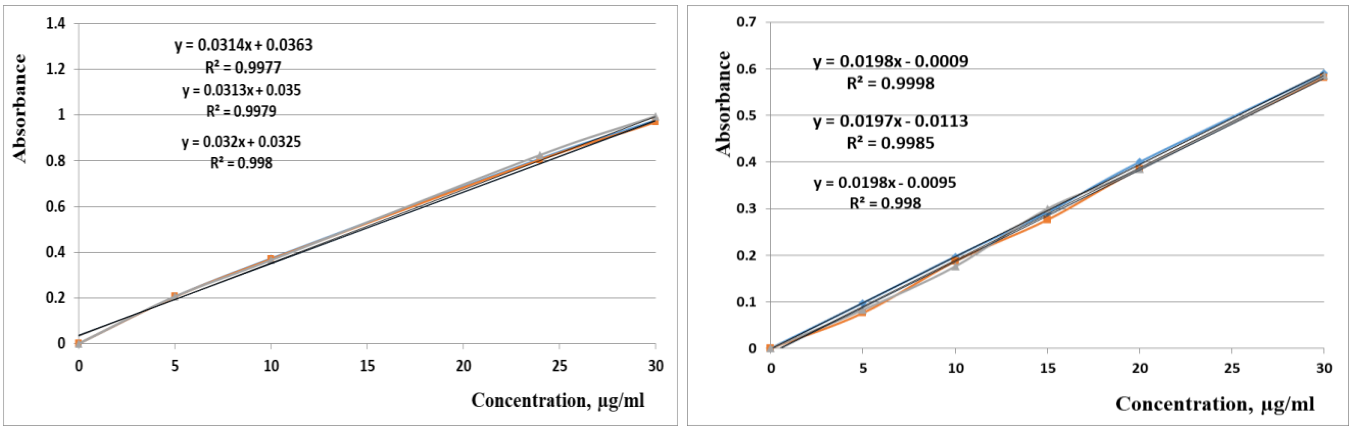

Fig. 4. Calibration curves of standard solutions of piracetam (left) and nicergoline (right) (n = 3 determinations)

The values of *LOD* calculated in accordance with the ICH guide were 1.737 µg / mL for nicergoline and 0.369 µg / mL for piracetam [32].

The values of *LOQ* calculated in accordance with the ICH guide were also calculated as 5.265 and 1.118 µg / mL for nicergoline and piracetam, respectively [32].

The method is *selective*, as placebo solution showed a very low absorption at the wavelength (272 nm) different from the absorption peaks of nicergoline and piracetam. Also, Hawthorn dry extract and excipients did not influence the results of the determinations.

Accuracy is expressed as the percentage of recovered analytes compared to actual values (tab. 1). The admissibility condition is at least 99% [32].

The developed UV-Vis spectrophotometric method for the assay of nicergoline and piracetam from combined capsules, is accurate, with an average recovery value ranging from 99.8% to 101.8%. RSD values were between 0.11 – 0.69%, as indicated in tab. 1.

Precision is generally expressed by standard deviation and standard relative deviation. As a result of studies the average values of substance concentrations were calculated after 6 determinations for each 3 capsule series (tab. 2).

Table 1. Recovery as a condition of admissibility

Nr.	The amount of Ciprofloxacin hydrochloride taken into work				Amount of Ciprofloxacin hydrochloride (mg/mL) (average for n=3)		Recovery		RSD
	(%)		(mg/mL)		(mg/mL)		(%)		(%)
	N	P	N	P	N	P	N	P	N/P
1	80	80	8.0	8.0	7.94	8.05	99.21	101.78	0.69/0.53
2	100	100	10.0	10.0	10.04	9.99	100.73	99.80	0.19/0.11
3	120	120	12.0	12.0	11.99	12.01	99.95	100.15	0.23/0.54

Note: N – Nicergoline, P – Piracetam, RSD – Relative standard deviation.

Table 2. Results of the repeatability determination and intermediate precision for piracetam and nicergoline in combinated capsules

Parameters	Repeatability: C, g						Intermediate precision: C, g					
Substances	P			N			P			N		
Series	S 01	S 02	S 03	S 01	S 02	S 03	S 01	S 02	S 03	S 01	S 02	S 03
1	0.2010	0.2001	0.2001	0.0046	0.0046	0.0046	0.2000	0.1997	0.2000	0.0046	0.0046	0.0046
2	0.2005	0.2001	0.2003	0.0045	0.0045	0.0046	0.2003	0.1997	0.1999	0.0045	0.0046	0.0046
3	0.2006	0.2004	0.2001	0.0046	0.0046	0.0045	0.2013	0.1999	0.2001	0.0045	0.0046	0.0045
4	0.2004	0.2006	0.2003	0.0046	0.0046	0.0045	0.1997	0.1994	0.1997	0.0045	0.0045	0.0045
5	0.1999	0.2004	0.2003	0.0045	0.0045	0.0045	0.1997	0.1992	0.2001	0.0045	0.0045	0.0045
6	0.1999	0.2001	0.2001	0.0046	0.0046	0.0046	0.2000	0.1999	0.1997	0.0045	0.0045	0.0045
Average	0.2004	0.2003	0.2003	0.0046	0.0046	0.0046	0.2002	0.1997	0.1999	0.0045	0.0046	0.0046
RSD, %	0.2122	0.1049	0.0703	0.5897	0.5160	0.8382	0.2940	0.1450	0.0940	0.7156	0.9528	0.7189

Note: N – Nicergoline, P – Piracetam, RSD – Relative standard deviation.

Table 3. Results of the robustness by spectrophotometric method for piracetam and nicergoline in combined capsules

	P			N		
λ, nm	203 nm	205 nm	207 nm	280 nm	282 nm	284 nm
A	0.85099	0.85247	0.85565	0.46128	0.46205	0.46201
A	0.85114	0.85152	0.85489	0.46119	0.4627	0.46174
A	0.85087	0.85089	0.85546	0.46208	0.46201	0.46189
Average	0.85100	0.85162667	0.85533333	0.46151667	0.462253333	0.46188
RSD, %	0.015896	0.09339568	0.04624122	0.10615694	0.083794175	0.02928845

Note: N – Nicergoline, P – Piracetam, A – absorbance, RSD – Relative standard deviation.

The results of the determinations showed that the method is accurate within acceptable limits, RSD values were ≤ 2% (between 0.07 and 0.95), so, the developed UV-Vis spectrophotometric method is precise.

Robustness is usually fixed to determine, if the results of the determinations will not be influenced by some insignificant variations in the established parameters, respectively, the validity of the analytical process will be maintained constant. The robustness of the developed method was determined by the variation of wavelengths ±2 nm (tab. 3).

The results obtained showed that the developed spectrophotometric method is robust, the relative standard deviation is less than 1.0%.

The results of the short-term *stability* were within the acceptance range. Also, the results showed that the samples were stable at room temperature for 24 hours. Acceptance criteria is: RSD ≤ 2% [34] (tab. 4).

Table 4. Results of the short-term stability determined by the proposed method (n=6)

Nr.	Concentration of piracetam and nicergoline from capsules, g			
	Declared		Found	
	P	N	P	N
1	0.2000	0.0045	0.2016	0.0046
2	0.2000	0.0045	0.2014	0.0045
3	0.2000	0.0045	0.2011	0.0046
4	0.2000	0.0045	0.2001	0.0046
5	0.2000	0.0045	0.1998	0.0045
6	0.2000	0.0045	0.1989	0.0046
Average	0.2000	0.0045	0.2005	0.0046
RSD, %			0.2016	0.5897

Note: N – N icergoline; P – Piracetam; RSD – Relative standard deviation

Conclusions

For the first time, the UV-Vis spectrophotometric method of concomitant dosing of piracetam and nicergoline in combined capsules was developed and validated. The proposed work technique is simple, accessible, fast, accurate, sensitive and reproductive. The developed spectrophotometric method serves as an alternative to the chromatographic method (HPLC), and can be included in the quality specification for capsules combined with piracetam, nicergoline and Hawthorn dry extract.

References

1. Putignano D, Orlando V, Monetti VM, et al. Fixed versus free combinations of antihypertensive drugs: analyses of real-world data of persistence with therapy in Italy. Patient Prefer Adherence. 2019;13:1961-1969. doi: 10.2147/PPA.S225444.
2. Ihm SH, Shin J, Park CG, et al. Efficacy of a fixed dose combination of irbesartan and atorvastatin (Rovelito((R))) in Korean adults with hypertension and hypercholesterolemia. Drug Des Devel Ther. 2019;13:633-645. doi: 10.2147/DDDT.S191973.
3. Beasley R, Fingleton J, Weatherall M. Restriction of LABA use to combination ICS/LABA inhaler therapy in asthma. Thorax. 2013;68 (2):119-120. doi: 10.1136/thoraxjnl-2012-202483.
4. Tee A, Chow WL, Burke C, et al. Cost-effectiveness of indacaterol/ glycopyrronium in comparison with salmeterol/fluticasone combination for patients with moderate-to-severe chronic obstructive pulmonary disease: a LANTERN population analysis from Singapore. Singapore Med J. 2018;59(7):383-389. doi: 10.11622/smedj.2018022.
5. World Health Organization. Guidelines for treatment of drug-susceptible tuberculosis and patient care, 2017 update [Internet]. Geneva: WHO; 2017 [cited 2021 Jul 2]. Available from: https://apps.who.int/iris/bitstream/handle/10665/255052/9789241550000-eng.pdf
6. Lima GC, Silva EV, Magalhães P, et al. Efficacy and safety of a four-drug fixed-dose combination regimen versus separate drugs for treatment of pulmonary tuberculosis: a systematic review and meta-analysis. Braz J Microbiol. 2017;48(2):198-207. doi: 10.1016/j.bjm.2016.12.003.
7. Mutemwa M, Peer N, de Villiers A, et al. Prevalence, detection, treatment, and control of hypertension in human immunodeficiency virus (HIV)-infected patients attending HIV clinics in the Western Cape Province, South Africa. Medicine (Baltimore). 2018;97(35):e12121. doi: 10.1097/MD.0000000000012121.
8. Jain RK. Empagliflozin/linagliptin single-pill combination therapy for patients with type 2 diabetes mellitus. Expert Opin Pharmacother. 2017;18(6):545-549. doi: 10.1080/14656566.2017.1299712.
9. Daniels SE, Atkinson HC, Stanescu I, et al. Analgesic efficacy of an acetaminophen/ibuprofen fixed-dose combination in moderate to severe postoperative dental pain: a randomized, double-blind, parallel-group, placebo-controlled trial. Clin Ther. 2018;40(10):1765-76.e5. doi: 10.1016/j.clinthera.2018.08.019.
10. Banek K, Webb EL, Smith SJ, et al. Adherence to treatment with artemether-lumefantrine or amodiaquine-artesunate for uncomplicated malaria in children in Sierra Leone: a randomized trial. Malar J. 2018;17(1):222. doi: 10.1186/s12936-018-2370-x.
11. European Medicines Agency. Guideline on clinical development of fixed combination medicinal products. London: EMA; 2017 [cited 2021 Jul 2]. Available from: https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-clinical-development-fixed-combination-medicinal-products-revision-2_en.pdf

12. Clarke PM, Avery AB. Evaluating the costs and benefits of using combination therapies. Med J Aust. 2014;200(9):518-20. doi: 10.5694/mja14.00199.
13. Llibre JM, de Lazzari E, Molina JM, et al. Cost-effectiveness of initial antiretroviral treatment administered as single vs multiple tablet regimens with the same or different components. Enferm Infecc Microbiol Clin. 2018;36(1):16-20. doi: 10.1016/j.eimc.2016.07.006.
14. Diez F, Kalafat J, Bhat J. Fixed dose combinations in capsules. Contract Pharma [Internet]. 2019 June 3 [cited 2021 Jul 6]. Available from: https://www.contractpharma.com/issues/2019-03-01/view_features/fixed-dose-combinations-in-capsules/
15. Hens B, Corsetti M, Bermejo M, Löbenberg R, González PM, Mitra A, Aceituno A. "Development of Fixed Dose Combination Products" Workshop report: Considerations of gastrointestinal physiology and overall development strategy. AAPS J. 2019;21(4):75. doi: 10.1208/s12248-019-0346-6.
16. Alshuaib WB, Al-Kandari JM, Hasan SM. Classification of hearing loss. In: Bahmad F, Jr., editor. Update on hearing loss. InTech; 2015. doi: 10.5772/61835.
17. Kuhn M, Heman-Ackah SE, Shaikh JA, Roehm PC. Sudden sensorineural hearing loss: a review of diagnosis, treatment, and prognosis. Trends Amplif. 2011 Sep;15(3):91-105. doi: 10.1177/1084713811408349.
18. Uncu L, Gadîrcă A, Parii S, Vislouh O, Suvorchina O. Medicamente combinate utilizate în tratamentul hipoacuziei neurosenzoriale [Combination drugs used in the treatment of neurosensory hearing loss]. Revista Farmaceutică a Moldovei. 2014;(3-4):52. Romanian.
19. Kiran Kumar K, Venkata Nadh R, Nagoji KEV. Extractive spectrophotometric determination of Nicergoline through ion-pair complexation reaction. Oriental J Chem. 2013;29(1):263-269. doi: 10.13005/ojc/290142.
20. Rahate K, Sivadas A, Sathi A, Sathi K. Development and validation of spectrophotometric methods for simultaneous estimation of citicoline and piracetam in tablet dosage form. J Pharm Bioallied Sci. 2013;5(3):202-207. doi: 10.4103/0975-7406.116818.
21. DrugBank Online. Nicergoline [Internet]. [cited 2021 Jul 6]. Available from: https://go.drugbank.com/drugs/DB00699
22. DrugBank Online. Piracetam [Internet]. [cited 2021 Jul 6]. Available from: https://go.drugbank.com/drugs/DB09210
23. Kiran Kumar K, Venkata Nadh R. Development and validation of HPLC method for the estimation of nicergoline in marketed formulations. Rasayan J Chem. 2011;4(4):885-889.
24. Walash MI, Belal F, El-Enany N, Abdelal A. Second-derivative synchronous fluorescence spectroscopy for the simultaneous determination of cinnarizine and nicergoline in pharmaceutical preparations. J AOAC Int. 2008 Mar-Apr;91(2):349-359.
25. Riahi S, Hariri M, Ganjali MR, Norouzi P. Rapid and direct spectrofluorometric and chemometrics methods for the simultaneous determination of two dansyl derivatives. Spectrosc Lett. 2010;43(3):226-234. doi: 10.1080/00387010903321737.
26. Zayed SI. Simultaneous determination of cinnarizine and nicergoline in a binary mixture using first derivative spectra, first derivative of ratio spectra and multivariate calibration techniques. J Anal Chem. 2010;65(9):921-928. doi: 10.1134/S1061934810090066.
27. Acharya M, Ak J, Garud N. Stability indicating reversed phase-high performance liquid chromatography method development and validation for simultaneous determination of related substances of citicoline and piracetam in pharmaceutical dosage form. Asian J Pharm Clin Res. 2016;9(2):292-7.
28. Dwi Lestari A, Tri Prasetyo A, Palupi T, Umayah E, Yuwono M, Indrayanto G. *HPLC* determination of piracetam in tablets; validation of the method. J Liq Chromatogr Relat Technol. 2005;28(9):1407-1416. doi: 10.1081/jlc-200054893.
29. Sahu K, Shaharyar M, Siddiqui AA, Sahu S. Establishment of inherent stability on piracetam by UPLC/HPLC and development of a validated stability-indicating method. Arabian J Chem. 2017;10:S576-S582. doi: 10.1016/j.arabjc.2012.11.003.
30. Siddiqui FA, Sher N, Shafi N, Wafa Sial A, Ahmad M, Mehjebeen, Naseem H. Development of new method for simultaneous analysis of piracetam and levetiracetam in pharmaceuticals and biological fluids: application in stability studies. BioMed Res Int. 2014;2014:1-8. doi: 10.1155/2014/758283.
31. El-Adl SM, El-sadek ME, Hasan MH. Determination and validation of piracetam in pharmaceuticals using quantitative nuclear magnetic resonance spectroscopy. Analytical Chem Lett. 2017;7(2):271-279. doi: 10.1080/22297928.2017.1320227.
32. ICH Harmonised tripartite guideline Q2(R1). Validation of Analytical Procedure: Text and Methodology. In: International Conference on Harmonisation of technical requirements for registration of pharmaceuticals for human use. [cited 2021 Jul 6]. Available from: https://database.ich.org/sites/default/files/Q2%28R1%29%20Guideline.pdf
33. Uncu L, Donici E, Valica V, Vîslouh O, Gonciar V, Parii S. Development and validation of an assay method for ciprofloxacin hydrochloride determination in combination ear drops. Chem J Mold. 2019;14(2):56-61. doi: http://dx.doi.org/10.19261/cjm.2019.607.
34. Bhowmick AA, Khandelwal KR, Mhaske DV, Swapnil K. Analytical method development and validation for piracetam as bulk and in pharmaceutical formulation. Int J PharmTech Res. 2010;2(1):201-204.

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LU designed the study; VE, EM conducted the laboratory work and performed the analytical part of the laboratory work; LU, ED interpreted the data; LU drafted, revised and approved the manuscript, VV revised and approved the manuscript.

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The research was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 45 of 26.02.2020).

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Optimisation of the magistral semisolid formulations with furazidine used in urogenital infections

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Abstract

Background: Urinary tract infections are the most common urogenital diseases, with an increased incidence in men and older people. Urogenital infections are caused by Gram-negative bacteria, in which *Escherichia coli* predominates with a share of 80%. The evolution of microbial resistance to preparations used in curative-prophylactic institutions, induces the need of the reintroduction of nitrofurans, noteworthy for their wide spectrum of antibacterial activity.
Material and methods: For the study, suppositories with furazidine were prepared by hand rolling and by melting and molding methods. Quantitative analysis was performed spectrophotometrically on a UV-VIS Perkin Elmer Lambda 40 spectrophotometer. All solvents and reagents had the degree of purity "pure for analysis" and "chemically pure".
Results: Duble cast method was applied to identify the exact mass of hydrophobic (cocoa butter, suppocire) and hydrophylic (polyethylene glycol mixtures) excipients. All the formulated suppositories were subjected to quality tests and showed acceptable physical characteristics and uniformity of drug contents. The UV-VIS spectrophotometric method for quantitiative determination of furazidine was developed and validated. The validation results showed that the developed method is simple, fast, accurate and robust.
Conclusions: Suppositories with furazidine were prepared by classic technological methods. Preparation of the suppositories with furazidine on cocoa butter excipient is a suitable alternative for individual medicinal prescriptions. The UV-VIS spectrophotometric dosing method for furazidine in suppositories was developed and validated.
Key words: suppositories, furazidine, PEGs, *in-vitro* dissolution test.

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Introduction

Urinary tract infections are inflammatory diseases of infectious etiology, affecting 150 million people each year worldwide [1]. Currently in the Republic of Moldova there is an increased number of people suffering from infectious diseases of the urinary tract [2], which occur in any part of the urinary system: in kidneys, ureters, bladder and urethra, more frequently infections involve the lower urinary tract – the bladder and the urethra [3]. The risk factors for its developing include urinary obstruction, renal failure, renal transplantation, immunosuppression, diabetes, obesity, genetic susceptibility, prolonged catheterization, sexual activity and older age. According to physicians the most common diseases are pyelonephritis, urolithiasis, glomerulonephritis and cystitis [4].

Most urinary tract infections are caused by Gram-negative bacteria, namely *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Enterococcus faecalis, Proteus mirabilis*, by Gram-positive bacteria as: *Staphylococcus aureus, Staphylococcus saprophyticus* and by yeast infections – *Candida spp.* For complicated urinary tract infections the order of prevalence for causative agents is *Enterococcus spp., K. pneumoniae, Candida spp., S. aureus* and *P. aeruginosa* [3]. Depending on the severity of the disease, the treatment for each case is complex and is selected individually.

Antibiotics are the most commonly recommended therapeutics however, increasing rates of antibiotic resistance and high recurrence rates threaten to greatly enhance the burden that these common infections place on society. Today, nitrofuran antibiotics, despite the long-term use in medicine (since the 1950) is a class of synthetic substances that are revival and reintroduced as "old" antibacterials for treating multidrug-resistant pathogens [5, 6]. Nitrofurans have a wide spectrum of antimicrobial activity, which acts by disrupting the process of cellular respiration of bacteria, inhibition the tricarboxylic acid cycle and causing disruption of nucleic acid synthesis and, ultimately, death of bacterial cells [7].

Furazidine (*furazidinum*) – is a nitrofuran derivative with properties analog to nitrofurantoin, used in the treatment of urinary tract infections. It is an imidazolidine-2.4-dione, an organonitrogen heterocyclic and an organooxygen heterocyclic antibiotic, derived from a semicarbazide [8].

Furazidine is a flavourless yellow or orange-yellow fine crystalline powder, bitter in taste. Very slightly soluble in water and ethanol, hardly soluble in dimethylformamide, slightly soluble in acetone, practically insoluble in chloroform and benzene. It is one of the most popular nitrofuran and is widely used in a large number of researches due to uses in medicine, in industrial and extemporaneous dosage forms and in cosmetology as well [9].

On Moldovan pharmaceutical market furazidine is present in industrial commercial brand names of Furasol, Furagin and Furamag in dosage forms of powder, tablets and capsules [10]. The survey of the compounding dosage forms with furazidine during the years of 2019-2021 in the production department of *Vasile Procopisin* University Pharmaceutical Center (UPhC), shows that the largest share is presented by semisolid pharmaceutical forms mostly in suppositories, followed by solid pharmaceutical forms as powders. Specialists select the rectal route of administration of drugs because it avoids the first hepatic passage, does not allow irritation of the mucosa of the gastrointestinal tract, in case of intolerance to some active substances, demonstrates good absorption of drugs, rapid therapeutic action and mostly tolerable for pediatric and geriatric patients [11, 12]. Thus, based on the importance of the study, the semisolid medicinal forms – suppositories containing furazidine only or in combination with other active components, such as benzocaine, dimexide, methylene blue and other, are frequently prescribed in the treatment of urogenital diseases [13]. Pharmaceutical forms prepared in pharmacy, whether magistral prescriptions prepared by the pharmacist on the basis of a medical prescription, or compounded dosage forms presented as stock elaborations prepared by the pharmacist on the basis of an official data from the pharmacopoeia, offer an effective alternative to industrial medicinal preparations. *Vasile Procopisin* UPhC of *Nicolae Testemitanu* SUMPh, is nowadays the leading compounding pharmacy from the Republic of Moldova and plays an important role in practic trainings of new generations of pharmacists concerning production, quality control and delivery of medicines [14].

The research was performed in order to develop the optimal composition of suppositories with furazidine prepared in the production department of *Vasile Procopisin* UPhC on hydrophilic and hydrophobic excipients using two preparation methods (hand rolling and molding), as well as to develop and validate a spectrophotometric dosing method for furazidine in suppositories. The paper aimed to highlight the comparative analysis of technological methods of suppositories prepared on water-soluble and fat-soluble excipients, quality control and quantitative determination according to the Analytical Standardization Documentation (ASD).

Material and methods

Reagents and chemicals. Furazidine – molecular formula $C_{10}H_8N_4O_5$, produced by Chengdu HuaXia Chemical Reagent Co. Ltd, 99% purity. N,N-dimethylformamide and other reagents of analytical grade have been purchased from Sigma-Aldrich Chemie GmbH and Merck (Germany). All solvents had the degree of purity "pure for analysis" and "chemically pure".

Preparation of suppositories. The preparation of the suppositories was performed in the Production Department of *Vasile Procopisin* UPhC. The elaboration of the composition and preparation of the suppositories on water-soluble excipients: PEG 400: 4000 (1:9); PEG 400: 1500 (0.5:9.5); PEG 400: 1500: 4000 (1:3:6), as well as on fat-soluble excipients: cocoa butter and suppocire was performed in accordance with the requirements of the European Pharmacopoeia [15].

Double cast technique. The amount of substance for a suppository was mixed with a part of the molten excipient and poured into the suppository mold. Calculations were made for 10 suppositories. Then the excess molten excipient was poured into each cavity, cooled and the excipient left outside the level of the mold cavities was scraped and removed. At the beginning, 10 suppositories obtained from the clean excipient were weighed, then 10 suppositories containing the excipient and each substance were weighed and the average mass was calculated for each.

Evaluation of suppositories. Visual characterization – twenty suppositories from each batch were randomly selected, longitudinally cut and examined through naked eyes for the assessment of physical characters. *Weight variation* – twenty suppositories were weighed and average weight was calculated. Each suppository was weighed individually on electronic balance (WLC 6/12 precision electronic pharmaceutical balance). No suppositories should deviate from average weight more than 7.5%. *Melting point* – melting range test was performed with the whole suppository. Suppository from each formulation was placed in a test tube with phosphate buffer pH 7.2 maintained at constant temperature 37± 0.5°C. The time required for the whole suppository to melt or disperse in the media was noted. *Penetration test* – this test was used to determine the temperature at which the suppository becomes sufficiently soft for a penetrating rod to drop through its length. Test apparatus Erweka PM 30, phosphate buffer pH 7.2 maintained at 37±0.5°C was used for this testing, the time taken for the penetration of entire suppository was recorded.

Drug content – was determined spectrophotometrically on Perkin Elmer Lambda 40 UV/VIS spectrophotometer, using solvents and reagents with a degree of purity "pure for analysis" and "chemically pure", various laboratory and pharmaceutical utensils.

In-vitro dissolution study. The study was carried out using dissolution apparatus USP Type II (Paddle) with apparatus Erweka DT6, dissolution medium – phosphate buffer, pH 7.2, the speed of paddle – 100 rpm, temperature of medium – 37 ± 0.5°C.

Validation of the UV-spectrophotometric method. The analytical parameters of linearity, accuracy, selectivity and robustness were evaluated for validation of the spectrophotometric method.

Statistical analysis. All measurements were carried out in triplicate and expressed as mean (n=3) ± standard deviation (n=3) of three replicates. Construction of curves and

graphical presentation were performed by MS Office Excel 2016, as well as identifying the differences between values. A probability value of p≤0.05 was considered to be significant.

Results and discussion

Preparation of suppositories. The active substance furazidine – a compound from the group of nitrofurans, with antimicrobial effect against specific microorganisms [16, 17], was used for the study. Due to the unwanted reactions that furazidine can manifest in the oral administration of medicinal preparations, suppositories are an effective and advantageous alternative. Furazidine – IUPAC name 1-[3-(5-nitrofuran-2-yl)prop-2-enylideneamino]imidazolidine-2.4-dione, has a pKa value of 8.23, characteristic for a substance with weak acidic properties. Calculating the percentage of non-ionized form [11] at the rectal average pH of 7.9 we can conclude that furazidine is satisfactorily absorbed at a neutral to slight basic pH, reported in adults [12].

Furazidine suppositories were prepared on a hydrophobic excipient of cocoa butter, by the method of manual modeling [18]. Subsequently the composition was developed for suppositories obtained by the melting and casting method prepared on suppocire and with polyethylene glycols (PEGs): PEG 400: 4000 (1:9); PEG 400: 1500 (0.5:9.5); PEG 400: 1500: 4000 (1:3:6). In order to efficiently and qualitatively achieve the method of preparing suppositories by melting and casting in patterns, the calculation of the displacement factors for each component of each assortment of suppositories with application of Double Casting Method was performed [13]. It is necessary to know the capacity of the forms and the value of the displacement factor, respectively the amount of displaced excipient of 1.0 g of active substance, mechanically dispersed in the excipient, according to the formula (1):

$$f = \rho \text{ excipient} / \rho \text{ active substance}, \quad (1)$$

where: *f – displacement factor; ρ – density.*

To compensate for the losses, an excess will be taken, depending on the number of suppositories to be prepared. The results obtained were used to calculate the mass of the excipients. The stages of the technological process of preparation of suppositories by the *method of melting and casting in molds*, were applied to hydrophobic base suppocire and for water-soluble excipients based on PEGs. The method consisted of pouring the mixture of active substances and excipient, hot fluidized, in forms corresponding to the subsequent cooling of suppositories. The stages of the technological flow of preparation of suppositories by the method of melting and molding in patterns are shown in fig. 1.

The preparation process depends on the properties of the active substances, so the furazidine was incorporated by suspension, in micronized form with dimensions between 50-100 μm. In the method of melting and casting into molds, melting of the excipient and mixing with the active substances is done in containers heated with water vapor, at a controlled temperature to avoid overheating [19]. Great attention has been paid to the homogeneity of the mixture when molding to avoid the tendency of sedimentation, therefore

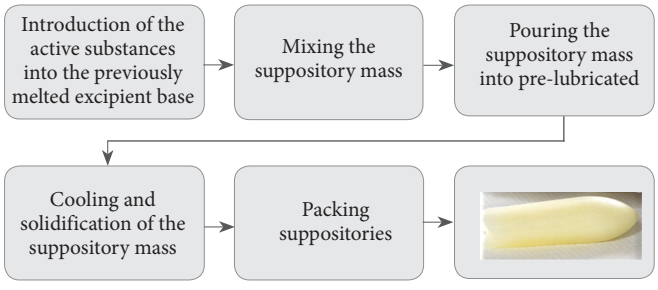

Fig. 1. Scheme of the technological flow of preparation of suppositories by the method of melting and molding in patterns

the mixture must be less fluid, stirring continuously during casting for a correct dosage. Drug has been suspended with the adjustable speed pistil, to ensure perfect homogenization in the mixture, with convenient viscosity, to maintain a homogeneous suspension of the incorporated active substance. Uniform flow in the molds was ensured.

The hand rolling method was applied for fat-soluble excipient cocoa butter. Suppositories were obtained by manual method, initially in the mortar furazidine was crushed in the presence of peach oil [20], the mixture was homogenized with cocoa butter until obtaining the appropriate consistency. Subsequently, the suppository mass was divided into appropriate doses, from which rectal suppositories were modeled, packaged in parchment paper, then in plastic boxes. The preparation was shaped, labeled according to the provisions of the ASD. The scheme of the technological process of preparation of suppositories by the method of hand rolling is presented in fig. 2.

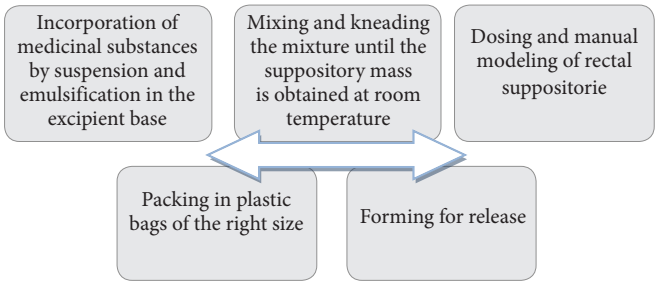

Fig. 2. The stages of the technological flow of obtaining suppositories by hand rolling method

Evaluation of suppositories. Twenty suppositories from each batch were randomly selected, longitudinally cut and examined through naked eyes for the assessment of physical characters like absence of fissuring, pitting, fat blooming, exudation and migration of active ingredients. Suppositories have a homogeneous appearance, yellowish in color, which retains its shape and consistency at room temperature.

Weight variation: twenty suppositories were weighed and average weight was calculated. Each suppository was weighed individually on WLC 6/12 electronic balance. No suppositories deviated from average weight by more than 5%, accordingly meeting the pharmacopoeia requirement [21].

Melting point: Macro melting range test was performed with the whole suppository. Suppository from each formulation was placed in a test tube with phosphate buffer pH

Table 1. Results of evaluation of furazidine suppositories for various parameters

Formulation	Weight* (average mass, gram)	Melting time** (minutes)	Penetration test** (minutes)	Drug content** (%)
Furazidine 0.1 g Cocoa butter q.s. ad 3.0 g	3.2±0.032	31.2±1.2°C	15.4±0.81	98.9% ±0.04
Furazidine 0.1 g Suppocire q.s. ad 3.0 g	3.0±0.032	36.8±0.12°C	17.25±0.73	97.14% ±0.83
Furazidine 0.1 g PEG 400: 4000 (1:9) q.s. ad 3.0 g	3.07±0.014	34.7±0.52°C	22.3±0.64	98.55%±0.112
Furazidine 0.1 g PEG 400:1500:4000 (1:3:6) q.s. ad 3.0 g	3.05±0.07	36.17±0.52°C	19.4±0.95	98.72±1.37
Furazidine 0.1 g PEG 400:1500 (0.5:9.5) q.s. ad 3.0 g	3.01±0.001	37.6±0.1°C	59.5±0.77	94.5±0.02

Note: *Average mass of 20 suppositories; **Average of 3 measurements; ± Standard Deviation.

7.2 maintained at constant temperature 37± 0.5°C. The time required by the whole suppository to melt or disperse in the media was noted. The melting time plays a crucial role in the release of active ingredient.

Penetration test: the study was carried out using apparatus Erweka PM 30, in medium of distilled water at temperature of 37±0.5°C. The average results are presented in table 1.

Quantitative determination of furazidine in suppositories was carried out by UV-VIS spectrophotometric method. Several solvents were selected for the elaboration of the technique for the extraction of medicinal substances from suppositories containing furazidine: purified water, dimethylformamide solution, acetate buffer and 96% ethyl alcohol. Repeated extraction with dimethylformamide met all the requirements for qualitative and quantitative analysis of furazidine, the extraction yield being 94%.

Extraction technique – 1 suppository (mass 3.0 g) was brought into a 100 ml beaker, 60 ml of dimethiformamide was added and mixed vigorously for 20 minutes. 30 ml of 96% ethyl alcohol was added, stirred, added 96% alcohol to the quota and mixed. The obtained mixture was filtered through filter paper; the first portions were discarded. 0.25 ml of the filtrate was placed in a volumetric flask and mixed with acetate buffer to the level.

The UV-VIS spectra of the 5 μg/ml furazidine samples recorded in the 230-450 nm region showed a maximum absorption for the standard furazidine solution at 292 nm, for the suppositories prepared on cocoa butter and suppocire at 286.11 nm, and for the suppositories with furazidine prepared on PEGs, the absorption spectrum at the wavelength of 286.04 nm was recorded, using the Perkin Elmer Lambda 40 UV/VIS spectrophotometer (fig. 3).

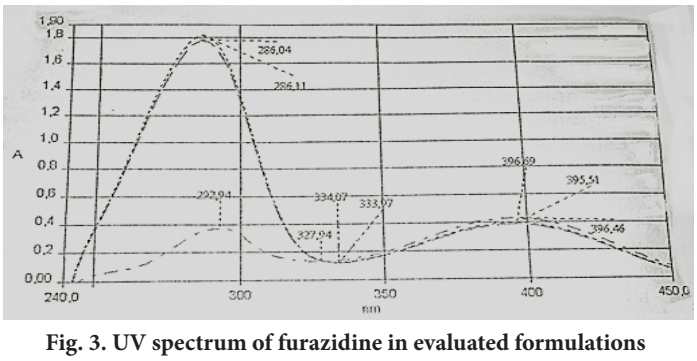

Fig. 3. UV spectrum of furazidine in evaluated formulations

The furazidine content was calculated according to the formula (2):

$$X=\frac{A_1 * 100 * 100 * a_o * 1 * C}{A_o * 1 * 1 * 100 * 100 * 100}$$

$$X=\frac{A_1 * 100 * 100 * a_o * 1 * C}{A_o * 1 * 1 * 100 * 100 * 100}, \quad (2)$$

where:

X - Furazidine content in the sample, g;

A_1 - Wavelength of the solution to be analyzed;

A_O - Wavelength of the standard furazidine solution;

a_O - mass of furazidine standard, g;

C – Amount of active substance, %.

The results for all assortments of suppositories meet the pharmacopeial requirements and are presented in table 1. All the suppositories prepared with furazidine showed acceptable physical characteristics and uniformity of drug contents.

The in-vitro drug release profile from different suppositories formulation is shown in fig. 4. The dissolution study showed that the suppositories melted in the dissolution medium maintained at 37±5^{0}C. All five formulations showed more than 50% drug release within 60 minutes.

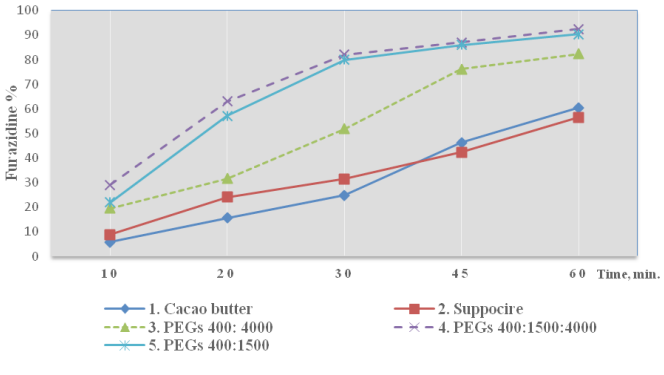

Fig. 4. Dissolution profiles of suppositories with furazidine using different types of excipients

In cocoa butter suppositories, the drug release was slightly slower due to high lipophilicity of the base and non-

Table 2. Results of validation of UV-VIS spectrophotometric dosing method for furazidine from suppositories

	Validation parameters	Suppositories with furazidine on hydrophobic base	Suppositories with furazidine on hydrophylic base
Repeatability	Substance content, g	0.108	0.989
	Coefficient of variation,%	0.550	0.467
Precision, day 1	Substance content, g	0.106	0.160
	Coefficient of variation,%	0.618	0.645
Precision, day 2	Substance content, g	0.107	0.108
	Coefficient of variation,%	0.589	0.542
Robustness	Coefficient of variation,%	286.04 nm: 0.005	286.11nm: 0.006

miscibility of the base with the dissolution media, characteristic for hydrophobic bases [22]. Furthermore, the lack of surfactants might have acted as a significant variable in the fat-based formulation in the release of furazidine [23, 24]. Suppositories prepared with the combination of PEG 400:1500:4000 (1:3:6) showed the maximum furazidine release (92.4%) within 60 minutes.

Validation of the UV-VIS dosing spectrophotometric method. UV-VIS spectrophotometric methods [25] for dosing furazidine in suppositories were developed and validated. Validation parameters were calculated according to SR ISO 8466 / 1-2016 European Pharmacopoeia Standard, Ed. 9.0 (2020) and ICH-Q2B-Validation of Analytical Procedure: Methodology (2005). The statistical parameters that were used to validate the method were: linearity, repeatability, accuracy and robustness. The first step was to select the solvent for the extraction of the active substances. Extraction technique used: a suppository (mass of 3.0 grams) was brought into a 100 ml beaker, 60 ml of dimethiformamide was added and mixed vigorously for 20 minutes, futher mixed with 30 ml of 96% alcohol and brought with 96% alcohol to the quota. The mixture obtained was filtered through filter paper, the first portions were discarded, and 0.25 ml of the filtrate was brought into a volumetric flask and mixed with acetate buffer up to 100 ml.

Preparation of the standard solution. 0.05 g (exact mass) preventively dried furazidine at a temperature of 100-105˚C, until the exact mass, is dissolved in 60 ml of dimethiformamide in a 100 ml volumetric flask, 30 ml of 96% ethyl alcohol are added, and mixed well.1 ml of solution was brought to a 100 ml volumetric flask, mixed with the acetate buffer to the level. The solutions were used ready-made.

The selectivity of the dosing method was determined by analysis of a control sample. At the wavelength of 292 nm corresponding to the furazidine determination, the control sample led to an absorbance value of 0.001. The accuracy of the developed methods was determined at 3 concentration levels: 80%, 100% and 120% by the standard addition method. The furazidine concentration was calculated using the linear regression equations established at the linearity parameter. The equation of linear regression for furazidine dosing was: $y = 0.238x + 0.014$, $R^2 – 0.999$.

The degree of accuracy, repeatability and intermediate accuracy was investigated. Repeatability was determined for 4 samples, at the concentration level of the drug substances of 100%, on the same day, respecting the same conditions. The degree of accuracy was investigated in 2 different days, under the same conditions, performing 5 determinations for each assortment of suppositories. Changes in the wavelength of absorption were performed to evaluate the robustness of the method. During the analysis of the pharmaceutical products, the levels found no significant difference and the relative standard deviation was below 0.93%, demonstrating the robustness of the proposed method, thus, furazidine in suppositories can be detected in low concentrations (tab. 2).

The UV-VIS spectrophotometric method for quantitiative determination of furazidine in evalueated suppositories was developed and validated. The validation results show that the developed method is simple, fast, accurate and robust.

Conclusions

In this paper, suppositories with furazidine were formed by hand rolling and molding methods and were subjected to physical evaluation, weight variation, content uniformity, melting point, penetration time test and *in-vitro* dissolution studies. All tests showed satisfactory results.

All five formulations showed more than 50% drug release within 60 minutes. Based on the *in-vitro* release rate studies, it can be concluded that PEG 400:1500:4000 can be used as a base which is easily soluble in aqueous medium, disperses rapidly and has higher rate of release for immediate release of furazidine and is recommended for bulk pharmaceutical elaborations.

Preparation of the suppositories with furazidine on cocoa butter excipient is a suitable alternative for individual medicinal prescriptions.

The UV-VIS spectrophotometric dosing method was developed and validated, this method could be included in the analytical procedures and documentations in the perspective of the quality evaluation of furazidine suppositories, the obtained results will serve as landmark for real-time stability studies.

References

1. Tan CW, Chlebicki MP. Urinary tract infections in adults. Singapore Med J. 2016 Sep;57(9):485-490. doi: 10.11622/smedj.2016153.
2. Guranda D, Ciobanu C, Diug E, et al. Perspectivele utilizării supozitoarelor cu extract purificat de Mumio în afecțiunile urogenitale [The opportunities of using suppositories with purified Mumiyo extract in urogenital diseases]. Revista Farmaceutică a Moldovei. 2020;(1-4):38-41. Romanian.
3. Flores-Mireles A, Walker J, Caparon M, et al. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol. 2015;13(5):269-284. https://doi.org/10.1038/nrmicro3432.
4. Öztürk R, Murt A. Epidemiology of urological infections: a global burden. World J Urol. 2020;38(11):2669-2679. https://doi.org/10.1007/s00345-019-03071-4.
5. Guay DR. An update on the role of nitrofurans in the management of urinary tract infections. Drugs. 2001;61(3):353-64. doi: 10.2165/00003495-200161030-00004.
6. Le VVH, Rakonjac J. Nitrofurans: revival of an "old" drug class in the fight against antibiotic resistance. PLoS Pathog 2021;17(7):e1009663. https://doi.org/10.1371/journal.ppat.1009663.
7. Babără D. Elaborarea tehnologiei și cercetarea biofarmaceutică a formelor medicamentoase cu izohidrafural [Elaboration of technology and biopharmaceutical research of isohydrafural drug forms]. [dissertation]. Chișinău: *Nicolae Testemitanu* State University of Medicine and Pharmacy; 1997. 124 p. Romanian.
8. PubChem [Internet]. National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Compound Summary for CID 146690, Furazidine; [cited 2021 Apr 9]. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/Furazidine.
9. Beliatskaia AV, Kashlikova IM, Elagina AO, et al. Nitrofurans for external use (review). Drug Dev Regist. 2019;8(2):38-47. doi: 10.33380/2305-2066-2019-8-2-38-47.
10. Agency for Medicines and Medical Devices, the Republic of Moldova. Nomenclatorul de stat al medicamentelor [State nomenclature of medicines] [Internet]. Chisinau: The Agency; 2021- [cited 2021 Jul 6]. Available from: https://amdm.gov.md/ro/nomenclator. Romanian.
11. Diug E, Guranda D, Ciobanu C. Biofarmacie și farmacocinetică [Biopharmacy and pharmacokinetics]. Chișinău: Print Caro; 2019. 204 p. Romanian.
12. Jannin V, Lemagnen G, Gueroult P, et al. Rectal route in the 21st century to treat children. Adv Drug Deliv Rev. 2014;73:34-49. https://doi.org/10.1016/j.addr.2014.05.012.
13. Diug E, Guranda D, Polișciuc T, Solonari R. Tehnologie farmaceutică extemporală [Extemporaneous pharmaceutical technology]. Chișinău: Universul; 2013. 160 p. Romanian.
14. Guranda D, Ciobanu C, Polișciuc T, Solonari R. Perspectives and optimization of semi-solid pharmaceutical forms in pediatric pharmacotherapy. In: Proceedings of the Romanian National Congress of Pharmacy. 17th ed. "21st century pharmacy – between intelligent specialization and social responsibility"; 2018 Sept 26-29; Bucharest, Romania. Bucharest; 2018. p. 88-92.
15. Council of Europe, European Directorate for the Quality of Medicine & HealthCare (EDQM). European Pharmacopoeia. 9th ed. Vol. 1. Strasbourg: Council of Europe; 2017.
16. Dighe NS, et al. Synthetic and pharmacological profile of furan. Asian J Biosci. 2014;1(1):7-14.
17. Guranda D, Polisciuc T, Ciobanu C, et al. Perspectivele utilizării nitrofuranilor în tratamentul plăgilor [Prospects for the use of nitrofurans in the treatment of wounds]. Revista Farmaceutică a Moldovei. 2019;(1-4):59-62. Romanian.
18. Todoran N, Redai E, Antonoaea P, Ciurba A. Ghidul activităților practice de tehnologie farmaceutică. Vol 3, Prepararea produselor medicamentoase semisolide și solide [Guide to practical pharmaceutical technology activities. Vol 3, Preparation of semi-solid and solid medicinal products]. Târgu-Mureș: University Press; 2016. 167 p. Romanian.
19. Popovici I, Lupuleasa D. Tehnologie farmaceutică [Pharmaceutical technology]. 4th ed. Vol. 1. Iași: Polirom; 2017. 720 p. Romanian.
20. Guranda D. Indicații metodice la lucrările de laborator pentru studenții anului III, Facultatea de Farmacie [Methodical indications for laboratory works for the 3rd year students, Faculty of Pharmacy]. Chișinău; 2011. 185 p. Romanian.
21. National Drug Agency. Farmacopeea Română [Romanian Pharmacopoeia]. 10th ed. Bucharest: Editura medicală; 2011. 1315 p. Romanian.
22. Nicoară AC, Cazacincu RG, Lupuleasa D, et al. Formulation and *in vitro* release testing of rectal suppositories containing nimesulide. Farmacia. 2015;63(1):111-117.
23. Belniak P, Świąder K, Szumiło M, et al. Comparison of physico-chemical properties of suppositories containing starch hydrolysates. Saudi Pharm J. 2017 Mar;25(3):365-369. doi: 10.1016/j.jsps.2016.09.004.
24. Asgari F, Amiri S, Ghiass M. Improved solubility of acetamidophenol from peg and witepsol suppositories via formation of inclusion complex by β-cyclodextrin with a controlled release profile. J Pharm Innov. 2019;14(1):57-65. https://doi.org/10.1007/s12247-018-9328-y.
25. Neagu AF. Principii generale ale spectrometriei atomice și moleculare [General principles of atomic and molecular spectrometry]. Bucharest: Editura Universitară „Carol Davila"; 2016. 131 p. Romanian.

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GD designed the study, performed some part of laboratory work, drafted the first manuscript; CC interpreted the data, revised the manuscript; NC conducted the laboratory work; SR conceptualized and performed a certain portion of laboratory work. All authors revised and approved the final version of the manuscript.

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Conflict of Interests

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Diastolic disorder inherent to doxorubicin cardiotoxicity

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Abstract

Background: The doxorubicin (Dx) cardiotoxicity is manifested by a marked heart failure evolution. The impact of Dx on lusitrop functions of the heart and the inherent diastolic disorders have a theoretical and practical value for the connection cardiology-oncology.
Material and methods: Dx cardiotoxicity was reproduced by its administration i/p in white rats in cumulative dose 16 mg/kg (Dx group n=9). Control group (n=9) received only physiological solution. The study was performed *in vitro* by using models of isolated heart perfusion in either isovolumic or exterior working regimens. The assayed indices of diastole functioning were: left ventricle (LV) end-diastolic pressure (LVEDP), diastolic stiffness, isovolumic relaxation velocity (-dP/dTmax) and protodiastolic pressure of LV (LVPDP).
Results: The indices of diastolic disorders induced by Dx were elevation of LVEDP, diastolic stiffness and LVPDP in a range of 97-168% comparing to control as well as diminution of -dP/dTmax in the physiological pattern of hemodynamics. LVEDP increased more in conditions of calcium overloading or endothelin-1 (ET-1) action that are involved in pathogenesis of diastolic rigidity. Dx action led to decrease of myocardium resistance to ischemia-reperfusion action resulting in the LVEDP elevation by 53% comparing to control.
Conclusions: 1. Diastolic disorders inherent to Dx cardiotoxicity are manifested by the increase of LVEDP and diastolic stiffness. 2. Diastolic disorders compromised the volume-pressure relationship of LV, the adaptation of the heart to effort with volume, being more pronounced during the action of calcium excess and ET-1.
Key words: doxorubicin cardiotoxicity, diastolic relaxation disorders.

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Introduction

The diastolic relaxation of the heart is an important phase of cardiac activity, which assures, by adequate fulfil of left ventricle cavity (LV), the feasibility of Frank Starling mechanism realizing (the length – force law) and of pumping function. But, on the other hand, the diastole is a vulnerable cardiac phase to the cardiovascular risk factors, but diastolic disorders are imposed as an early predictor of heart failure [1, 2]. Conceptually, the cardiac lusitrope function is dependent on different factors and conditions, like turnover of calcium into cardiomyocyte, indispensable for working of ionic pumps (first of all SERCA2a), as well as structural changes which develop at extracellular matrix and cardiac myocytes level [3, 4].

Among natural and synthetic factors with cardiotoxic action, the anthracyclines (election drugs in neoplasm treatment, e. g. doxorubicin) are positioned in a special format in the interdisciplinary plan of collaboration and mutual interest, oncology-cardiology. Cardiotoxicity of doxorubicin (Dx) may already progress to cumulative sub-therapeutic doses of the drug (<500 mg/m2), manifested by the onset and rapid progression of heart failure inherent to dilated cardiomyopathy with a marked risk of mortality indifferent of patient age.

Thus, there is a need to stop the administration of the drug, which reduces the chances of survival of the cancer patient, especially with leukemia and lymphoma. In this aspect, it is obvious that oncology and cardiology have a common task: the early detection of Dx cardiotoxicity and application of measures to limit the characteristic myocardial injuries and dysfunction.

The evaluation of Dx cardiotoxicity in terms of estimation the nature of diastolic disorders is authentic and important from several points of view. First of all it is needed to highlight the pathogenetic mechanisms of triggering and exacerbation of heart failure. Secondly, the demarcation of functional predictors of Dx cardiotoxicity is important. And thirdly, it is important to determine the targets of pathogenetic treatment of heart failure.

The aim of the study was to evaluate *in vitro* the disturbances of diastolic relaxation characteristic for Dx cardiotoxicity.

Material and methods

Myocardial Dx disorder was reproduced in white rats by i/p administration of Dx (cumulative dose 16 mg/kg in 2 weeks, 2 injections/per week at a dose of 4.0 mg/kg). The animals were sacrificed by euthanasia (sodium thiopental,

0.4 mg/kg) 10 days after the last anthracycline injection, as doxorubicin is a drug that, based on reduced clearance, accumulates in the body. This model of Dx-induced cardiotoxicity has been used by other authors, aiming to highlight the link between the action of anthracycline and the inflammatory response of the myocardium [5].

The isolated heart was perfused in isovolumic or working regime respectively according to the Langendorff and Neely-Rovetto method, the functional indices of LV being estimated by technical device for recording real-time parameters „Bio-Shel" (Australia) or the autograph Linearcorder MARK WR3101 (Germany) connected to the mechanical sensor. For estimation of diastolic relaxation peculiarities together with the performance of the LV pump function, were used the following functional indices:

- End-diastolic pressure (LVEDP),
- Protodiastolic pressure (LVPDP),
- Maximum isovolumic relaxation rate (-dP/dTmax),
- Diastolic stiffness,
- Aortic jet velocity,
- Coronary flow,
- Cardiac output.

Functional reserves of diastole were estimated in various exercise stress tests:

- Modification of filling pressure of the left atrium (effort with volume),
- Increasing the filling volume of the LV cavity (volume-pressure relationship),
- Increasing the retrograde perfusion pressure (coronary pressure – diastolic compliance relationship),
- Stimulation of isolated heart with calcium and Endothelin 1 (ET-1),
- The ischemia-reperfusion impact.

The obtained data, exposed by the value M±m (mean and standard error), were compared and statistically analyzed, according to the t-Student criteria, with the control group (intact animals) or with indices attested before the exercise stress test. The error margin less than 5% was considered admissible and the deviation from the reference value was significant ($p < 0.05$).

Results

Dx cardiotoxicity was imposed by marked impairment of diastolic relaxation, that already has been attested in the optimal perfusion regime of the isolated heart, when the filling pressure of the left atrium (LAFP) was 15 cmH_2O, and the pressure in the aorta – 80 cmH_2O (tab. 1).

One of the main important indices, estimating the feasibility of diastolic relaxation, PTDVS, was increased by 168% in the group with Dx compared to the control one, which was associated with increased of more than 109% the value of diastolic stiffness. The diastolic disorder is based on disturbance of relaxation isovolumic phase of the heart, as key parameter of it, -dP/dT max, demonstrates a significant decline of 24.81%. Impaired diastolic relaxation compromises systolic contraction due to the inefficiency of the Frank-Starling mechanism. Thus, the protodiastolic pressure, which means LV pressure at the end of systole, is elevated by 97% in the group with Dx.

Table 1. Indices of diastolic relaxation of the isolated heart perfused in physiological regime

Indices	Group		p
	Control (n=9)	Dx (n=9)	
End-diastolic pressure (LVEDP)	4.7±0.26	12.6±0.78 +168% vs control	<0.001
-dP/dT max, mm Hg/sec	6710±174	5045±120 -24.81% vs control	<0.05
Diastolic stiffness of LV, mm Hg/ml	29.8±1.7	62.5±4.4 +109.73% vs control	<0.01
Protodiastolic pressure of LV, mm Hg	0.78±0.06	1.53±0.08 +97% vs control	<0.01

The manifestations of the heart lusitrop function disturbances at administration of Dx can be determined by estimating the diastolic stiffness of the LV, which was clearly increased by 109% compared to the control index. Diastolic stiffness shows the ratio between rate of increasing LV pressure during diastolic filling to the stroke volume or systolic volume. As a consequence, the parameters of the LV pump function were significantly reduced compared to the control group (tab. 2).

The aortic jet decreased by 40.47%, which determined the decreased cardiac output by 36.9%, caused by decreased coronary flow by 31.65%.

Manifestations of impaired diastolic relaxation in the group with Dx become more pronounced in the hemodynamic effort tests, with calcium, ET-1, as well as in the impact of ischemia-reperfusion.

Table 2. The values of indices of the isolated heart pump function

Indices	Group		p
	Control (n=9)	Dx (n=9)	
Aortic jet velocity, ml/min	21.5±1.4	12.8±0.8 -40.47% vs control	<0.01
Coronary flow, ml/min	15.9±1.2	10.8±1.2 -31.65% vs control	<0.01
Cardiac output, ml/min	37.4±1.9	23.6±1.3 -36.9% vs control	<0.01

In the exercise effort test with minimal filling pressure of the left atrium (LAFP) (5 cm H_2O) the depreciation of the index -dP/dT max compared to the control prototype increased from 24.81% to 32.77% (tab. 3).

Compared to the index attested in the perfusion of the isolated heart in physiological comfort regime, its value increased in both groups, but the enhancement was different: in the control group -dP/dT max was increased by 19% and in the group with Dx – only by 7 %. In conditions of decreasing the venous return to the heart, the phase of isovolumic relaxation of the heart has a decisive significance in order to ensure as much as possible the filling of the left ventricular cavity based on developing the adequate pressure gradient.

Table 3. Value of functional indices of the cord isolated at the minimum level of LAFP

Indices	Group		p
	Control (n=9)	Dx (n=9)	
Aortic jet velocity, ml/min	5.7±0.3	1.6±0.1 -71.93% vs control	<0.001
End-diastolic pressure of LV, mm Hg	2.2±0.15	3.9±0.19 +78% vs control	<0.001
Protodiastolic pressure of LV, mm Hg	0.22±0.02	0.48±0.04 +119% vs control	<0.01
-dP/dT max, mm Hg/sec	7966±188	5356±149 -32.77% vs control	<0.05

The pumping function of LV appreciated after the aortic jet had a decline of 87.5% (in the control group – 73.5%) reaching an average value of 1.6 ml/min that is 71.93% lower than control index (5.7±0.3 ml/min). The end-diastolic pressure decreased intelligibly in both groups, but its value remaining significantly higher in the group with Dx, the difference being 78%. The protodiastolic pressure decreased simultaneously with the decreasing of filling pressure of the left atrium, the smaller rebound being characteristic for Dx cardiotoxicity (68.83 vs 71.8%), which indicates a weaker systole compared to the control group.

In the sample with maximum LAFP (25 cmH2O) the LV filling is facilitated due to an artificial gradient installed between the left atrium and the left ventricle. However, the reduced diastolic compliance of the myocardium in the group with Dx conditioned a higher elevation of LVEDP compared to the control pattern: 5.6 vs 2.1 mm Hg (tab. 4).

As a consequence, the value of LVEDP in this volume stress test became 184% higher than the index in the control group.

The reduced diastolic compliance was manifested by increasing the value of diastolic stiffness by 129%, this being higher than the enhancement attested to the perfusion of isolated heart in physiological regime (109.73%). Limiting the filling of the LV cavity due to increased diastolic stiffness of the myocardium led to lower values of the pump function indices. The aortic jet, for example, was found to be reduced by 42.6%, and stroke volume – by 25%.

Table 4. Values of functional indices of the heart isolated at the maximum level of LAFP

Indices	Groups		p
	Control (n=9)	Dx (n=9)	
Aortic jet velocity, ml/min	31.7±2.5	18.2±1.4 -42.6% vs control	<0.001
Stroke volume, ml	0.2±0.009	0.15±0.009 -25% vs control	<0.01
End-diastolic pressure of LV, mm Hg	6.8±0.52	19.3±1.83 +184% vs control	<0.001
Diastolic stiffness, mm Hg/ml	31.86±1.8	72.95±4.5 +129% vs control	<0.001
-dP/dT max, mm Hg/sec	7718±156	5296±141 -31.39% vs control	<0.05

Evidence of impaired diastolic relaxation at Dx administration was also confirmed on the retrograde perfusion model without recirculation of the isolated heart (Langedorff method, to unify the initial values of LVEDP) in the conditions of increased filling (tab. 5).

The impact of the gradual increasing of LV filling volume on LVEDP hemodynamics is different between batches, taking into account that initial volume was similar (0.2 ml) and LVEDP was calibrated at 14 mm Hg level. In the control group, the elevation of LVEDP, in conditions of increasing LV volume from 0.2 up to 0.4 ml, was found in a range of 7-51%. The cardiotoxicity of doxorubicin increased the LVEDP to 95.8%, which led to a significant 30% lag of the index at the end of the test: 27.4±1.6 vs 21.2±1.2 mm Hg.

Due to a higher elevation of the end-diastolic pressure of LV in the group with Dx, a more conclusive depreciation of coronary flow was attested. Compared to the control prototype, the decline of coronary flow in conditions of doubling the filling volume of LV was with 46% higher (45.33 vs 31.21%), and the final rebound of absolute value of the CF (coronary flow) was 21.65% (7.6±0.5 vs 9.7±0.3 ml/min).

Table 5. The hemodynamics of end-diastolic pressure and coronary flow in condition of gradual increasing of left ventricular filling volume

Indices	Groups	Initial	Left ventricular filling volume of isovolumic heart			
		LVFV =0.20 ml	0.25 ml	0.30 ml	0.35 ml	0.40 ml
LVEDP mm Hg	Control, n=9	14.0±0.3	15.1±0.7 +7%	16.4±0.9 * +17%	17.8±1.1 * +27%	21.2±1.2 ** +51%
	Dx, n=8	14.0±0.3	15.7±0.7 +12%	17.2±1.1 * +22%	20.5±1.3 * +46%	27.4±1.6 ** +95.8% p<0.05
CF, ml/min	Control, n=9	14.1±0.4	13.7±0.4 -3%	12.4±0.3 * -13%	11.0±0.4 ** -22%	9.7±0.3 ** -31.21%
	Dx, n=8	13.9±0.5	13.1±0.4 -6%	11.2±0.3 * -20%	9.3±0.4 -34% p<0.05	7.6±0.5 ** -45.33% p<0.05

Note: LVFV – left ventricular filling volume; CF – coronary flow; +/-% – relative deviations of the indices compared to their initial value; p – the significance of the discrepancy versus the respective index; * – p<0.05 versus initial value;
** – p <0.01 versus initial value.

An important intrinsic factor that influences the functionality of diastolic relaxation is the pressure of the coronary perfusion, which on the retrograde perfusion model of the isolated isovolumic heart is dependent on the perfusion volume of the coronary system. In this context, there was estimated the hemodynamics of LVEDP modification in conditions of increasing the pressure in the aortic estuary from 80 up to 120 cmH2O (tab. 6).

Table 6. The LVEDP hemodynamics in conditions of increased coronary pressure of the isolated isovolumic heart

Groups	Coronary perfusion pressure of the isolated isovolumic heart (cmH_2O)		
	80	100	120
Control (n=9)	14 mm Hg	15.6±1.2	16.3±1.4
Dx (n=9)	14 mm Hg	18.3±1.3 +18% vs control	19.8±1.5 +22% vs control
p		<0.05	<0.05

It should be mentioned that LVEDP was measured at the level of the initial or basal aortic pressure (80 cmH2O) equal in both batches with 14 mmHg, by the latex balloon placed in the cavity of the left ventricle. The progression of coronary hypertension has led in both groups to a natural effect – the elevation of LVEDP. But, in the group with Dx, the increase of the end-diastolic pressure of LV was much more pronounced. At the level of aortic pressure of 100 cmH2O, the LVEDP value increased by 31% compared to the control increment of 12%. At the maximum level of coronary pressure (120 cmH2O) the enhancement of LVEDP was 42%, which is 147% higher than the control increment equal to 17%. As a consequence at both levels of coronary blood pressure LVEDP in doxorubicin disorder of the heart was significantly higher compared to the control pattern by 18% and 22%, respectively.

The lusitrop function of the heart is influenced by several neuroendocrine factors (e.g., ET-1, catecholamines, angiotensin II), which normally had a positive inotropic effect due to the increased calcium concentration in the sarcoplasm. In order to estimate the character of the diastole change under the action of ET-1 and excess calcium, was appreciated the basal value, as well as the value of LVEDP, as well as the index value at the peak of stimulation of the isolated heart perfused in working regime (tab. 7).

Table 7. Modification of LVEDP to the action of ET-1 and excess of calcium

Groups	ET-1(10^{-7} M)		Calcium (3.0 mM)	
	Initial	Peak	Initial	Peak
Control (n=9)	4.9±0.29	6.1±0.55 +25% vs initial	4.8±0.29	6.9±0.61 +44% vs initial
Dx (n=9)	12.5±0.87	17.7±1.26 +42% vs initial	12.4±0.87	21.2±1.87 +71% vs initial
p (vs control)	<0.001	<0.001	<0.001	<0.001

The action of both factors on the isolated heart was imposed by elevating LVEDP, both in the control group and in the group with Dx, the more conclusive effect being characteristic for the excess of calcium. Remarkably, that enhancement of LVEDP was significantly higher in the doxorubicin group. Thus, under the action of ET-1 the rate of LVEDP elevation was imposed by the ratio of 42 vs 25%, and under the action of calcium excess (increasing the concentration of Ca^{2+} in perfusion from 2.5 up to 3.0 mM) – 71 vs 44%.

As a result of these quantitatively distinct changes, the cardiotoxicity of Dx was imposed by a clearly higher stimulation of LVEDP than the control, by 191% under the action of ET-1 and by 208% under the action of calcium excess. These differences are obviously larger compared to the initially estimated patterns, so till stimulation of isolated heart.

A cardinal factor that detrimentally influences the diastolic functionality is myocardial ATP deficiency resulting in the progressive accumulation of calcium in the cardiomyocyte during diastole relaxation due to the incompetence of ionic pumps, primarily of the ATP-ase of sarcoplasmic reticulum (SERCA2a). This phenomenon affects the dissociation of actin-myosin complex and led to development of diastolic stiffness, which to a severe degree is noted as diastolic contracture. Because myocardial ischemia represents a major condition of impaired bioenergy synthesis and calcium metabolism, the severity of its impact on the LVEDP hemodynamics and capacity of lusitrope to restore function during reperfusion was estimated (tab. 8).

It should be mentioned that in both groups the basal value of LVEDP, until the action of ischemia, was installed at equal levels: 14 ± 0.1 mm Hg. According to the estimated hemodynamics of LVEDP, cardiac doxorubicin disorder was manifested by a considerable reduction in myocardial tolerance to ischemic impact. Thus, LVEDP has increased during ischemia period of 30 min to the average value of 63.7 mm

Table 8. The LVEDP hemodynamics during ischemia and isovolumic isolated heart reperfusion

Groups	Ischemia (min)				Reperfusion (min)			
	5	10	20	30	5	10	30	45
Control (n=9)	16.3±0.8	27.2±1.7	36.2±1.9	41.7±2.3	36.3±2.5	27.9±2.3	20.7±1.8	14.9±1.1
Dx (n=9)	21.5±1.4	38.8±2.1	55.6±3.6	63.7±4.4	58.2±4.3	46.5±3.3	30.6±2.5	22.8±1.4
p	<0.05	<0.05	<0.01	<0.01	<0.05	<0.05	<0.05	<0.01

Hg, and in the control group the index reached 41.7 ± 2.3 mm Hg, the final difference being 53%. Moreover, the quality of LVEDP recovery in the Dx group was much poorer. If in the control group the index at 45 minutes of reperfusion practically reached the basal level (14.9±1.1 mm Hg), then in the group with Dx it remains clearly higher (22.8±1.4 mm Hg), the difference remaining at the levels of the final period of ischemia, 53%.

Discussion

The researchers *in vitro* on isovolumic isolated and working heart perfusion models (Langedorff and Neely-Rovetto methods) have demonstrated that the cardiotoxicity of doxorubicin is imposed by significantly affecting diastolic relaxation. Already in physiological hemodynamic regime, LVEDP values and diastolic stiffness were considerably increased more than double (109-168%) compared to the control group indices. Increased diastolic stiffness was associated with a significant 24.8% reduction in isovolumic relaxation rate (-dP/dTmax).

The diastolic disorder in the Dx group is one of the main causes of impaired pump function of the left ventricle, manifested by a 36.9% decline in cardiac output, as worsening lusitrop function indices in volume effort tests were accompanied by increased rebound of the pump function parameters. In conditions of reducing the venous return to the heart to the minimal value (5 cmH2O) the difference of the - dP/dTmax index compared to the control increased by 32%, and the aortic jet rebound increased by 78%. At maximum LV filling the increased diastolic stiffness of the myocardium led to elevation of LVEDP by about 2.3 times compared to the control, and the hemodynamics of the volume-pressure relationship gradually reproduced on the isovolumic isolated heart perfusion model has the pattern of an upward curve compared to the control prototype. The increased diastolic stiffness is conceptually viewed as an intrinsic factor of increasing myocardial mechanical stress that limits coronary perfusion especially in the subendocardial layer [6, 7].

In this study, the doubling of the filling volume of the isolated isovolumic heart was manifested in the Dx disorder of the myocardium by decreasing the coronary flow more accentuated compared to the control pattern by 46%, which correlated with an end-diastolic pressure of LV well above the control level. Impairment of coronary perfusion is an important pathogenetic factor in the disorder of the heart's adaptation to overload with volume and resistance, especially when myocardial dysfunction is directly related to impaired endothelium-dependent vascular relaxation, which can be compensated by the feasibility of the Vanhoutte phenomenon based on repolarization mechanism of vascular muscle media [8].

It should be mentioned that in the interface of dependence the coronary perfusion on the diastolic myocardial rigidity, it is also valid the inverse relation: the coronary flow influences the diastolic relaxation. In the presented research this aspect was confirmed. Thus, the increase of the retrograde perfusion pressure of the isolated isovolumic heart by 50% led to the elevation of LVEDP, while in the group with Dx the index increase was 147% higher compared to the control increase.

One of the mechanisms highlighted in this study on the impairment of diastolic relaxation in the repeated administration of Dx is the incompetence of the control system of calcium ion turnover in the cardiomyocyte during the systole-diastole cycle. The increasing calcium concentration in perfusion by 0.5 mM led to a more conclusive elevation of the LVEDP value by 62% compared to the rate of increase of the control index, which indicates the dysfunction of the calcium pumps, first of all the ATP-ases of the sarcoplasmic reticulum (SERCA2a) together with myocardial energy deficit. A similar phenomenon was observed in the isolated heart stimulation with ET-1, which increases the influx of calcium following the activation of ETA receptors. The diastolic disorder at the peak of stimulation of the isolated heart perfused in working regime with ET-1 was imposed by negative inotropic effect, manifested by decreased LV systolic pressure and cardiac output, evidences associated with a more pronounced elevation of LVEDP [9].

The role of excess calcium in diastolic disorder under the cardiotoxic impact of Dx was confirmed in a previous study, in which the chronic or acute action of taurine (natural modulator of calcium metabolism and bioenergetics) significantly improved the lusitrop function of the heart as well as parameters of LV pump function [10]. The similar results were obtained by other authors, who demonstrated the benefit of taurine administered for 14 days in the model of Dx myocardial disorder in rats on echocardiographic indicators, the first on the reduction of LV end-diastolic volume as a true sign of diastole improvement [11]. Taurine also has a remarkable antioxidant effect, and Dx cardiotoxicity, on the contrary, is marked by a prooxidant effect and, respectively, the activation of oxidative stress [12].

The diastolic disorder in the group with Dx was characterized by a more pronounced ischemic contracture vs control. Moreover, during the reperfusion period, the restoration of the lusitrop function of the heart in the group with Dx was frankly compromised, so that LVEDP remains 53% above the control index. The pathophysiological substrate of the ischemia-reperfusion impact is determined by the rapid energetic decline, the accumulation of calcium in the heart cells, as well as the excess of oxygen free radicals. These pathogenetic mechanisms are the main elements of the pathophysiological interface regarding the disturbance of diastolic relaxation and the evolution of doxorubicin heart failure.

The impairment of diastole under the cardiotoxic action of Dx may also be related to the detrimental action of anthracycline on the extracellular matrix. In the context of the latest literature data is to be highlighted the effect of Dx to induce heart cell death by apoptosis, pyroptosis and ferroptosis [13].

Pyroptosis is triggered by the inflammatory response of the myocardium induced by Dx, and the increased expression of proinflammatory cytokines and chemokines remains during the cessation of its action. The inflammatory responses together with oxygen free radicals influence the extracellular matrix by activating metalloproteinases, fibroblasts and

interstitial macrophages. As a result, the extracellular matrix increases due to fibrillar collagen type I and III, as well as degraded collagen. These changes in the interstitium obviously decrease the diastolic compliance of the myocardium caused by reducing cardiomyocyte counts.

Ferroptosis under the action of Dx is mediated by the accumulation of iron ions in the mitochondria of the cardiomyocyte, which has a negative effect on the synthesis control of ATP molecules and oxygen free radicals, which justifies this phenomenon as a mechanism responsible for Dx cardiotoxicity [14], as well as a heart protection target in cancer patients exposed to the anthracyclines action [15].

An important aspect of the clinical applicability of the data obtained in cardiology-oncology is the proof of the key role of isovolumic diastolic relaxation disorder of the heart in disturbance of the LV pump function, and on the other hand elucidating the mechanism of early growth of the Tei index in cardiac patients exposed to cardiotoxic effect of anthracyclines [16]. Therefore, the increase of the Tei index values as an early predictor of cardiac dysfunction under the action of Dx is based primarily on increasing the time of isovolumic relaxation of the heart resulting in reduced ejection time of the left ventricle.

Conclusions

1. Impairment of diastolic relaxation inherent to Dx cardiotoxicity is manifested mainly by significant increase of LVEDP and diastolic stiffness in association with reducing the isovolumic diastolic relaxation velocity.

2. Diastolic disorders compromise the volume-pressure relationship of LV, the adaptation of the heart to effort with volume and become more pronounced during the action of calcium excess and ET-1.

References

1. Nishimura RA, Borlaug BA. Diastology for the clinician. J Cardiol. 2019;73(6):445-452. doi: 10.1016/j.jjcc.2019.03.002.
2. Kim IC, Hong GR. Intraventricular flow: more than pretty pictures. Heart Fail Clin. 2019;15(2):257-265. doi: 10.1016/j.hfc.2018.12.005.
3. Gillebert TC, De Pauw M, Timmermans F. Echo-Doppler assessment of diastole: flow, function, and haemodynamics. Heart. 2013;99(1):55-64. doi: 10.1136/heartjnl-2012-301961.
4. Nagueh SF. Left ventricular diastolic function: understanding pathophysiology, diagnosis, and prognosis with echocardiography. J Am Coll Cardiovasc Imaging. 2020;13(1 Pt 2):228-244. doi: 10.1016/j.jcmg.2018.10.038.
5. Argun M, Uzum K, Sonmez M, et al. Cardioprotective effect of metformin against doxorubicin cardiotoxicity in rats. Anatol J Cardiol. 2016;16(4):234-241. doi: 10.5152/akd.2015.6185.
6. Popovici M, Cobeț V, Popovici I, et al. Perfuzia coronariană și stresul miocardic: relații reciproce [Coronary perfusion and myocardial stress: reciprocal relation]. Buletinul Academiei de Științe a Moldovei. Științe Medicale. 2008;4(18):23-32. Romanian.
7. Galderisi M, Cicala S, De Simone L, et al. Impact of myocardial diastolic dysfunction on coronary flow reserve in hypertensive patients with left ventricular hypertrophy. Ital Heart J. 2001;2(9):677-684.
8. Tacu L, Ivanov M, Cobet V, et al. Vanhoutte and Bowditch phenomena in heart failure: the relation with ischemia-reperfusion impact. Eur J Heart Fail. 2018;20(Suppl 1):335.
9. Cobeț V. Reglarea tonusului coronarian prin hiperpolarizare: mecanism compensator în disfuncția endotelială [The regulation of coronary tonus by hyperpolarisation: compensatory mechanism in endothelial dysfunction]. Buletinul Academiei de Științe a Moldovei. Științe Medicale. 2010;2(25):65-72. Romanian.
10. Tacu L, Cobeț V. Efectele endotelinei 1 în afecțiunea doxorubicinică a cordului [The endothelin-1 effects in doxorubicin disorder of the heart]. Buletinul Academiei de Științe a Moldovei. Științe Medicale. 2020;1(65):145-151. Romanian.
11. Cobet V, Tacu L, Hajawi O, et al. Taurine blunts the doxorubicin cardiotoxicity: chronic and acute effects. EP Europace. 2021;23(Suppl 3):iii585. https://doi.org/10.1093/europace/euab116.533.
12. Baris VO, Gedikl E, Yersal N, et al. Protective effect of taurine against doxorubicin-induced cardiotoxicity in rats: echocardiographical and histological findings. Amino Acids. 2019;51(10-12):1649-1655. doi: 10.1007/s00726-019-02801-7.
13. Wang Y, Ren K, Lu Q, et al. Inhibiting effects of taurine on doxorubicin-induced oxidative stress in rats. Adv Mater Res. 2014;912-914:214-216. https://doi.org/10.4028/www.scientific.net/AMR.912-914.214.
14. Christidi E, Brunham LR. Regulated cell death pathways in doxorubicin-induced cardiotoxicity. Cell Death Dis. 2021;12(4):339. https://doi.org/10.1038/s41419-021-03614-x.
15. Tadokoro T, Ikeda M, Ide T, et al. Mitochondria-dependent ferroptosis plays a pivotal role in doxorubicin cardiotoxicity. JCI Insight. 2020;5(9):e132747. doi: 10.1172/jci.insight.132747.
16. Fang X, Wang H, Han D, et al. Ferroptosis as a target for protection against cardiomyopathy. Proc Natl Acad Sci USA. 2019;116(7):2672-2680. doi: 10.1073/pnas.1821022116.

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Authors' contributions

LT interpreted the data and performed the analytical part of the laboratory work, drafted the first manuscript; VC conceptualized the project, designed the research and revised the manuscript critically.

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Ethics approval and consent to participate

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Conflict of Interests

No competing interests were disclosed.

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The general condition of patients requiring the alveolar bone crest reconstruction

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Abstract

Objectives: To present the survey questionnaire used for the medical assessment of patients requiring alveolar ridge reconstruction as well as the study of concomitant pathologies and their influence on the healing process.
Material and methods: The study involved 173 patients aged between 18 and 69 years. All patients have been assessed according to the survey questionnaire developed to determine the patient's general condition, life anamnesis, and medical history.
Results: Out of the total of 173 patients, 72 (41.6%) had no concomitant pathologies, 8 patients (4.6%) had concomitant pathologies that did not interfere with the operation, 40 (23.1%) had pathologies that might affect the outcome of alveolar ridge reconstruction but still were admitted to surgery, and 53 patients (30.6%) had pathologies that could affect the outcome of the reconstruction procedure and were not admitted to bone grafting.
Conclusions: Assessment of patients with alveolar ridge defects during preparation and planning of preimplantation bone reconstruction identified a number of concomitant pathologies, more or less noticed by the patient, which may remain unclear due to superficial study of the patient's condition that is able to influence the surgical treatment outcome. These issues can be both intraoperative and postoperative and lead to failure of surgical treatment and further reoperation.
Key words: alveolar ridge reconstruction, systemic conditions.

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Introduction

In our days, implant treatment of toothless is the most elected method. Chele et al. propose the immediate post extraction implant placement [1], however, in cases of alveolar ridge deficiency additional bone growth is needed. In 2016, Knofler W. et al. showed that bone growth procedures associated with dental implants are required in 58.2% of cases [2]. Postoperative healing after bone grafting is directly influenced by the general condition of the body. Thus, in addition to the thorough study of the affected area, the assessment of the patient's general condition and concomitant diseases are equally important for the rehabilitation of edentulous patients with severe mandibular atrophy.

A multitude of alveolar grafting methods are proposed in preimplantation. In literature, there are studies on guided bone regeneration, which is the most widely used method of alveolar grafting showing high efficacy [3].

Guided bone regeneration (GBR) is an extremely delicate procedure that relies on body's ability of self-healing. Therefore, patients with poor healing potential cannot undergo GBR procedures. Systemic conditions that may impede the patient to undergo surgery include a number of general medical conditions such as: uncontrolled diabetes mellitus, tumors, recent radiation of the head and neck region, acquired immunodeficiency syndrome (AIDS) or other conditions that cause immunosuppression, decompensated systemic conditions, cardio-vascular diseases, etc. [4]. Cardiovascular diseases are systemic conditions that highly affect tissue regeneration as they disrupt the nutrition and oxygenation of tissues through vascular damage. These types of diseases are extensively evaluated while preparing patients for preimplantation. Cardiovascular diseases are among the most common systemic conditions. Patients with cardiovascular diseases may develop medical conditions such as: congestive heart failure, angina pectoris, myocardial infarction, cardiac arrhythmia, heart valve prosthesis, pacemaker, hypertension, anticoagulant addiction, and post-extraction dental hemorrhage (PDH) [5].

Patients may also suffer from general diseases and conditions such as: infectious endocarditis, diabetes mellitus, osteoporosis, hormonal medication, radiotherapy, gastroesophageal reflux disease (GERD), systemic lupus erythematosus, HIV infection, smoking status, and stress. All these diseases may have a different impact on alveolar ridge reconstruction. Often, because of the focus on local diseases doctors neglect the assessment of body's general condition. A general pathology may go unnoticed if the patient does not communicate about it. All causes mentioned above, as well as superficial examination of the patient can generate poor

results and failure of clinical outcomes. Thus, it is imperative to detect concomitant pathologies and study their impact on alveolar ridge reconstruction.

This study is to assess the general medical condition of patients requiring alveolar ridge reconstruction. To study the concomitant pathologies and their influence on the healing process after bone grafting.

Material and methods

The study took place between 2016 and 2020. There were assessed 173 patients aged between 18 and 69 years and the mean age constituted 46.7±0.3 years (84 men and 89 women).

All patients have been assessed according to a survey questionnaire that was developed to determine the patient's general condition, life anamnesis, and medical history. The first part contains information about the patient's personal data. The second part is filled in by the doctor and contains information about oral hygiene, smoking status, smile line and aesthetic expectations of the patient. The third part of the survey questionnaire contains a set of questions to which the patient answers by ticking the options, if necessary; additionally it states information about the disease.

List of patient's questions:

- Do you suffer/ have you suffered from any acute or chronic diseases?
- Do you suffer/ have you suffered from: disorders of the immune system; allergies or drug/nonmedicinal intolerances; hypertension; vascular diseases; heart disease; respiratory diseases; gastrointestinal diseases; hepatobiliary diseases; kidney and/or urinary diseases; neurological diseases; mental illness; eye diseases; hematological diseases; endocrine diseases; diseases of the skeleton; diseases of the skin and mucous membranes; tumors; obstructive sleep apnea?
- Are you following any treatment (medicinal, homeopathic, phytotherapy)?
- Have you been on any treatment with: antibiotic in the last month, anticoagulants, or bisphosphonates?
- Are you having difficulty breathing?
- Do you have / have you had any vicious habits?
- Have you undergone any surgery in your lifetime?
- Have you had dental treatment before?
- Have accidents/incidents or complications occurred in previous dental treatments?
- Have you ever donated blood?
- Are you pregnant?

In the study all patients have had severe alveolar ridge atrophy requiring reconstruction and further implant-prosthetic rehabilitation. By using the survey questionnaire, the presence or absence of general pathologies was evaluated and some of the patients were accepted for bone grafting. Other patients were not engaged in bone reconstruction surgery because of concomitant pathologies occurrence associated with absolute contraindications. The selected patients were assessed according to evaluating postoperative healing parameters at 7 days, 21 days, and 6 months (implantation stage). The influence of general pathology on healing process was also taken into account. The healing parameters were: wound dehiscence, mucosal erosion, and graft exposure. These parameters have been analyzed after clinical and paraclinical aspects by using pictures. The data obtained was entered into a Microsoft Excel table and analyzed statistically using IBM SPSS Statistics 22.

Results and discussion

Out of the total 173 patients, 72 (41.6%) had no concomitant pathologies, 8 patients (4.6%) had concomitant pathologies that did not interfere with the operation, 40 (23.1%) had pathologies that could affect the outcome of alveolar ridge reconstruction but still were admitted to intervention, and 53 patients (30.6%) had pathologies that could affect the outcome of the reconstruction and were not admitted to bone grafting. 53 (100%) patients who were not admitted to bone reconstruction had the following general pathologies and vices (tab.1.): osteoporosis treated with intravenous bisphosphonates (3-5.7%), cardiopathies or angiopathies treated with oral anticoagulants (7-13.2%), compensated or decompensated diabetes mellitus (14-26.4%), long-term treatment of pemphigus with corticosteroids that decreases osteogenesis and creates immunosuppression (1-1.9%), long-term treatment of osteoarthritis with corticosteroids that decreases osteogenesis and creates immunosuppression (2-3.8%), radiotherapy performed no less than 12 months after the last cure (5-9.3%), chemotherapy performed no less than 6 months after the last cure (3-5.7%), rheumatoid arthritis treated with cytostatic (7-13.2%), hemophilia (2-3.8%), non-compliant heavy smokers (more than 10 cigarettes/day) (8-15.1%), after use of intravenous phosphate drugs (current consumption in suspension) (1-1.9%).

Table 1. Patients with general pathologies and unaccepted vices for bone reconstruction

General pathologies and vices	Patient No	Percentage
Osteoporosis treated with intravenous bisphosphonates	3	5.7%
Cardiopathies or angiopathies treated with oral anticoagulants	7	13.2%
Compensated or decompensated diabetes mellitus	14	26.4%
Long-term treatment of pemphigus with corticosteroids	1	1.9%
Long-term treatment of osteoarthritis with corticosteroids	2	3.8%
Radiotherapy /no less than 12 months after the last cure	5	9.3%
Chemotherapy /no less than 6 months after the last cure	3	5.7%
Rheumatoid arthritis treated with cytostatic	7	13.2%
Hemophilia	2	3.8%
Non-compliant heavy smokers	8	15.1%
After use of intravenous phosphate drugs	1	1.9%
Total	53 patients	100%

40 patients (100%) had pathologies and vices that could affect the outcome of alveolar ridge reconstruction but still were admitted to intervention (tab. 2). These pathologies are: hyperparathyroidism (osteoporosis) (1-2.5%), hypothyroidism (osteoporosis) (4-10%), Crohn's disease (osteoporosis caused by malabsorption of D2 and calcium in the intestine) (2-5%), gastroesophageal reflux disease (5-12.5%), compensated diabetes (18-45%), and light smokers (up to 10 cig/day) (10-25%).

Table 2. Patients with general pathologies and vices admitted to bone reconstruction

General pathologies and vices	Patient No	Percentage	Local complications	
			Wound dehiscence	Erosion of the mucosa with graft exposure
Hyperparathyroidism	1	2.5%	0 – 0%	1 – 2.5%
Hypothyroidism	4	10%	1 – 2.5%	0 – 0%
Crohn's disease	2	5%	0 – 0%	0 – 0%
Gastrointestinal reflux disease	5	12.5%	2 – 5%	1 – 2.5%
Compensated diabetes	18	45%	6 – 15%	1 – 2.5%
Light smokers	10	25%	3 – 7.5%	1 – 2.5%
Total	40	100%	12 – 30%	4 – 10%

Other 8 (100%) patients had the following concomitant pathologies that did not interfere with alveolar ridge reconstruction (tab. 3): drug allergy (3-37.5%), hepatitis B infection (2-25%), chronic bronchitis (2-25%), and renal lithiasis (1-12.5%).

Table 3. Patients with concomitant pathologies that do not interfere with bone reconstruction surgery

General pathologies	Patient No	Percentage	Local complications	
			Wound dehiscence	Erosion of the mucosa with graft exposure
Drug allergy	3 – 37.5%	37.5%	0	0
Hepatitis B	2 – 25%	25%	0	0
Chronic bronchitis	2 – 25%	25%	0	0
Renal lithiasis	1 – 12.5%	12.5%	0	0
Total	**8 patients**	**100%**	**0%**	**0%**

The majority of patients (72) admitted to surgery did not have concomitant pathologies or vices (tab. 4).

Table 4. Patients without concomitant pathologies and complications admitted to bone reconstruction

General pathologies	Patient No	Percentage	Local complications	
			Wound dehiscence	Erosion of the mucosa with graft exposure
No pathologies	72	100%	1 – 1.4%	5 – 7%

The presence of complications was studied in patients who underwent alveolar ridge reconstruction. Concomitant pathology which is influencing the outcome of alveolar ridge reconstruction can lead to high rate of exacerbations. Thus, out of 72 patients (100%), 1 patient (1.4%) had wound dehiscence and 5 patients (7%) had mucosal erosion with graft exposure. All these patients, who experienced complications were completely healthy without concomitant pathologies. Patients with general pathologies that did not influence bone reconstruction (8 patients) had no postoperative complications. 40 (100%) patients who had pathologies that could affect the outcome of alveolar ridge reconstruction showed wound dehiscence in 12 cases (30%) and erosion of the mucosa with graft exposure in 4 cases (10%).

The positive outcome of bone reconstruction surgery is closely related to bone structure characteristics. Bone tissue is a dynamic highly organized structure that can be remodeled according to mechanical stress and hormonal activity. The resorbed bone is replaced by the forming cells and the bone neoformation, which lasts approximately 3 months [6]. Another component of the positive outcome of bone reconstruction procedures is related to body condition when various intra- and postoperative complications may occur but they are not directly related to bone healing. These conditions and their management are described below [7].

Some cardiovascular pathologies are treated with anticoagulant medication. These can lead to immediate and delayed complications with harmful consequences or alveolar ridge reconstruction failure. Zanoaga O. et al. showed that dental extractions can be carried out without cancelling the administration of antithrombotic drugs [8]. Two classes of new oral anticoagulants are currently available for this purpose: thrombin inhibitors (dabigatran) and factor Xa inhibitors (rivaroxaban, apixaban, edoxaban). Unlike vitamin K inhibitors (acenocoumarol, warfarin), which block the formation of several active vitamin K-dependent factors (factors II, VII, IX and X), new oral anticoagulants block the activity of a single factor in the blood clotting cascade [8]. These new oral anticoagulants are: Dabigatran etexilate (brand name Pradaxa), Rivaroxaban (brand name Xarelto), Apixaban (brand name Eliquis), and Edoxaban (brand name Lixiana).

Cardiac pathology and increased risk of endocarditis should be considered in the oral surgical patient. Patients with valvular prostheses, history of infectious endocarditis, as well as those with cyanogenic congenital heart disease may develop infectious endocarditis, which remains a severe form of valvular dysfunction associated with poor prognosis and high mortality. In 2018, Zanoaga O. showed that dentoalveolar surgery requires prophylaxis of the infective endocarditis [9].

Glucose metabolism disease influences the tissue healing. Uncontrolled diabetes was associated with greater variability and increased rate of infectious complications in alveolar ridge reconstructions; insulin-mediated metabolic control can reverse these adverse effects [10].

The high rate of complications, such as resorption, non-

integration and delayed healing of bone graft, especially in the upper jaw, can occur in patients with osteoporosis. Generally, osteoporosis is not a contraindication for bone augmentation and placement of dental implants. Although, an increase of risk factors and complications can be expected in patients with osteoporosis [11]. A number of therapeutic approaches have been proposed to accelerate bone healing and prevent complications in bone grafting. These treatments include bisphosphonates, hormone replacement therapy (HRT), calcitonin, diet high in vitamins and calcium supplements [12]. The cornerstone of osteoporosis prevention and treatment is the provision of adequate intake of calcium, vitamin D and weight-bearing exercises. It is known that mechanical demand produces an increase of the cellular metabolism and collagen synthesis. There are studies showing that physical exercises are beneficial for preventing bone loss in postmenopausal women. Smoking is a risk factor for osteoporosis and is also associated with high rate of implant failure [13]. A combination of osteoporosis and tobacco intake leads to a high complication rate, thus, quitting smoking is imperative for a positive outcome of bone reconstruction in patients with osteoporosis. Other risk factors for osteoporosis are the use of corticosteroids and increased consumption of alcohol and caffeine. Patients with osteoporosis should be encouraged to reduce alcohol and caffeine intake before dental implant surgery or bone grafting.

Currently, it is unknown if the use of systemic bisphosphonates for the treatment of osteoporosis influences directly or indirectly the capacity of guided bone regeneration (GBR) techniques [14]. Bisphosphonates are the synthetic analogues of inorganic pyrophosphate. The most common oral drugs used in the treatment of osteoporosis are: alendronate, risedronate and ibandronate. They have been shown to reduce osteoclast activity and therefore bone resorption [15]. Recently, bisphosphonates have been associated with jaw osteonecrosis. Most cases of necrosis are, however, associated with intravenous administration of high doses of pamidronate and zoledronate, which are commonly used for bone metastases treatment in multiple myeloma and breast cancer. Treatment with oral bisphosphonates may be considered as risk factor for osteonecrosis [16].

In 2008, Radzichevici M. showed that the excess of phosphorus accumulates in tissues. Phosphorus binds with calcium and is retained in the bone in large quantities sclerosing and destroying bone vascular and nerve endings. Thus, the oral cavity appears to be "the gate of infection" that communicates with the jaw bones through the periodontium [17]. In this way the mechanism of overinfection of jaw bones can be explained. The high risk of failure or occurrence of postreconstruction osteonecrosis in patients who administer or have administered narcotic phosphorus substances emphasizes the importance of a full preoperative assessment.

Long-term administration of corticosteroids within systemic pathologies has negative impact on the body, i.e. on marginal periodontium and alveolar ridge reconstruction. Corticosteroid therapy has an adverse effect on the bone system and can induce osteoporosis [18]. Regarding the alveolar ridge reconstruction in long-term corticosteroid therapy it is reasonable to assess the risk induced by corticosteroid-induced osteoporosis [19].

After radiotherapy osteoblasts and osteoclasts decrease quantitatively and the terminal differentiation of osteoblasts is accelerated. Data published showed that mesenchymal stem cells in the bone marrow are quantitatively reduced [20]. Vascular sclerosis and fibrosis are characteristic features of radiation injury. Spontaneous bone healing is highly compromised and reduced in irradiated areas and the physiology of bone regeneration is modified as well [21]. One of the most important life-threatening adverse effect is osteoradionecrosis. Ionizing radiation limits the vascularization, increases the incidence of fibrous union, and leads to high morbidity rate. Bone grafts cannot be used when vascular system is in poor condition [22]. Therefore, alveolar ridge reconstruction should be considered from 12 months after the last course of radiotherapy.

Gastric pathologies have a great impact on the oral cavity state, as well they affect the rehabilitation after oral surgery. Gastroesophageal reflux disease GERD constitutes the risk factor for chronic marginal periodontitis; therefore, it can influence the outcome of alveolar ridge reconstruction. The most reasonable explanation is the reduction of salivary gland functioning. Mixed saliva covers all vital internal anatomical surfaces with secretions rich in mucin that provide a diffuse protective barrier against mechanical, thermal, chemical and microbial damage. Saliva also acts as an endogenous antacid agent that acts against symptomatic gastroesophageal reflux disease. Therefore, the decrease of salivary secretion leads to insufficient acid neutralization [23]. Hyposalivation in patients with GERD has been demonstrated in several studies. It was found an association between reduced salivary flow and periodontal disease among the elderly [24]. Therefore, we can conclude that hyposalivation in GERD may have an influence on the development of chronic periodontitis by allowing the proliferation of intraoral bacteria, thus increasing the risk of infection of grafts and materials used in alveolar ridge reconstructions. Therefore, dental surgeons should manage GERD in patients requiring alveolar ridge reconstruction.

In 2016, Adachi et al. showed that the degree of endoscopic atrophy of the gastric mucosa in patients with marginal periodontitis was significantly higher compared to those without periodontitis [25]. Long-term *H. pylori* infection causes not only atrophy of the gastric mucosa, but also marginal periodontitis. Therefore, it is reasonable to treat *H. pylori* before alveolar ridge reconstruction. This measure will help to control periodontopathic bacteria and prevent complications after reconstruction.

HIV-positive patients may be candidates for alveolar bone reconstruction. These immunocompromised patients may be incapable for sustained, controlled and effective immune response to exogenous trauma that constitutes a high risk for developing further postoperative complications. Thus, HIV-positive patients have a high postoperative infection rate after rehabilitation of maxillofacial trauma (11.8%

vs 4.4% HIV-negative) [26]. The hypothesis of a higher risk for HIV-infected patients after oral surgery has been presented [27]. No relationships between complications and virologic or immunological laboratory parameters were found.

A special category of patients requiring alveolar ridge reconstruction are smokers. Although, smokers may not have general pathologies that could influence the outcome of alveolar ridge reconstruction there is a risk associated with smoking consequences on tissues and structures of the oral cavity. Smoking has been associated with increased accumulation of bacterial plaque; high incidence of gingivitis and periodontitis; high rate of tooth loss and increased resorption of the alveolar bone; and poor mucogingival healing after surgery due to frequent occurrence of refractory periodontitis. Several studies have shown that smoking was a risk factor for marginal bone loss or implant failure [28]. Some authors suggest that better results can be achieved in smokers if an aggressive antimicrobial regimen is followed [29], however, the results obtained after keeping this regimen are not as favorable as those obtained in non-smokers.

Particular attention should be paid to patients aged 60 years old or over (geriatrics) while performing alveolar ridge reconstruction. Being a geriatric patient is not a contraindication for implant treatment and alveolar ridge reconstruction. Healthy elderly patients without systemic conditions can have dental implants and there is no evidence that geriatric changes of bone metabolism affect directly the osseointegration. According to Hyo-Jung Lee et al. implant therapy in geriatric patients should not be considered a risk factor [30]. It is imperative to assess the general state of the geriatric patient that is a candidate for oral reconstructive surgery and take a therapeutic decision according to obtained results.

The study allowed us to identify some steps (fig. 1) for the guidance of specialists that have to elect patients with general diseases for alveolar ridge reconstruction [29].

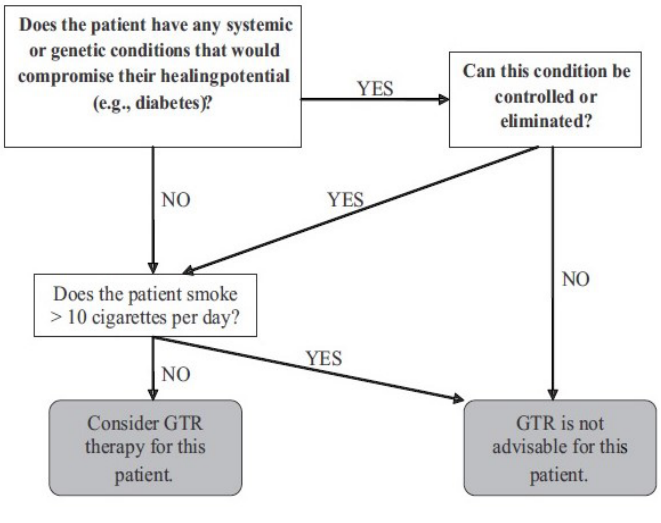

Fig. 1. Decision tree for determining whether the patient is a candidate for GBR, based on their systemic conditions [29]

The fact that 10% of all patients presented concomitant pathologies that can influence the alveolar ridge reconstruction highlights the importance of anamnesis and a thorough preoperative preparation in order to maximize the positive outcome of alveolar ridge reconstruction.

Conclusions

The study has shown the existence of: high rate of postoperative complications after alveolar ridge reconstruction in patients with concomitant pathologies, which are able to influence the healing process; low complication rate or its absence when concomitant pathologies are lacking; pathologies that cannot influence the healing process. Assessment of patients with alveolar ridge defects during preparation and planning of preimplantation bone reconstruction identified a number of concomitant pathologies, more or less noticed by the patient, which may remain unclear due to superficial study of the patient's condition that is able to influence the surgical treatment outcome. These issues can be both intraoperative and postoperative and lead to failure of surgical treatment and further reoperation.

References

1. Chele N. Implantarea dentară imediată: riscuri şi beneficii [Immediate dental implantation: risks and benefits]. Chişinău; 2017. 272 p. Romanian.
2. Knofler W, Barth T, Graul R, Krampe D. Retrospective analysis of 10000 implants from insertion up to 20 years-analysis of implantations using augmentative procedures. Int J Implant Dent. 2016 Dec;2(1):25. doi: 10.1186/s40729-016-0061-3.
3. Jensen SS, Terheyden H. Bone augmentation procedures in localized defects in the alveolar ridge: clinical results with different bone grafts and bone-substitute materials. Int J Oral Maxillofac Implants. 2009;24:218-236.
4. Cooper LF, Masuda T, Yliheikkila PK, Felton DA. Generalizations regarding the process and phenomenon of osseointegration. Part II. *In vitro* studies. Int J Oral Maxillofac Implants. 1998 Mar-Apr;13(2):163-174.
5. Lusk KA, Snoga JL, Benitez RM, Sarbacker GB. Management of direct-acting oral anticoagulants surrounding dental procedures with low-to-moderate risk of bleeding. J Pharm Pract. 2018 Apr;31(2):202-207. doi: 10.1177/0897190017707126.
6. Sîrbu D, Topalo V, Chele N, et al. Regenerarea osoasă în reabilitarea implanto-protetică a pacienţilor cu defecte ale oaselor maxilare [Bone regeneration in the rehabilitation process of patients with defects of the maxillary bones]. Med Stomatol. 2016;1(38):33-42. Romanian.
7. Gheţiu A, Sîrbu D, Chele N, Bran S, Jurjiu V, Nosaci A. Crearea ofertei osoase a maxilarului superior cu atrofie avansată în reabilitarea implanto-protetică prin grefă osoasă autogenă din creasta iliacă [Reconstruction of the upper jaw bone volume with advanced atrophy in the implant-prosthetic rehabilitation by autogenous bone graft from the iliac crest]. Med Stomatol. 2019;4(53):9-18. Romanian.
8. Zănoagă O. Anticoagulantele orale noi în practica medicului stomatolog [New oral anticoagulants in dental practice]. Med Stomatol. 2019;4(53):46-52. Romanian.
9. Zănoagă O, Frăsineanu D, Zgîrcea A, Mostovei A, Hachi G. Antibioticoprofilaxia în stomatologie la pacienţii cu risc sporit de endocardită infecţioasă [Antibiotic prophylaxis in dentistry in patients with an increased risk of infectious endocarditis development]. Med Stomatol. 2018;1(46):45-49. Romanian.
10. Donos N, Dereka X, Mardas N. Experimental models for guided bone regeneration in healthy and medically compromised conditions. Periodontol 2000. 2015 Jun;68(1):99-121. doi: 10.1111/prd.12077.
11. Sîrbu D, Topalo V, Voloc A, Corcimari E, Voloc C. Studiul radiologic al osteoporozei la pacienţii de sex feminin cu reabilitare implanto-protetică

la mandibulă [Radiological study of osteoporosis in female patients with rehabilitation for mandibular implant prosthesis]. Mold J Health Sci. 2018;1(15):44-55. Romanian.
12. Mofid MM, Inoue N, Atabey A, Marti G, Chao EYS, Manson PN, Vander Kolk CA. Callus stimulation in distraction osteogenesis. Plast Reconstr Surg. 2002 Apr;109(5):1621-1629. doi: 10.1097/00006534-200204150-00020.
13. Erdogan O, Shafer DM, Taxel P, Freilich MA. A review of the association between osteoporosis and alveolar ridge augmentation. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007 Dec;104(6):738.e1-738.e13. doi: 10.1016/j.tripleo.2007.04.008.
14. Greenspan SL, Emkey RD, Bone HG, Weiss SR, Bell NH, Downs RW, McKeever C, Miller SS, Davidson M, Bolognese MA, Mulloy AL, Heyden N, Wu M, Kaur A, Lombardi A. Significant differential effects of alendronate, estrogen or combination therapy on the rate of bone loss after discontinuation of treatment of postmenopausal osteoporosis. A randomized, double-blind, placebo-controlled trial. Ann Intern Med. 2002 Dec 3;137(11):875-883. doi: 10.7326/0003-4819-137-11-200212030-00008.
15. Fini M, Giavaresi G, Torricelli P, Borsari V, Giardino R, Nicolini A, Carpi A. Osteoporosis and biomaterial osteointegration. Biomed Pharmacother. 2004 Nov;58(9):487-493. doi: 10.1016/j.biopha.2004.08.016.
16. Marx RE, Sawatari Y, Fortin M, Broumand V. Bisphosphonate-induced exposed bone (osteonecrosis/ osteopetrosis) of the jaws: risk factors, recognition, prevention and treatment. J Oral Maxillofac Surg. 2005 Nov;63(11):1567-1575. doi: 10.1016/j.joms.2005.07.010.
17. Radzichevici M. Osteomielita toxică a maxilarelor, particularităţile clinice şi paraclinice, metode de tratament [Toxic osteomyelitis of the maxilla, clinical and paraclinical features, treatment plan]. Med Stomatol. 2019;4(53):110-114. Romanian.
18. McCabe LR. Understanding the pathology and mechanisms of type I diabetic bone loss. J Cell Biochem. 2007 Dec 15;102(6):1343-1357. doi: 10.1002/jcb.21573.
19. Chatzopoulos GS, Cisneros A, Sanchez M, Wolff LF. Systemic medical conditions and periodontal status in older individuals. Spec Care Dentist. 2018 Nov;38(6):373-381. doi: 10.1111/scd.12319.
20. Gungor T, Hedlund T, Hulth A, Johnell O. The effect of irradiation on osteoclasts with or without transplantation of hematopoietic cells. Acta Orthop Scand. 1982;53(3):333-337. doi: 10.3109/17453678208992225.
21. Lerouxel E, Moreau A, Bouler JM, Giumelli B, Daculsi G, Weiss P, Malard O. Effects of high doses of ionising radiation on bone in rats: a new model for evaluation of bone engineering. Br J Oral Maxillofac Surg. 2009 Dec;47(8):602-607. doi: 10.1016/j.bjoms.2008.12.011.
22. Sciubba JJ, Goldenberg D. Oral complications of radiotherapy. Lancet Oncol. 2006 Feb;7(2):175-183. doi: 10.1016/S1470-2045(06)70580-0.
23. Song JY, Kim HH, Cho EJ, Kim TY. The relationship between gastroesophageal reflux disease and chronic periodontitis. Gut Liver. 2014;8(1):35-40. doi: 10.5009/gnl.2014.8.1.35.
24. Hirotomi T, Yoshihara A, Ogawa H, Ito K, Igarashi A, Miyazaki H. Salivary spinability and periodontal disease progression in an elderly population. Arch Oral Biol. 2008;53(11):1071-76. doi: 10.1016/j.archoralbio.2008.05.009.
25. Adachi K, Mishiro T, Tanaka S, Yoshikawa H, Kinoshita Y. A study on the relationship between reflux esophagitis and periodontitis. Intern Med. 2016;55(18):2523-2528. doi: 10.2169/internalmedicine.55.6898.
26. Martinez-Gimeno C, Acero-Sanz J, Martin-Sastre R, Navarro-Vila C. Maxillofacial trauma: influence of HIV infection. J Craniomaxillofac Surg. 1992 Oct;20(7):297-302. doi: 10.1016/s1010-5182(05)80399-3.
27. Campo J, Cano J, Romero J, Hernando V, Rodriguez C, Bascones A. Oral complication risks after invasive and non-invasive dental procedures in HIV-positive patients. Oral Dis. 2007;13(1):110-116. doi: 10.1111/j.1601-0825.2006.01262.x.
28. Noda K, Arakawa H, Kimura-Ono A, Yamazaki S, Hara ES, Maekawa K, Matsuka Y. A longitudinal retrospective study of the analysis of the risk factors of implant failure by the application of generalized estimating equations. J Prosthodont Res. 2015 Jul;59(3):178-184. doi: 10.1016/j.jpor.2015.04.003.
29. Bashutski J, Oh TJ, Chan HL, Wang HL. Guided tissue regeneration: a decision-making model. J Int Acad Periodontol. 2011 Jul;13(2):48-57.
30. Hyo-Jung L, Young-Kyun K, Jin-Young P, Su-Gwan K, Myung-Jin K, Pil-Young Y. Short-term clinical retrospective study of implants in geriatric patients older than 70 years. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2010 Oct;110(4):442-446. doi: 10.1016/j.tripleo.2010.02.019.

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AG conceptualized the idea, conducted literature review, wrote the manuscript, revised and finalized the text.

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Mast cells in luminal *vs* non-luminal breast cancers

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Abstract

Background: Tumor growth and development is determined by the mutual interaction between the cancer cells themselves and the microenvironment. It contains various elements, including immune cells. Of all, mast cells have one of the most controversial roles. The aim of the present study was to evaluate the expression of mast cell tryptase in the luminal and non-luminal subtypes of breast cancer and establish a possible link between infiltration with MCs and expression of hormone receptors.
Material and methods: The experimental study included 80 cases of breast carcinomas that were analyzed immunohistochemically in order to establish the molecular profile and the expression of tryptase, a specific marker of mast cells. The data were processed using the SPSS program. Pearson's coefficient (r) and the other values were considered statistically significant in case of $p \leq 0.05$.
Results: Both intratumoral mast cells (MCit) and peritumoral mast cells (MCpt) correlated with the expression of hormone receptors for estrogen (ER) and progesterone (PR). Thus, the following relations were established: MCit and ER (r=0.343, p=0.002), MCpt and ER (r=0.394, p=0.000295) and MCpt and PR (r=0.386, p=0.000409). Statistically significant correlations between HER2 expression and mast cells content have not been established.
Conclusions: Mast cells invasion, peri- and intratumoral, is strongly influenced by the expression of hormone receptors. The luminal subtypes of breast cancer are characterized by a higher density of mast cells.
Key words: breast carcinoma, tryptase, mast cells, molecular subtypes

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Introduction

Mast cells (MCs) are bone marrow derived cells characterized by their sensitivity to IgE-dependent stimulation and unique cytoplasmic granule contents, such as histamine, tryptase, chymase. MCs intricately associate with blood vessels and nerves in most vertebrates. They are frequent at sites that interface with the external environment such as the skin, respiratory and gastrointestinal tracts. As front-line cells at the host-environment interfaces, MCs promote host defense against pathogens by facilitating the initiation of appropriate immune responses. MCs are renowned contributors to hypersensitivity reactions and participate in tissue remodeling events and are considered pro-angiogenic, promoting vessel formation through both constitutive and immunologically mediated release of angiogenic substances [1].

The contribution of MCs to tumor development and progression has proven to be a controversial area of research. Clinical studies have suggested a link between elevated IgE or the presence of allergic disease and reduced development of melanoma, breast cancer, and some types of brain tumor [2]. The ability of MCs to promote angiogenesis is viewed as a key process in promoting tumor development [3].

MCs infiltrations have been described in a variety of human cancers, including non-small-cell lung cancer and pulmonary adenocarcinoma [4], breast cancer [5], colorectal cancer [6], and basal cell carcinoma [7]. Several studies have reported links between disease progression and survival and MC density [1, 8]. It has been proposed that MCs could display a protective effect before tumor onset, but sustain its development at later stages [9]. Tryptase, the most abundant secretory granule-derived neutral serine proteinase contained in MCs, can degrade components of the extracellular matrix and has been used as a specific marker for MCs [10]. Tryptase+ MCs are often observed in peritumoral areas in early-stage breast cancers without evidence of degranulation [1]. Historically, MC tryptase is renowned for its pro-tumorigenic role via enhancement of angiogenesis [11].

Some MCs proteases are stored in complexes with heparin. Heparin suppresses proliferation and reduces the number of breast cancer cell colonies. It was hypothesized that heparin might interrupt interactions between tumor-associated fibroblasts and cancer cells, thus impairing tumor development. Some scholars noticed that Mcs are enriched in the tumor bed and invasive margin of late-stage breast cancers, especially in case of luminal subtypes [12]. In breast

cancer, as in cutaneous human tumors, the local impact of MCs on tissue remodeling and cell recruitment events, and their effect on draining lymph nodes/systemic immunity need to both be carefully considered and may not have similar disease impacts [1].

The aim: Evaluation of the expression of mast cell tryptase in the luminal and non-luminal subtypes of breast cancer and establishing a possible link between infiltration with MCs and expression of hormone receptors.

Material and methods

This study included patients with breast carcinomas who were treated by surgery at Arad Clinical Hospital, Romania during 2013-2016. The patients did not undergo chemo- or radiotherapy before surgery.

The histological technique was described in the previous work which also regarded MCs [13]. All samples were routinely processed: fixed in 10% formalin and embedded in paraffin (Paraplast High Melt, Leica Biosystems). The blocks were then used to create tissue microarrays by TMA Grand Master (3DHISTECH Ltd., Budapest, Hungary) and cut into 4-μm-thick sections which were placed on glass slides (Surgipath X-tra Adhesive, Leica Biosystems, Newcastle UponTyne, UK). The sections were automatically colored using Mayer's hematoxylin (Merck, Germany), aqueous eosin (Merck, Germany) and analyzed by 3 independent pathologists. The appropriate cases were selected for immunohistochemical staining.

Immunohistochemistry for estrogen receptor (ER), progesterone receptor (PR), HER2 and tryptase was performed automatically by Leica Bond-Max (Leica Biosystems, Newcastle UponTyne, UK) in order to establish the molecular subtype and to detect the MCs. Antigen retrieval was performed by incubating the slides in the Bond Epitope Retrieval Solution 1 (pH 6) and 2 (pH 9) (Leica Biosystems, Newcastle UponTyne, UK). Primary antibodies used in this study were the following: PR (clone 16), ER (clone 6F11), mast cell tryptase (10D11) and HER2 (clone CB11), all from Leica Biosystems (Newcastle UponTyne, UK), and all ready-to-use. The Bond Epitope Retrieval Solution 2, pH9, Leica Biosystems (Newcastle UponTyne, UK) was applied for 20 minutes and time of incubation was 30 minutes. The system of detection was Bond Polymer Refine Detection System. Mayer's hematoxylin was used for counterstaining (5 minutes).

ER, PR and HER2 scoring was done by standard method (according to Allred score and according to the recommendations of the American Society of Clinical Oncology, respectively) [14, 15]. The cut-off for ER and PR was 10%. The cases were classified into molecular subtypes as follows: luminal A (ER+ and/or PR+, HER2−), luminal B/ HER2− (ER+, HER2− with PR <20%/or PR-), luminal B/HER2+ (ER+ and/or PR+, HER2+), HER2+ (ER− and PR−/HER2+), and triple-negative breast cancer (ER−/PR−/HER2−) [12].

The slides stained for tryptase were scanned on Axio Imager A2 microscope (Carl Zeiss, Germany) at low magnification (×100), and the areas with the highest number of positive cells were chosen. The number of MCs located in the tumor and peritumoral stroma was counted. Mast cells were subdivided into intratumoral (MCit) and peritumoral (MCpt). There were analyzed 3 microscopic fields for each type of localization, at ×400 magnification.

Data was stored in a MS Excel 2010 database and analyzed statistically by the SPSS statistical software package (SPSS Statistics 23.0; IBM, Chicago, IL, USA). Pearson's coefficient and other values were considered statistically significant in case of $p \leq 0.05$.

Results

80 cases of breast carcinomas were analyzed. The mean age of patients was 66.04, ranging from 37 to 84. Most tumors were moderately differentiated (45 cases). There was found only one well differentiated case and 34 were poorly differentiated. According to the histological type, 74 cases (92.5%) were ductal invasive, 3 cases (3.8%) – lobular invasive, 2 cases (2.5%) – lobular *in situ* and 1 case (1.3%) – ductal *in situ*. The most frequent molecular subtype was luminal B/HER2+ (fig. 1).

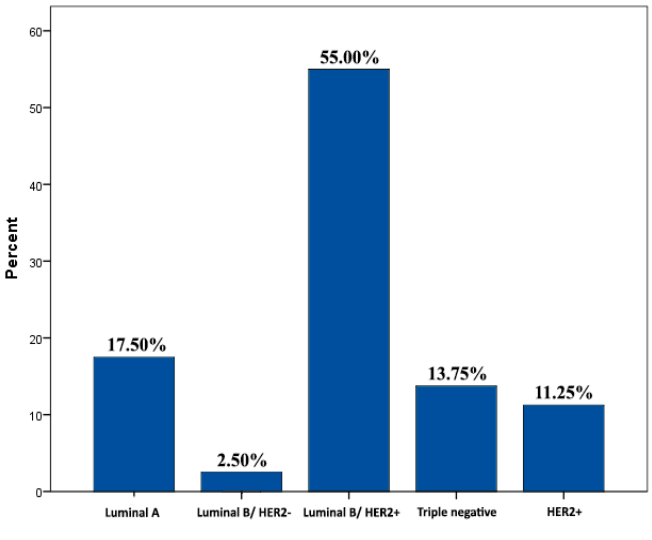

Fig.1. The distribution of molecular subtypes, n=80

Then was analyzed the distribution of MCs in different molecular subtypes. Higher numerical values of both MCit and MCpt were determined in luminal subtypes (tab. 1).

Table 1. The distribution of mast cells

Molecular subtype	Maximum numerical values	
	MCit	MCpt
Luminal B/HER2+	27.6	65.0
Luminal A	20.0	27.6
HER2+	6.0	11.3
Triple-negative	2.3	16.6

These data were also supported by statistically significant correlations. Both MCit and MCpt correlated with the expression of hormone receptors for estrogen and progester-

one. Thus, the following relations were established: MCit and ER (r=0.343, *p*=0.002), MCpt and ER (r=0.394, *p*=0.000295) and MCpt and PR (r=0.386, *p*=0.000409). Moreover, statistically significant correlations between HER2+ expression and MCs have not been established (MCit and HER2+: r=-0.026, *p*=0.820; MCpt and HER2+: r=0.199, *p*=0.077).

Mann-Whitney U test was done to compare MCit and MCpt in luminal and non-luminal subtypes. Its results supported the previous findings (tab. 2 and 3).

Table 2. The differences between MCit and MCpt in luminal and non-luminal breast carcinomas

	Subtype	N	Mean Rank	Sum of Ranks
MCit	Luminal	60	43.99	2639.50
	Non-luminal	20	30.03	600.50
	Total	80		
MCit	Luminal	60	44.48	2669.00
	Non-luminal	20	28.55	571.00
	Total	80		

Table 3. The statistics of Mann-Whitney U test

	MCit	MCpt
Mann-Whitney U	390.500	361.000
Wilcoxon W	600.500	571.000
Z	-2.340	-2.657
Asymp.Sig. (2-tailed)	0.019	0.008

Discussion

Tumor growth and development are determined by both, cancer cell–autonomous and microenvironmental mechanisms, including the contribution of infiltrating immune cells and the complexity of these phenomena is well recognized [4, 8, 9]. Of all, MCs have one of the most controversial roles.

Thus, several lines of evidence suggest that MCs are responsible for mediating angiogenesis [1, 16, 17]. Imada A. et al. compared survivals in the low and high MC count groups in patients with stage I lung adenocarcinoma. The members in the high MC count group had significantly worse prognosis than those in the low mast cell count group ($p<0.05$). In the well- and moderate-differentiation subgroups of lung adenocarcinoma, members in the high MC count group had extremely significantly worse prognosis than those in the low MC count group ($p<0.01$) [4]. Suzuki S. et al. concluded that high peritumoral MCs infiltration predicts poor prognosis in patients who underwent hepatectomy for colorectal liver metastases. The number of MCs in metastatic lesions is important for predicting the prognosis of colorectal liver metastases patients and as an indication of therapy [11]. Hu G. et al. showed in a meta-analysis including 28 published studies with 4224 patients identified from PubMed and EBSCO that tryptase+ MC infiltration significantly decreased overall survival and disease-free survival in all types of solid tumors and significantly correlated with lymph node metastasis of solid tumor [10].

However, elevated MCs at tumor sites or within draining lymph nodes have also been connected with improved outcomes [1]. Rajput AB. et al. conducted a study on tissue microarrays containing 4.444 cases and showed that the presence of stromal MCs was a favorable prognostic factor in the training set ($p=0.001$) [18]. Similar to our findings, Majorini MT. et al. proved that MCs influence the phenotype of breast cancer cells by stimulating a luminal phenotype which has a better outcome. They increased expression of ER, PGR, BCL2, and CK8. Simultaneously, MCs reduce the activation of HER2 and basal drivers such as EGFR, and prevent the expression of the basal marker CK5, thus potentially affecting the behavior of neighboring cancer cells [9].

Conclusions

Mast cells invasion, peri- and intratumoral, is strongly influenced by the expression of hormone receptors. The luminal subtypes of breast cancer are characterized by a higher density of mast cells.

References

1. Hanes MR, Giacomantonio CA, Marshall JS. Mast cells and skin and breast cancers: a complicated and microenvironment-dependent role. Cells. 2021;10(5):1-21. doi: 10.3390/cells10050986.
2. Wulaningsih W, Holmberg L, Garmo H, et al. Investigating the association between allergen-specific immunoglobulin E, cancer risk and survival. Oncoimmunology. 2016;5(6):e1154250. doi: 10.1080/2162402X.2016.1154250.
3. Ribatti D, Finato N, Crivellato E, et al. Angiogenesis and mast cells in human breast cancer sentinel lymph nodes with and without micrometastases. Histopathology. 2007;51(6):837-842. doi: 10.1111/j.1365-2559.2007.02869.x.
4. Imada A, Shijubo N, Kojima H, Abe S. Mast cells correlate angiogenesis and poor outcome in stage I lung adenocarcinoma. Eur Respir J. 2000;15(6):1087-1093. doi: 10.1034/j.1399-3003.2000.01517.x.
5. Aponte-López A, Fuentes-Pananá EM, Cortes-Muñoz D, Muñoz-Cruz S. Mast cells, the neglected member of the tumor microenvironment: role in breast cancer. J Immunol Res. 2018;2018:2584243. doi: 10.1155/2018/2584243.
6. Malfettone A, Silvestris N, Saponaro C, et al. High density of tryptase-positive mast cells in human colorectal cancer: a poor prognostic factor related to protease-activated receptor 2 expression. J Cell Mol Med. 2013;17(8):1025-1037. doi: 10.1111/jcmm.12073.
7. Varricchi G, Galdiero MR, Marone G, Granata F, Borriello F, Marone G. Controversial role of mast cells in skin cancers. Exp Dermatol. 2017;26(1):11-17. doi: 10.1111/exd.13107.
8. Rigoni A, Bongiovanni L, Burocchi A, et al. Mast cells infiltrating inflamed or transformed gut alternatively sustain mucosal healing or tumor growth. Cancer Res. 2015;75(18):3760-3770. doi: 10.1158/0008-5472.CAN-14-3767.
9. Majorini MT, Cancila V, Rigoni A, et al. Infiltrating mast cell–mediated stimulation of estrogen receptor activity in breast cancer cells promotes the luminal phenotype. Cancer Res. 2020;80(11):2311-2324. doi: 10.1158/0008-5472.CAN-19-3596.
10. Hu G, Wang S, Cheng P. Tumor-infiltrating tryptase+ mast cells predict unfavorable clinical outcome in solid tumors. Int J Cancer. 2018;142(4):813-821. doi: 10.1002/ijc.31099.
11. Suzuki S, Ichikawa Y, Nakagawa K, et al. High infiltration of mast cells positive to tryptase predicts worse outcome following resection of colorectal liver metastases. BMC Cancer. 2015;15:840. doi: 10.1186/s12885-015-1863-z.
12. Glajcar A, Szpor J, Pacek A, et al. The relationship between breast cancer molecular subtypes and mast cell populations in tumor microenvironment. Virchows Arch. 2017;470(5):505-515. doi: 10.1007/s00428-017-2103-5.

13. Carpenco E, Ceauşu RA, Cimpean AM, et al. Mast cells as an indicator and prognostic marker in molecular subtypes of breast cancer. In Vivo (Brooklyn). 2019;33(3):743-748. doi: 10.21873/invivo.11534.
14. Allred DC, Harvey JM, Berardo M, Clark GM. Prognostic and predictive factors in breast cancer by immunohistochemical analysis. Mod Pathol. 1998;11(2):155-168.
15. Wolff AC, Hammond MEH, Allison KH, et al., Human epidermal growth factor receptor 2 testing in breast cancer : American Society of Clinical Oncology / College of American Pathologists Clinical Practice Guideline Focused Update. J Clin Oncol. 2019;36(20):2105-2122. doi: 10.1200/JCO.2018.77.8738.
16. Raica M, Cimpean AM, Ceausu R, Ribatti D. Lymphatic microvessel density , VEGF-C , and VEGFR-3 expression in different molecular types of breast cancer. Anticancer Res. 2011;31(5):1757-1764.
17. Raica M, Cimpean AM, Ceauşu R, Ribatti D, Gaje P. Interplay between mast cells and lymphatic vessels in different molecular types of breast cancer. Anticancer Res. 2013;33(3):957-964.
18. Rajput AB, Turbin DA, Cheang MC, et al. Stromal mast cells in invasive breast cancer are a marker of favourable prognosis: a study of 4444 cases. Breast Cancer Res Treat. 2008;107(2):249-257. doi: 10.1007/s10549-007-9546-3.

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EC designed the research, collected the data, performed statistics study and interpreted the data, drafted the manuscript; VF conducted the laboratory work, revised the manuscript critically; VD conducted/performed the laboratory work; EF interpreted the data; LS conceptualized the project and designed the research, revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Structure of emergencies at the prehospital stage in Moldova from 2019-2020 years

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Abstract

Background: In the last 2 years, some changes have occurred in the structure of emergencies at the prehospital stage in the Republic of Moldova, being largely influenced by the COVID-19 pandemic.
Material and methods: Retrospective analysis of the Prehospital Emergency Medical Service (PEMS) Request Sheets of the National Centre of Prehospital Emergency Medicine (NCPEM) of the Republic of Moldova for the years 2019-2020.
Results: According to the nosological profile in 2020 at the prehospital stage, respiratory emergencies were on the first place with 23% of the 761.416 of total number of requests. On the second – cardiovascular (20.6%), on the third – neurological (13.3%), infectious – 3.1%. In 2019, on the first place – cardiovascular with 22.3% of the 845.572 of total number of requests, followed by respiratory – 19.3%, neurological – 13.6%, infectious – 2.2%. In 2020, on the first place were respiratory emergencies, which compared to 2019 increased practically by 4% and the infectious emergencies by almost 1%. The number of endotracheal intubation procedures and medical-assisted transportations practically doubled in y.2020 compared to y.2019.
Conclusions: All these changes: increased respiratory and infectious emergencies, intubation procedures, and assisted-medical transportations, have occurred exeptionally due to COVID-19 infection.
Key words: emergency, prehospital, nosological, respiratory, infectious.

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Introduction

Prehospital Emergency Medical Service (PEMS) is an integrated part of the Health System of the Republic of Moldova (RM), which provides the population of the entire country with Emergency Medical Care (EMC), based on the principles of universal accessibility. PEMC in the Republic of Moldova is provided free of charge to all citizens of the RM, as well as stateless persons, refugees and international travelers. The chain of emergency services at the prehospital stage begins with the request received at the single emergency calls service 112, sent to the single medical dispatcher, followed by medical assistance at the place of request, and finalized (if necessary) with transporting patients to the hospital and referral.

On September 4, 1944, in Chisinau city was opened the first EMC Station in the RM. Initially, the station had only a two-horse cart, which served the critical patients at home or in public places. In 1948, 3 teams were formed to serve prehospital emergencies: 2 teams of doctors and 1 team of emergency nurses. In 1956, the first pediatric team was created. Between 1960 and 1971 years, the specialized teams of cardiology, toxicology, traumatology, neurology, adults reanimation, and psychiatry were formed. In 1978, the first pediatrics reanimation team was organized. In 1993, based on *Nicolae Testemitanu* State University of Medicine and Pharmacy was founded the Emergency Medicine Department. According to the Government Decision No 377 of 16.06.2015 [1] and the Order of the Ministry of Health No 537 of 26.06.2015 [2], on October 1, 2015, the EMC System was reorganized into the National Centre of Prehospital Emergency Medicine (NCPEM) – Public Medical-Sanitary Institution (PMSI) of strategic importance, with legal personality, which is subordinated directly to the Ministry of Health, Labor and Social Protection (MHLSP) of the Republic of Moldova, accredited by the National Council for Evaluation and Accreditation (fig. 1).

The reorganization of the EMC Service in a unified system (PMSI NCPEM), which until the reform was divided into 5 legal entities contributed to:

- ✓ Significant improvement of the institutional management system, both vertically and horizontally;
- ✓ The number of administrative functions has been reduced;
- ✓ The conditional boundaries between the served territories by the PEMC subdivisions have been eliminated;
- ✓ The opening of the new EMC Points (in the rural area) led to the improvement of the population's access and the increase of the operativity;

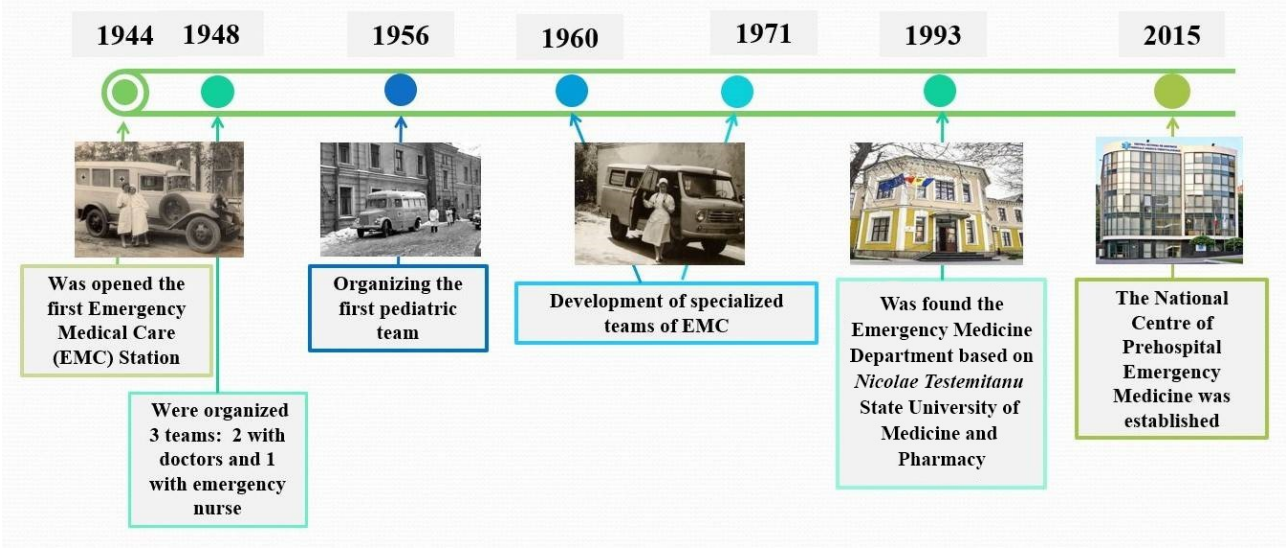

Fig. 1. History of Emergency Medical Service of the Republic of Moldova

- ✓ Unification of key processes and creation of new fields of activity;
- ✓ Mobilization of the necessary resources to solve the operative tasks.

In the last 2 years, some changes have occurred in the structure of emergencies at the prehospital stage in the Republic of Moldova, being largely influenced by the COVID-19 pandemic.

Material and methods

Retrospective analysis of the Prehospital Emergency Medical Service (PEMS) Request Sheets of the National Centre of Prehospital Emergency Medicine (NCPEM) of the Republic of Moldova for the years 2019-2020.

Results and discussion

In 2020, the PMSI NCPEM teams served 761.416 emergency calls (2019 – 845.572) and Prehospital EMC was given to 734.912 people with various medical-surgical emergencies (2019 – 820.813). The share of served calls in urban areas in 2020 was 53.1% or 390.102 served patients, and in rural areas – 46.9% or 344.810 served patients. The rate of the served calls in urban areas was 52.3% or 429.437 served patients, and in rural areas – 47.7% or 391.376 served patients – in 2019. Due to the service environment, there was an increase of 0.8% of the rate of the served Prehospital EMC requests (PEMC Calls) in the urban population in 2020 compared to 2019, which is due to the demographic processes in the Republic of Moldova through the continuous concentration of the population in cities. In 2020 the share of false alarm calls or unnecessary requests, out of the total number of served calls, was 3.5% or 26.504 cases. In the year 2019 – 2.9% or 24.759 cases. The average number of the served prehospital emergency medical care requests in 24 hours in y.2020 was 2.080 and in 2019 – 2.317 (fig. 2) [3].

Were performed 73 endotracheal intubations in the year 2020, while in 2019 – only 44. In 2020 year were performed 114.339 glucometries, and in 2019 – 107.506. Bladder catheterization in 2020 was performed for 1.392 patients, and in 2019 – in 1.304 cases (tab. 1) [3].

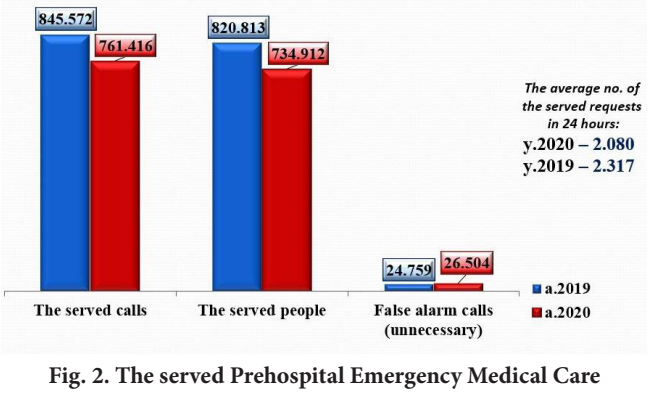

Fig. 2. The served Prehospital Emergency Medical Care Calls in years 2019 – 2020 [3]

Table 1. The served Prehospital EMC Calls and interventions for diagnosis and treatment performed by PEMC teams of the RM in years 2019 – 2020 [3]

Parameter	Year 2019	Year 2020
The served calls	845.572	761.416
The served people	820.813	734.912
Of them: Urban	429.437 / 52.3%	390.102 / **53.1%**↑
Of them: Rural	391.376 / 47.7%	344.810 / 46.9%
False alarm calls	24.759 / 2.9%	26.504 / 3.5%
Average No of served requests in 24 hours	2.317	2.080
Endotracheal intubations	44	**73**↑
Glucometries	107.506	**114.339**↑
Bladder catheterization	1.304	**1.392**↑

Table 2. Structure of Calls by the degree of urgency and the Medical-assisted transportations performed by PEMC teams of the RM in years 2019 – 2020 [3]

Parameter	Year 2019			Year 2020		
Degree of emergencies	% of served people	Abs. served people	% of trans-portations	% of served people	Abs. served people	% of trans-portations
Major	***26.5***	217.333	71.6	**28.6** ↑	210.087	68.9
Second-degree	69.1	567.509	29.1	65.5	481.576	25.8
Third-degree	3.2	25.945	14.1	3.0	21.705	9.3
Medical-assisted transportations	***1.2***	**10.026**	99.7	**2.9**↑	**21.544**	81.4

In 2020, of the total number of served people, by the degree of emergencies, the major ones constituted 28.6% (served patients being 210.087), grade II emergencies – 65.5% (served patients – 481.576), grade III emergencies – 3.0%, or 21.705 cases. Medical-assisted transportations were in 2.9% or 21.544 cases. In 2019, major emergencies constituted 26.5% (217.333 served patients), grade II emergencies – 69.1% (567.509 served patients), grade III emergencies – 3.2% (25.945 served patients). Medical-assisted transportations were in 1.2% or 10.026 cases (fig. 3 and 4, tab. 2) [3].

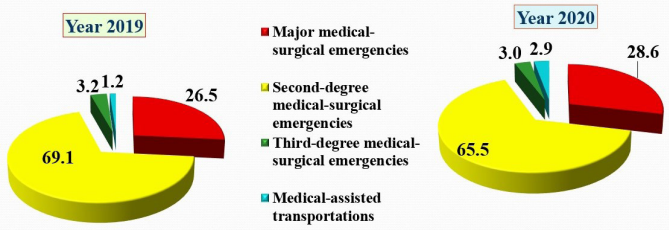

Fig. 3. Structure of emergency calls by the degree of urgency based on the Prehospital Emergency Medical Care (PEMC) Request Sheets for the years 2019-2020 (%) [3]

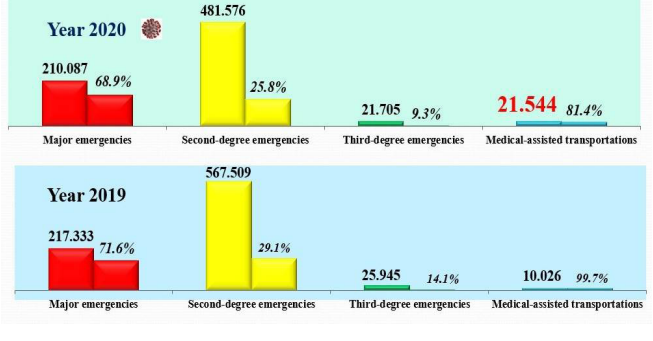

Fig. 4. Medical-assisted transportations in 2019-2020 performed by PEMC teams [3]

In the RM, many of the patients with major emergencies were left at home, because they categorically refused to be hospitalized [3]. The most common reason for being non-transported was the refusal of transportation by the patient (74.15% – 76.38%), followed by treatment of patients at the scene (15.41% – 15.74%) [4]. There is a scarcity of research on EMS reattendance to non-transported, low acuity patients. A systematic review indicated that 2-6% of non-transported patients re-contacted the EMS, and 5-19% of non-transported patients independently presented to the emergency department within 48 hours [5, 6]. In a recent UK study, 9% of non-transported patients re-contacted the EMS provider within 3 days of the initial non-transport event [5, 7]. While reattendance to the same patient within two or three days of initial presentation might suggest that ambulance crews 'got it wrong' on the first assessment, the reality is that patients with minor presentations will sometimes deteriorate or other complications arise. Reattendance has been observed internationally [5, 8-10], and may be unavoidable to some degree.

By the nosological profile of the structure of prehospital EMC requests, in the RM, in y.2020, the respiratory emergencies were on the first place with a rate of 23% of the 761.416 total number of requests, or 168.866 served patients. On the second place were cardiovascular emergencies with a rate of 20.6% or 151.509 served patients. On the third place were the neurological emergencies with 13.3% or 97.727 cases. Trauma emergencies constituted 9.9% or 72.724 cases. The rate of infectious emergencies was 3.1%, that is, 22.863 served patients. In 2019, cardiovascular emergencies ranked first – 22.3% with a total number of 845.572 PEMC requests or 183.131 served patients. They were followed (second place) by the respiratory emergencies with 19.3% or 158.674 cases. Neurological emergencies ranked third with a rate of 13.6% or 111.953 neurological served patients. Trauma emergencies accounted for 10.8% or 88.902 cases. The share of infectious emergencies in y.2019 was 2.2% or 17.882 infectious served patients (tab. 3 and fig.5) [3].

Table 3. The structure of prehospital emergency calls by the nosological profile, years 2019-2020 (% & abs.) [3]

Parameter	Year 2019		Year 2020	
Nosological profile of emergency calls	%	Abs. served people	%	Abs. served people
Cardiovascular	22.3	183.131	**20.6** ↓	151.509
Respiratory	***19.3***	158.674	**23.0** ↑	**168.866**↓
Neurological	13.6	111.953	13.3	97.727
Trauma	10.8	88.902	**9.9** ↓	72.724
Infectious	***2.2***	17.882	**3.1** ↑	**22.863**↑

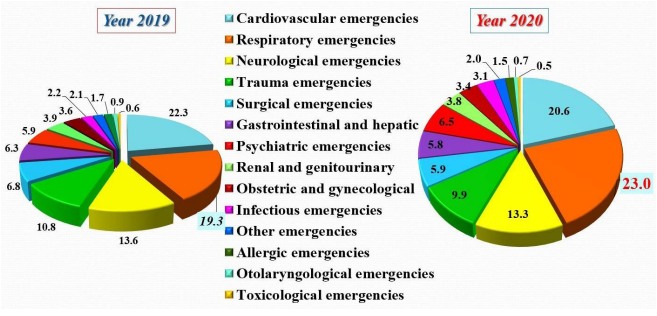

Fig.5. The structure of prehospital medical-surgical emergencies by the nosological profile, years 2019–2020 (%) [3]

In 2020, in the RM, on the first place were respiratory emergencies, which compared to 2019 increased practically by 4% and the infectious emergencies – by almost 1% [3]. After the first reported case of SARS-CoV-2, there were significant increases in complaints, chiefly of fever (211% increase, $p<0.001$) and respiratory symptoms (245% increase, $p<0.001$) [11]. The data for the period 2013–2017 from Italy showed that, with some exception due to environmental differences, the highest proportion of incoming emergency calls is not acute or urgent and could be more effectively managed in other settings than in an Emergency Department (ED). Better management of dispatch can reduce crowding and save hospital emergency department's time, personnel, and health system costs. This data highlights the importance of promoting policies to increase the availability of ambulances with staff who can manage problems on site, reducing the admissions of less appropriate EDs [12].

In the RM, the share of prehospital cardiovascular emergencies decreased in y.2020, reaching 20.6%, compared to 22.3% in y.2019. Trauma emergency in 2020 declined and accounted for 9.9%, compared to 10.8% in 2019 [3]. In Saudi Arabia the cardiac cases showed a smaller difference at 26.61%, whereas trauma cases showed a decline of 1.641 (a change of – 6.11%). Some of the medical or cardiac cases related to COVID-19 complications could not be determined from the data collected [4].

In 2020, according to nosologies, in the RM was determined the following structure of cardiovascular emergencies:

- Hypertension (HTN). Hypertensive urgencies – 56.095 served patients or a share of 37% of the total number of 151.509 cardiovascular emergencies.
- Essential HTN and tension jumps – 27.462 cases or 18.1%.
- HTN. Hypertensive emergencies – 20.185 served patients or a rate of 13.3%.
- Acute coronary syndrome (ACS) without ST-segment elevation – 3.359 cases or 2.2%.
- ACS with ST-segment elevation – 1.800 cases or 1.2%.
- Sudden cardiac death – 194 cases or 0.1%.

In y.2019, according to nosologies, in the RM was the following structure of cardiovascular emergencies:

- Hypertension (HTN). Hypertensive urgencies – 65.286 cases or a rate of 35.6% of the total number of 183.131 cardiovascular emergencies.
- Essential HTN and tension jumps – 33.732 cases or 18.4%.
- HTN. Hypertensive emergencies – 23.570 served patients or a share of 12.9%.
- ACS without ST-segment elevation – 4.705 cases or 2.6%.
- ACS with ST-segment elevation – 2.022 cases or 1.1%.
- Sudden cardiac death – 244 cases or 0.1% (tab. 4) [3].

According to the Prehospital EMC Request Sheets of the NCPEM, of the RM, during 2019-2020, which were the basis of this research, it was determined that in 2020 the number of investigations increased, such as bladder catheterizations and glucometries, and the number of endotracheal intubations practically has doubled. In y.2020, it can be noted an increase of the share in major emergencies by 2.1% and medically-assisted transportations by 1.7% compared to 2019. Also, in 2020 increased the share of emergencies grade I (majors), hypertensive urgencies (commons), hypertensive emergencies (extremes), and ACS with ST-segment elevation.

In 2020 were registered 102.529 pediatric medical-surgical emergencies or a share of 14.0% from the total no. of served people in the RM. The structure of pediatric emergencies according to the nosological profile was:

- Respiratory – 50.701 cases or 49.5%.
- Trauma – 14.766 or 14.4%.

Table 4. The structure of cardiovascular emergencies by the nosological profile based on the Prehospital Emergency Medical Care Request Sheets for the years 2019-2020 (% & abs.) [3]

Parameter	Year 2019		Year 2020	
Structure of cardiovas-cular emergencies	%	Abs. served people	%	Abs. served people
Hypertension (HTN). Hypertensive urgencies.	35.6	65.286	**37.0** ↑	56.095
Essential HTN and tension jumps.	18.4	33.732	***18.1*** ↓	27.462
HTN. Hypertensive emergencies.	12.9	23.570	**13.3** ↑	20.185
ACS without ST-segment elevation.	2.6	4.705	**2.2** ↓	3.359
ACS with ST-segment elevation.	1.1	2.022	**1.2** ↑	1.800
Sudden cardiac death	0.1	244	0.1	194

- Infectious – 4.740 (4.6%).
- Neurological – 2.204 (2.1).
- Cardiovascular – 1.287 (1.3%).

In 2019 year, 159.309 medical-surgical emergencies were registered in children or a share of 19.4% from the total number of served people. By the nosological profile, the structure of pediatric emergencies was:

- ❖ Respiratory – 82.951 cases or 52.1%.
- ❖ Trauma – 19.750 or 12.4%.
- ❖ Infectious – 8.464 (5.3%).
- ❖ Neurological – 3.011 (1.9).
- ❖ Cardiovascular – 2.92 (1.3%).

It can be noticed, that in 2020 compared to 2019, the percentage of respiratory and infectious emergencies decreases, which is the opposite for adults. Instead, pediatric trauma and neurological emergencies are increasing in the RM (tab.5) [3].

Table 5. Structure of pediatric emergencies by the nosological profile (%, abs.) 2019–2020 [3]

Parameter	Year 2019		Year 2020	
Pediatric emergencies in RM	% of served people	Total, Abs.	% of served people	Total, Abs.
	19.4	159.309	↓ *14.0*	102.529
Of them: respiratory	52.1	82.951	↓ *49.5*	50.701
trauma	12.4	19.750	**14.4** ↑	14.766
infectious	5.3	8.464	↓ *4.6*	4.740
neurological	1.9	3.011	**2.1** ↑	2.204
cardiovascular	1.3	2.092	1.3	1.287

Conclusions

In 2020, compared to 2019, at the prehospital stage, increased the share of emergencies grade I (majors), hypertensive urgencies (commons), hypertensive emergencies (extremes), and ACS with ST-segment elevation, likewise, increased the number of bladder catheterizations, glucometries, endotracheal intubations, medical-assisted transportations, which, in many cases was due to the worsening of health in patients infected with the SARS-CoV-2 coronavirus (COVID-19 disease), and with comorbidities.

Many of the patients with major emergencies were left at home, because they categorically refused to be hospitalized for several reasons, one of which, in 2020, was the fear of infection with the SARS-CoV-2 coronavirus. However, the refusal of hospitalization in emergencies requires raising the level of health education of the population.

By the nosological profile of prehospital EMC requests structure, compared to 2019, the respiratory emergencies in 2020 were on the first place, and the rate increased practically by 4% and the infectious ones – by almost 1%. The number of endotracheal intubation procedures and medical-assisted transportations practically doubled in 2020 compared to 2019. All these changes: increased respiratory and infectious emergencies, the intubation procedures, and assisted-medical transportations, exeptionally have occurred due to COVID-19 infection.

References

1. [Government of the Republic of Moldova]. [Decision No 377 of 16.06.2015, on the establishment of the National Centre of Prehospital Emergency Medicine]. Monitorul Oficial al Republicii Moldova. 2015 Jun 19;(150-159):art. no. 420. Romanian.
2. [Ministry of Health of the Republic of Moldova]. [Order No 537 of 26.06.2015, regarding the implementation of the Government Decision No 377 of 16.06.2015, on the establishment of the National Centre of Prehospital Emergency Medicine]. Chisinau: The Ministry; 2015. Romanian.
3. The Prehospital Emergency Medical Service Request Sheets of the National Centre of Prehospital Emergency Medicine of the Republic of Moldova during 2019-2020. Chisinau: National Centre of Prehospital Emergency Medicine; 2020.
4. Al-Wathinani A, Hertelendy AJ, Alhurishi S, Mobrad A, Alhazmi R, Altuwaijri M, Alanazi M, Alotaibi R, Goniewicz K. Increased Emergency Calls during the COVID-19 Pandemic in Saudi Arabia: a national retrospective study. Healthcare. 2021;9(1):14. https://doi.org/10.3390/healthcare9010014.
5. Todd VF, Swain A, Howie G, Tunnage B, Smith T, Dicker B. Factors associated with emergency medical service reattendance in low acuity patients not transported by ambulance. Prehosp Emerg Care. 2021:1-17. doi: 10.1080/10903127.2020.1862943.
6. Ebben RHA, Vloet LCM, Speijers RF, Tonjes NW, Loef J, Pelgrim T, Hoogeveen M, Berben SAA. A patient-safety and professional perspective on non-conveyance in ambulance care: a systematic review. Scand J Trauma Resusc Emerg Med. 2017;25(1):71. doi: 10.1186/s13049-017-0409-6.
7. Coster J, O'Cathain A, Jacques R, Crum A, Siriwardena AN, Turner J. Outcomes for patients who contact the emergency ambulance service and are not transported to the Emergency Department: a data linkage study. Prehosp Emerg Care. 2019;23(4):566-77. doi: 10.1080/10903127.2018.1549628.
8. Hoglund E, Andersson-Hagiwara M, Schroder A, Moller M, Ohlsson-Nevo E. Characteristics of non-conveyed patients in emergency medical services (EMS): a one-year prospective descriptive and comparative study in a region of Sweden. BMC Emerg Med. 2020;20(1):61 doi: 10.1186/s12873-020-00353-8.
9. O'Cathain A, Knowles E, Bishop-Edwards L, Coster J, Crum A, Jacques R, James C, Lawson R, Marsh M, O'Hara R, et al. Understanding variation in ambulance service non-conveyance rates: a mixed methods study. Health Serv Deliv Res. 2018;6(19):1-192. doi: 10.3310/hsdr06190.
10. Ebben RHA, Castelijns M, Frenken J, Vloet LCM. Characteristics of non-conveyance ambulance runs: A retrospective study in the Netherlands. World J Emerg Med. 2019;10(4):239-43. doi: 10.5847/wjem.j.1920-8642.2019.04.008.
11. Saberian P, Conovaloff JL, Vahidi E, Hasani-Sharamin P, Kolivand P. How the COVID-19 epidemic affected prehospital emergency medical services in Tehran, Iran. West J Emerg Med. 2020;21(6):110-116. http://dx.doi.org/10.5811/westjem.2020.8.48679.
12. Campagna S, Conti A, Dimonte V, Dalmasso M, Starnini M, Gianino MM, Borraccino A. Trends and characteristics of emergency medical services in Italy: a 5-years population-based registry analysis. Healthcare. 2020;8(4):551. https://doi.org/10.3390/healthcare8040551.

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Authors' contribution
BG conceptualized, designed and conducted the research, drafted the manuscript, revised the manuscript critically; MP collected the data, designed the research, did statistics and interpreted the data, drafted the manuscript, revised the manuscript critically; TB designed the research, interpreted the data, revised the manuscript critically; SL designed the research, interpreted the data, revised the manuscript critically; LP revised the manuscript critically; ND designed the research, interpreted the data, revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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Not applicable.

Conflict of Interests
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Current inconsistencies in the reporting of cases of intraabdominal retained textile foreign bodies

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Abstract

Background: It is assumed that the incidence of textile foreign bodies (TFB) unintentionally left in abdominal cavity is underreported, mostly due to the legal implications of their detection.
Material and methods: One hundred thirty-five responses were received to a specially developed anonymous questionnaire on the TFB problem, including medico-legal aspects. Of the total number of respondents, 81 were surgeons and 54 – gynecologists.
Results: Over 80% of respondents consider that if TFB was removed from abdominal cavity during the surgical intervention, it should be indicated in the final diagnosis. At the same time, the fact of detecting and removing TFB retained in abdomen in the real cases known by respondents was reflected in the surgical report and in diagnosis in only 49.1%. False description in case of detection and removal of intra-abdominal TFB admits 29.6% from total number of respondents, but only 24.5% with a shorter length of work (<15 years), and 40.7% – with a work experience over 15 years.
Conclusions: Surprisingly, about 20% of respondents consider it justified not to indicate retained TFB in the final diagnosis. Moreover, the real frequency of TFB diagnosis concealment is 1.6 times higher and sharply contradicts the declared intentions about the need to report the true cause of pathology. Almost half of surgeons with a long lasting work experience allow a false description of intraoperative findings and, as a result, the official diagnosis.
Key words: textile foreign body, surgical report, underreporting, false description.

Cite this article
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Introduction

Threat of medical errors is omnipresent in the work of health professionals. In modern days the reduction of medical errors is becoming main priority of different medical organizations as well as healthcare policy makers and different patients' rights organizations. It is generally considered that the incidence and different outcomes of specific types of surgical errors are, in most cases, well described, but the knowledge of why these errors occur remains unanswered [1]. Considered as one of the most baffling examples of serious surgical errors is the manifestation of retained surgical foreign bodies (sponges, meshes, instruments, needles and other surgical consumables) [1, 2]. Textile foreign bodies (TFB) can cause serious physical complications as well as moral harm to the patient and can also lead to serious professional and medico-legal consequence [2-4]. TFB events are generally believed to occur once from 1000 up to 18000 surgeries [5-9] of which 0.3-1 events in 1000 abdominal surgical interventions [3], however this is highly likely an underestimation or underreporting [8, 10-12]. In the general population, the rates of TFBs are mostly identified in abdominal surgery followed by gynecologic, vascular, and urologic procedures [5, 6, 13, 14]. This study examines the experience of surgeons in the understanding, reporting, problem identification, preventive strategies, legal status of TFBs on surgical practice.

Material and methods

This study is a part of a PhD study in abdominal textilomas and was approved by the Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy, protocol No 48 of 12.02.2020. It was an online and live survey study. The questionnaire consists of 21 questions and was created using Microsoft Office Word and converted to online survey using Google Forms. The questionnaire was sent over emails to the members of *Nicolae Anestiadi* Surgeons Association of the Republic of Moldova as well as to Obstetrics and Gynecology surgeons, Urology, Vascular, Cardiothoracic, Pediatric and Oncologic surgeons. All the responses were voluntary, anonymous and confidential. The questionnaire was sent repeatedly so as to gather the most number of total possible answers by surgeons. There was no time limit of completion the survey.

The questionnaire was created in such a way so as to gather as much information as possible from surgeons and

consists of general questions, confirmation of a known case/cases of TFB, management of those cases and legal and organizational management of those and potentially other cases.

Results and discussion

Of 543 surgeons that were eligible to participate in the survey, 145 questionnaire answers were received. The respondents comprised 79 general surgeons (54.4%), 56 obstetrics and gynecologists (38.6%), 3 thoracic surgeons (2.0%) and one from urologists, pediatric surgeons, orthopedics and traumatology, vascular, oncologist, cardiac and ICU specialists (4.8%). Of all the respondents, 22 surgeons have work experience of less than 5 years (15.17%), 32 surgeons have work experience between 5-15 years (22.06%), 32 – between 15-25 years (22.06%), 33 – between 25-35 years (22.75%) and 26 have work experience of more than 35 years (17.93%). Institutional level of respondents work place is regional hospitals in 37 answers (25.51%), municipal hospitals in 41 answers (28.37%), republican hospitals in 59 answers (40.68%), departmental hospitals in 7 answers (4.82%) and private hospitals in one answer (0.68%).

Of all respondents, 97 (66.89%) encountered an event when a TFB was left unintentionally in the abdominal cavity, of these 8 (5.15%) respondents were with surgical experience of less than 5 years, 15 (15.46%) respondents with surgical experience of 5-15 years, 22 (22.68%) respondents with surgical experience of 15-25 years, 30 (30.92%) respondents with surgical experience of 25-35 years and 22 (22.68%) respondents with surgical experience of more than 35 years.

When the respondents were asked how many cases of TFB they encountered during surgery, 28 (19.31%) said they had never experienced textile foreign body during surgery, 44 (30.34%) respondents encountered at least one such case, 38 (26.20%) encountered two cases, 12 (8.27%) – 3 cases, 11 (7.58%) – 4 cases and 6 (4.13%) – 5 or more such cases and 2 (1.37%) – 10 cases. In one case the surgeon was aware of such case from a fellow surgeon and 5 (3.44%) did not answer.

In the questionnaire the answer of some surgeons that encountered different time span from the time a TFB was retained to the time of its extraction constituted "Some days" in 44 cases, "Some weeks" in 37 cases, "Some months" in 45 cases, "Some years" in 25 cases, and 30 did not give an answer.

Regarding the second surgery (extraction of TFB) 39 respondents answered that the foreign body was found accidentally during surgery, 53 respondents answered that the second surgery was performed because of complications that were caused by the TFB. In 33 answers the TFB was symptomatic and diagnosed using Computed Tomography (CT) or Ultrasonography (USG) and in 15 answers TFB was asymptomatic and diagnosed using CT or USG. In 15 answers the diagnosis of TFB was known before any diagnostic procedures. There were 29 respondents who did not answer.

In those known cases of repeated surgery for TFB the outcome was simple extraction with no postoperative complications or prolonged hospital stay in 79 answers. Prolonged hospital admission and pharmaceutical treatment was present in 55 answers. The necessity of organ resection caused by TFB was present in 9 answers, with development of severe postoperative complications was present in 24 answers. Death of the patient caused by TFB was present in one answer only and 30 respondents did not answer.

There was an effort to ask surgeons their point of view of how they can describe, as an event, a case of TFB and the answers can be found in table 1.

Table 1. Distribution of surgeons' point of view of TFB based on proposed answer type

Types of answers	Answers (n=145), 100%
Medical accident	25 (17.24%)
Medical incident	29 (20%)
Adverse event	4 (2.75%)
Postoperative complication	11 (7.58%)
Medical deficiency	7 (4.82%)
Medical error	24 (16.55%)
Organizational error	11 (7.58%)
Medical neglect	22 (15.17%)
Malpractice	8 (5.51%)
Other	2 (1.37%)
No answer	2 (1.37%)

Interesting as well as worrying results were obtained when respondents were asked to answer the question if the detection, removal and final postoperative diagnosis were reflected in official postoperative protocol/documentation. In 57 (39.31%) answers by the respondents the official report on TFB extraction, description and diagnosis was absent in postoperative documentation, partially in 6 (4.13%) answers. The description of intraoperative findings, extraction and postoperative diagnosis were present in 60 (41.37%) answers. In 3 (2.06%) answers the surgeons did not know if the description was present in official documentation and 19 (13.10%) respondents did not wish to answer.

Another worrying result based on questionnaire answers was if the surgeons admit intentional, false description in case of TFB detection during surgery. In case of such an event, 48 (33.10%) respondents admit possible intentional falsification of documentation if such a case is detected, 91 (62.75%) respondents did not admit false postoperative description in official documentation and 7 (4.82%) respondents had doubts or partially admit potential falsifying of the report.

Unlike official documentation the reluctance to admit a TFB finding during surgery to the patient or patient's relatives is not as high as with official documentation. Nonetheless, 35 (24.13%) respondents consider not to inform the patient or his relatives of a TFB finding, 44 (30.34%) respondents consider to officially inform the patient or his representatives. A very big group of 63 (43.44%) respondents consider other circumstances that may or may not lead to informing the patient or his relatives and 3 (2.06%) respondents have other opinions.

A series of questions regarding who is responsible when

a TFB is retained unintentionally inside the abdominal cavity and what sanctions for those responsible should be applied are presented in table 2 and 3.

Table 2. Distribution of surgeons' point of view on whom lies the responsibility of TFB event

Types of answers	Answers (n=145, 100%)
Operating surgeon	36 (24.82%)
Scrub nurse	12 (8.27%)
Both (operating surgeon and scrub nurse)	36 (24.82%)
Entire surgical team	54 (37.24%)
Medical institution	4 (2.75%)
Nobody	2 (1.37%)
Other	1 (0.68%)

Table 3. Distribution of surgeons' point of view on which sanctions should apply in case of TFB event

Types of answers	Answers (n=145, 100%)
Internal professional discussion	118 (81.37%)
Disciplinary sanction	18 (12.41%)
Civil liability	2 (1.37%)
Criminal liability	0 (0 %)
Other	6 (4.13%)
No answer	1 (0.68%)

Surgeons that knew cases of TFB were asked to answer about the consequences that members of the surgical team who accidentally left a TFB during surgery suffered when this event was uncovered: 28 respondents answered that there were no consequences, 94 respondents answered that the medical team had an internal professional trial and discussion, 11 respondents answered that administrative sanctions were applied against the surgical team, 3 respondents answered that civil liability was initiated against the surgical team, 2 respondents answered that criminal liability was initiated against the surgical team, 6 respondents did not know what consequences the surgical team suffered and 19 respondents did not answer.

When asked if the medical institution should also be responsible together with the surgical team when a petition or legal complaint arrives, 119 (82.06%) respondents agree that the medical institution should also be responsible whenever such actions occur, 25 (17.24%) respondents don't agree and one respondent was hesitant to answer.

A sensible topic such as retained foreign bodies needed an anonymous way to try to collect data from surgeons and as such a questionnaire was selected as the best way to obtain such information especially it was proven as the best and risk-free modality [15]. It is a well established fact that the incidence of TFB is underestimated and underreported and generally the only reliable source of these events comes from legal cases or medical case reports [1, 16, 17, 18]. Overall it is easy to understand as to why these events are so rarely discussed, it is difficult to establish a clear diagnosis [17, 19], it brings great legal consequences [1, 19] and these cases are unlikely to be reported because of fear of malpractice repercussions [15-21]. In most cases the victims of such event is the patient, the physician and the medical institution [22].

The effect that physicians suffer from adverse surgical events is termed as the "second victim syndrome" [9, 23, 24]. Because TFB are classified as "never events" according to the National Quality Forum, physicians can suffer significant professional reputation damage and even the risk of indefensible litigation [5, 12, 25]. This leads to psychological and emotional burden on the surgeon and contributes to unwillingness to disclose RSI events [26].

A cross-sectional multi-center survey of surgeons revealed that intraoperative unfavorable events do cause serious emotional distress in 84% of respondents. Anger, embarrassment/shame, anxiety, guilt, sadness are the most frequently reported emotions expressed by physicians, with no correlation with years of experience [23]. Another survey demonstrated that fellow residents have a much greater risk of adverse consequences from their emotional suffering, in part due to greater self-perceived responsibility and fear of repercussions [24].

In an event when an unaccounted surgical item is lost or not retrieved during surgery and discovered later, surgeons may face a difficult decision of whether to perform a repeated surgery to remove the TFB or observe the patient. The operative surgeon may ask for intraoperative consults from a fellow surgeon that can be valuable help, but the decision to ask for assistance is ignored by the hesitation to publicize their medical error. Because of professional reputation concerns, many surgeons do not have the necessary motivation to report their surgical errors because of the risk of legal proceeding against them [12, 27].

A critical step in finding a way of preventing events with TFBs is accurate and sincere reporting of all such cases and also "near miss" events would also allow for a real evaluation of the efficiency of new possibilities in identifying TFBs. The present environment is lawsuit-motivated or lawsuit-oriented and as such created a real barrier for transparency, and because of it highlights the necessity for major shift in the system that TFB events are handled [23]. In majority of countries the blame of TFB events has, traditionally, been placed on the operating surgeon, however, there are reports that 90% of TFB events are the result of system or team error [9, 29]. As such a need arises for a more proactive system approach for prevention of TFB and should be implemented through continuous quality improvement with multiprofessional teams that participate in a meticulous review and careful observation of the event without attributing blame [30-32]. In changing the focus from blame assignment to finding of different strategies for prevention of such events, a more transparent environment may be created [33]. Standard protocols must involve the entire operating team and as such will improve outcomes and encourage to a more team-based mindset and spirit [9, 28, 33].

Another barrier to transparency that has been identified is the lack of standardized reporting [23]. The introduction of a standardized reporting system should be done in such a

way as to encourage reporting. These systems should have a considerable emphasis on promoting a supportive learning conditions and resolving safety issues, rather than being accusatory and hostile oriented. It is important as these unfortunate events have multiple origins rather than due to "one man" failures, incompetence or negligence [12, 23, 34, 35].

Conclusions

The healthcare system must be shifted towards a more proactive approach rather than to a more reactive one towards medical errors. For a continuous and stable reduction in the incidence of TFB events will be demanded improved preventative and recovery strategies. Such events are categorized as "never events", which in return suggest that they are totally preventable. However, their ongoing occurrence in spite of many new protocols and regulations development proves what a complex and multifactorial this problem is. Present day literature leans on improving vigilance as well heavily backs historical methods of prevention. In present day reality TFB events prevention requires a serious system-based approach that depends on the entire surgical team and even then, human error and imperfections cannot be excluded and will always be present, and as such require the implementation of technological support.

References

1. Gawande AA, Studdert DM, Orav EJ, et al. Risk factors for retained instruments and sponges after surgery. N Engl J Med. 2003;348(3):229-235. doi: 10.1056/NEJMsa021721.
2. Tacyildiz I, Aldemir M. The mistakes of surgeons: "gossypiboma". Acta Chir Belg. 2004;104(1):71-75. doi: 10.1080/00015458.2004.11679521.
3. Hyslop JW, Maull KI. Natural history of the retained surgical sponge. South Med J. 1982;75(6):657-660. doi: 10.1097/00007611-198206000-00006.
4. Imran Y, Azman MZ. Asymptomatic chronically retained gauze in the pelvic cavity. Med J Malaysia. 2005;60(3):358-359.
5. Al-Qurayshi ZH, Hauch AT, Slakey DP, Kandil E. Retained foreign bodies: risk and outcomes at the national level. J Am Coll Surg. 2015;220(4):749-59. doi: 10.1016/j.jamcollsurg.2014.12.015.
6. Stawicki SPA, Moffatt-Bruce SD, Ahmed HM. Retained surgical items: a problem yet to be solved. J Am Coll Surg. 2013;216(1):15-22. doi: 10.1016/j.jamcollsurg.2012.08.026.
7. Lovrec VG, Cokan A, Lukman L, Arko D, Takač I. Retained surgical needle and gauze after cesarean section and adnexectomy: a case report and literature review. J Int Med Res. 2018;46(11):4775-80 doi: 10.1177/0300060518788247.
8. Hacivelioglu S, Karatag O, Gungor AC, et al. Is there an advantage of three dimensional computed tomography scanning over plain abdominal radiograph in the detection of retained needles in the abdomen? Int J Surg. 2013;11(3):278-81. doi: 10.1016/j.ijsu.2013.01.009.
9. Corrigan S, Kay A, O'Byrne K, Slattery D, Sheehan S, McDonald N, et al. A Socio-technical exploration for reducing & mitigating the risk of retained foreign objects. Int J Environ Res Public Health. 2018;15(4):714. https://doi.org/10.3390/ijerph15040714.
10. Williams TL, Tung DK, Steelman VM, Chang PK, Szekendi MK. Retained surgical sponges: findings from incident reports and a cost-benefit analysis of radiofrequency technology. J Am Coll Surg. 2014;219(3):354-64. doi: 10.1016/j.jamcollsurg.2014.03.052.
11. Stawicki SP, Evans DC, Cipolla J, et al. Retained surgical foreign bodies: a comprehensive review of risks and preventive strategies. Scand J Surg. 2009;98(1):8-17. doi: 10.1177/145749690909800103.
12. Birolini DV, Rasslan S, Utiyama EM. Unintentionally retained foreign bodies after surgical procedures. Analysis of 4547 cases. Rev Col Bras Cir. 2016;43(1):12-7. https://doi.org/10.1590/0100-69912016001004.
13. Jayadevan R, Stensland K, Small A, Hall S, Palese M. A protocol to recover needles lost during minimally invasive surgery. J Soc Laparoendosc Surg. 2014;18(4):e2014.00165. doi: 10.4293/JSLS.2014.00165.
14. Wang B, Tashiro J, Perez EA, Lasko DS, Sola JE. Hospital and procedure incidence of pediatric retained surgical items. J Surg Res. 2015;198(2):400-5. doi: 10.1016/j.jss.2015.03.054.
15. Scriven A, Smith-Ferrier S. The application of online surveys for workplace health research. J R Soc Promot Health. 2003;123(2):95-101. doi: 10.1177/146642400312300213.
16. Teixeira PG, Inaba K, Salim A, et al. Retained foreign bodies after emergent trauma surgery: incidence after 2526 cavitary explorations. Am Surg. 2007;73(10):1031-1034.
17. Gonzalez-Ojeda A, Rodriguez-Alcantar DA, Arenas-Marquez H, Sanchez Perez-Verdia E, Chavez-Perez R, Alvarez-Quintero R, et al. Retained foreign bodies following intra-abdominal surgery. Hepatogastroenterology. 1999;46(26):808-12.
18. Lincourt AE, Harrell A, Cristiano J, Sechrist C, Kercher K, Heniford BT. Retained foreign bodies after surgery. J Surg Res. 2007;138(2):170-4. doi: 10.1016/j.jss.2006.08.001.
19. Yildirim S, Tarim A, Nursal TZ, Yildirim T, Caliskan K, Torer N, et al. Retained surgical sponge (gossypiboma) after intraabdominal or retroperitoneal surgery: 14 cases treated at a single center. Langenbecks Arch Surg. 2006;391(4):390-5. doi: 10.1007/s00423-005-0581-4.
20. Kaiser CW, Friedman S, Spurling KP, Slowick T, Kaiser HA. The retained surgical sponge. Ann Surg. 1996;224(1):79-84. doi: 10.1097/00000658-199607000-00012.
21. Jones SA. The foreign body problem after laparotomy. Personal experiences. Am J Surg. 1971;122(6):785-6. doi: 10.1016/0002-9610(71)90446-6.
22. Weprin S, Crocerossa F, Meyer D, et al. Risk factors and preventive strategies for unintentionally retained surgical sharps: a systematic review. Patient Saf Surg. 2021;15(1):24. doi:10.1186/s13037-021-00297-3.
23. Han K, Bohnen JD, Peponis T. The surgeon as the second victim? Results of the Boston Intraoperative Adverse Events Surgeons' Attitude (BISA) study. J Am Coll Surg. 2017;224(6):1048-56. doi: 10.1016/j.jamcollsurg.2016.12.039.
24. Marmon LM, Heiss K. Improving surgeon wellness: The second victim syndrome and quality of care. Semin Pediatr Surg. 2015;24(6):315-8. doi: 10.1053/j.sempedsurg.2015.08.011.
25. Parelkar SV, Sanghvi BV, Shetty SR, Athawale H, Oak SN. Needle in a haystack: intraoperative breakage of pediatric minimal access surgery instruments. J Postgrad Med. 2014;60(3):324-6. doi: 10.4103/0022-3859.138823.
26. Mahran MA, Toeima E, Morris EP. The recurring problem of retained swabs and instruments. Best Pract Res Clin Obstet Gynaecol. 2013;27(4):489-95. doi: 10.1016/j.bpobgyn.2013.03.001.
27. Luu S, Leung SOA, Moulton C. When bad things happen to good surgeons: reactions to adverse events. Surg Clin North Am. 2012;92(1):153-61. doi: 10.1016/j.suc.2011.12.002.
28. Steelman VM, Alasagheirin MH. Assessment of radiofrequency device sensitivity for the detection of retained surgical sponges in patients with morbid obesity. Arch Surg. 2012;147(10):955-60. doi: 10.1001/archsurg.2012.1556.
29. Stawicki SP, Cook CH, Anderson HL. Natural history of retained surgical items supports the need for team training, early recognition, and prompt retrieval. Am J Surg. 2014;208(1):65-72. doi: 10.1016/j.amjsurg.2013.09.029.
30. Fencl JL. Guideline implementation: prevention of retained surgical items. AORN J. 2016;104(1):37-48. doi: 10.1016/j.aorn.2016.05.005.
31. American College of Surgeons (ACS) Committee on Perioperative Care. Revised statement on the prevention of unintentionally retained surgical items after surgery. Bull Am Coll Surg. 2016;101(10):50-1.

32. Moynihan M, Moinzadeh A. Retained needle in the AirSeal trocar during robot-assisted laparoscopic radical prostatectomy: lessons learned. J Endourol Case Rep. 2018;4(1):105-7. doi: 10.1089/cren.2018.0034.
33. Goldberg JL, Feldman DL. Implementing AORN recommended practices for prevention of retained surgical items. AORN J. 2012;95(2):205-16. doi: 10.1016/j.aorn.2011.11.010.
34. Zejnullahu VA, Bicaj BX, Zejnullahu VA, Hamza AR. Retained surgical foreign bodies after surgery. Open Access Maced J Med Sci. 2017;5(1):97-100. https://doi.org/10.3889/oamjms.2017.005.
35. Sigakis C, Lantow B, Windham K, Meddings K, Lind KE, Suby-Long T. Sometimes it takes a village-reducing retained surgical items through multidisciplinary collaboration. J Am Coll Radiol. 2016;13(6):709-12. https://doi.org/10.1016/j.jacr.2015.10.022.

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Author's contributions
SG conceptualized the idea, designed the study, conducted literature review, designed the survey, interpreted the data and wrote the manuscript.

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The screening by isoelectric focusing of transferrin for the diagnosis of congenital disorders of glycosylation

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Abstract

Background: Congenital Disorders of Glycosylation (CDG) are a group of inherited metabolic disorders caused by the defect in various steps in the biosynthesis of glycoproteins and other glycoconjugates.
Material and methods: 40 patients under clinical suspicions for CDG at the Institute of Mother and Child were examined by isoelectric focusing of transferrin (IEFT) in collaboration with RadboudUMC, Netherlands and U.S.A. The spectrum of clinical presentations of these patients was multisystem damage, predominantly neurological manifestations.
Results: Most of the patients (55%) had early neurological manifestations from the birth, such as hypotonia, psychomotor disability, cerebral MRI abnormalities, seizures (25%), cutis laxa (17.5%), total alopecia (2.5%), abnormal fat pads (2.5%), myopia (7.5%), nystagmus (5%), strabismus (2.5%), stroke-like episodes (2.5.%), ataxia (7.5%), abnormal coagulation (10%), hepatomegaly (35%) and liver cirrhosis (2.5%). Serum samples analyzed by IEFT showed the results: 37 normal, 2 questionable and 1 abnormal patterns. Two samples questionable belongs to the patients with Galactosemia and Fructosemia, which give the false-positive results. The last positive sample is performed additionally for glycomics profiling. In some cases, with IEFT negative profile was performed genetic test and were diagnosed other diseases, mimicking CDG, such as: NARP syndrome, late diagnosed PKU, GSD, Manosidoses, Prader-Willi Syndrome and chromosomal aberrations.
Conclusions: The CDG is a rare metabolic disease with multisystem impairment and variety of symptoms which determine overlapping of phenotype with other genetic disorders. The process of diagnosis is very complex and can take several years.
Key words: congenital disorders of glycosylation, multisystem affections, isoelectric focusing of transferring.

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Introduction

Congenital disorders of glycosylation are a rare group of inherited metabolic disorders caused by genetic defects in various steps in the biosynthesis of glycoproteins and other glycoconjugates. First case of Congenital Disorders of Glycosylation (CDG) was described in 1980 by Jaeken J. at the twin-sister with marked psychomotor retardation [1]. The incidence and prevalence of CDG have not been well established because of clinical heterogeneities and complexity of diagnosis. Currently, more than 140 different types of CDG are reported in over 1350 patients diagnosed at molecular level. According to the latest reports, CDG is classified in protein N- or protein O-glycosylation defects, glycosphingolipids and glycosylphosphatidylinositol anchor glycosylation defects, and multiple glycosylation pathway defects [2]. The most common group of the pathologies are N-glycosylation disorders responsible for more than 70 types of CDG. PMM2-CDG (CDG-1a) is the most frequent diagnosed type of CDG which is identified in 62% of the patients with the ranges from 1/20000 in Dutch population and 1/77000 in Estonia based on isolated reports [3, 4]. This ultra-rare group of pathologies shows an exponential growth due to the development of sequencing technologies, so that in 2013 every 11 days a new type of CDG was confirmed and other 5 new forms in the first half of 2017 have been described [3]. The glycoproteins exist in whole body and the incorrect process of protein glycosylation disrupts the function of many organs and tissues leading to multisystem impairment and clinical heterogeneity involving 80% of neurological manifestations, 22% – jg-hepatic disorders, 20% – cardiac damage, 20% – dermatological and 10% – immunological troubles and others [5-9]. Often, the variability of clinical manifestation is mimicking other pathologies which determine the non-recognition and underdiagnosis of CDG. The first step in diagnosis of this group of pathologies is the isoelectric focusing of transferrin (IEFT) – a screening method considered as a "gold standard" in the diagnosis of CDG. The abnormal isoelectric profile of transferrin may divide the CDG into two large groups – CDG I and CDG II. During the process of the diagnosis the high necessary moment is to exclude the secondary abnormalities of glyco-

sylation leading to false-positive profiles as in Fructosemia, Galactosemia, severe liver disease, alcoholism and others. As a final step in research of CDG the clinical glycomics and genetic diagnosis are used.

Material and methods

Following the medical genetic counseling in the Laboratory of Prevention of Hereditary Pathologies of the Centre of Reproductive Health and Medical Genetics (the Institute of Mother and Child, Chisinau) there were collected 40 samples of serum from suspected for CDG patients. The children with multisystem impairment and a predominantly undefined diagnosis associated with other suspicion criteria for CDG (tab. 1) have been selected. To characterize CDGs forms and differentiate other metabolic errors which mimic CDG, the basic first- and second-line metabolic work-up has been done. For excluding chromosomal aberrations, the karyotype and CGH-array was performed. In order to complete the metabolic investigations the spectrum of urinary organic acids was analyzed by ^{1}H-NMR spectroscopy at "Petru Poni" Institute of Macromolecular Chemistry of Romanian Academy, Iasi, Romania. The screening of all patients with clinical phenotype compatible with CDG was performed by serum transferrin isoelectric focusing in collaboration with Translation Metabolic Laboratory.

Table 1. Suspicion criteria for CDG included in the study

Suspicion criteria for CDG
Age from 3 months to 18 years
Psychomotor disability
Hypotonia
Failure to thrive
Seizures
Dysmorphic features excluding chromosomal aberrations

Radboundumc, Nijmegen, The Netherland and Mayo clinic, U.S.A. In some cases with very specific clinical manifestations the genetic testing was done by genes panel or Whole Exome Sequencing in collaboration with partners from Cytogenomic Medical Laboratory, Bucharest, Romania.

Results

The cohort of patients selected for screening by IEFT were children of various age (2mo-15y) with an unexplained multisystem involvement, predominantly including nervous system. Most of the patients (55%) had early neurological manifestations from birth: hypotonia, psychomotor disability, brain MRI abnormalities as cerebellar and/or cortical atrophy, hypogenesia of corpum callosum, mega cisterna mag-

Table 2. Neurological manifestation in the suspected CDG patients

Serum	Hypotonia	IEFT profile	Cerebelar MRI					Neurological finding						Serum	Hypotonia	IEFT profile	Cerebelar MRI					Neurological finding					
			Hypogenesia of corpus calosum	Cerebelar atrophy	Cortical atrophy	Encephalomalacia	Mega cisterna magna	Stroke-like episodes	Strabismus	Nistagmus	Seizures	Ataxia	Mental retardation				Hypogenesia of corpus calosum	Cerebral atrophy	Cortical atrophy	Encephalomalacia	Mega cisterna magna	Stroke-like episodes	Strabismus	Nistagmus	Seizures	Ataxia	Mental retardation
S1	x	-	x						x		x		x	S21		+											
S2	x	-											x	**S22**	x	-			x								
S3	x	-												**S23**	x	-									x		x
S4	x	-										x	x	S24		-									x		
S5		+												**S25**	x	-	x		x					x	x	x	
S6	x	-	x										x	**S26**	x	-											
S7		-											x	**S27**	x	-						x		x		x
S8	x	-											x	**S28**	x	-								x		
S9	x	-	x				x						x	**S29**	x	-						x	x			
S10	x	-					x						x	**S30**	x	-					x		x		x	
S11	x	-												**S31**	x	-	x		x				x			x
S12	x	-											x	**S32**	x	-										
S13	x	-												**S33**	x	-				x		x				
S14	x	-												S34		-								x		
S15	x	-				x					x		x	**S35**	x	+										
S16		-				x					x			S36		-								x		
S17		-							x					**S37**	x	-							x			x
S18	x	-	x							x			x	**S38**	x	-										
S19	x	-		x			x				x		x	S39		-								x		
S20	x	-		x								x	x	**S40**	x	-									x	x

na, encephalomalacia and leukomalacia (tab. 2), reported in other described cases of CDG [10, 11]. The seizures associated with abnormal EEG were present at 25% of patients, in 3 children the abnormal EEG was identified without clinical convulsions.

The phenotype evaluation of investigated patients paid attention to dysmorphic features as inverted nipples (15%), microcephaly (7.5%) and "doll face" (5%). The dermatological examination gave importance in presence of cutis laxa (17.5%), total alopecia (2.5%), ichthyoid exfoliative dermatitis (2.5%) and abnormal fat pad (2.5%). The cardiological involvement as dilative (2.5%) and hypertrophic (5%) cardiomyopathy was found.

The important clinical signs directly suggest CDG were responsible for the failure to thrive and growth retardation which have been found in this cohort respectively in 17.5% and 20% of patients. Other affected system in CDG is ophthalmic and the manifestations detected in these subjects were: myopia (7.5%), nystagmus (5%), strabismus (2.5%) and optic atrophy (2.5%). The impairment of liver in CDG is one of the most important clue to suspect these pathologies and in the present studied cases hepatomegaly was in 35% and liver cirrhosis in 2.5 %. Other symptoms of no less importance presented by our patients were hypoglycemia (16%), stroke-like episodes (2.5%), ataxia (7.5%) and abnormal coagulation (10%).

The screening by IEFT was made to all patients who were suspected for CDG. 37 analyzed serums had no modification on IEFT, but in other 3 samples a positive transferrin profile on IEFT was identified, that allowed to suspect CDG. In all 3 cases were recommended to exclude secondary abnormalities of glycosylation. In S-5, one of the positive IEFT patients presented hepatomegaly, failure to thrive, high transaminases, high postprandial lactate, cataracts, galactose and galactitol in urine by ^{1}H NMR spectroscopy which were determined by metabolic work-up – features suggestive for Galactosemia, confirmed then by the mutation P.E203L/E203L in homozygous status in *GALT* gene. So, the Galactosemia is reported as a condition leading to false-positive IEFT profile [12] and should be excluded obligatory in suspected CDG subjects. The second abnormal profile on IEFT was in S-21, but the molecular analysis in correlation with phenotype of patient revealed that positive profile of transferrin was caused by fructose intolerance as a disease that determines secondary abnormality at IEFT, as well. The last positive serum S-35 was one of the patients who manifested hepatomegaly, elevated transaminases, cutis laxa, failure to thrive, dysmorphic features, hypoglycemia, unstable stool with frequent diarrhea, abnormal coagulation. The IEFT profile revealed the presence of disialo- and asialotransferrin that leads to the suspicion for CDG type I (fig. 1).

Initially, there were excluded the secondary abnormalities of glycosylation as Galactosemia and Fructosemia. His urinary NMR profile was negative for galactose and galactitol. The used fructose-free diet did not change the IEFT profile, confirmed by ALDOB negative result then. Next steps

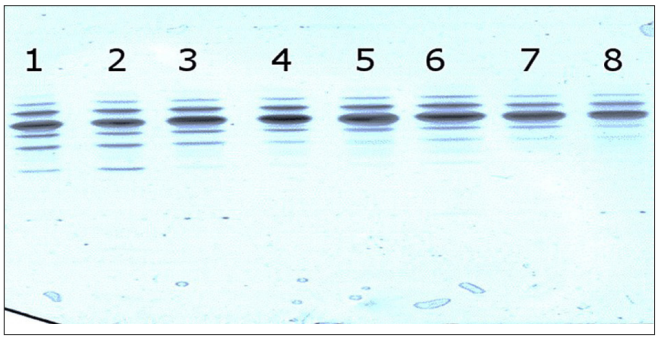

Fig. 1. IEFT profile: 1-3 positive profile, 4-5 negative profile

for the research and diagnosis of CDG in this case there will be clinical glycomics, that comprises a spectrum of different analytic methodologies which provide insights into the mechanisms of glycosylation that will be performed in collaboration with RadboudUMC, Nijmegen, The Netherlands [13].

In the rest of reported cases with normal IEFT the diagnosis tests continued to reveal other diseases those that mimic CDG (tab. 3). For the first, in the cases with severe hypotonia SMA and Pompe Disease were excluded. After metabolic investigations in some cases there were found hyperlactacidemia, hypoglycemia, hyperammonemia, high Anion Gap and acidosis that is highly suggestive for the inborn errors of metabolism, especially for glycogen storage disorders that were diagnosed in 2 cases. The diagnosis of other groups of inborn errors of metabolism will involve the specific tests which will be performed in future.

Many genetic tests were used to differentiate other genetic disorders mimicking CDG. As a consequence, in the TL1gene of mitochondrial genome was revealed the presence of m.3243A>G mutation associated with Leigh Syndrome.

This is a mitochondrial disorder that usually overlaps with clinical features of CDG.

In other two cases (S 9 and S 12), the genetic tests revealed mutations in PAH gene, that confirmed PKU. The studied patients with PKU developed a non-classical more severe phenotype of disease, possibly due to late diagnosis.

In those patients with unspecific for CDG dysmorphic features and severe hypotonia, the molecular karyotype (CGH array) was performed. So, in 1 case a pathogenic variant of microdeletion on chrs 16 p11.2 was found and in 2 other cases the Prader Willi Syndrome patterns were identified. In one case with severe development delay, seizures, tetraparesis, dysmorphic features and osteoporosis, the enzyme assay suggested the presence of Mannosidosis, which subsequently required genetic confirmation.

At the same time, the negative profile of IEFT does not exclude a CDG form and it is necessary to consider other types of glycosylation troubles as O-, GPI-anchor-, lipid- and multiple defects of glycosylation needing to be investigated by other specific tests.

Table 3. The diseases that mimicked CDG in reported cases

No	Clinical manifestation	IEFT profile	Other disease that mimic CDG
1.	Nephrosclerosis, hypertension, failure to thrive, periodic hypoglycemia, elevated transaminases	Negative	Mutation in SLC9A3R1 gene c.328C<G
2.	Psychomotor retardation, hypotonia,, cutis laxa, dysmorphic features, episodic opistatonus, knee joint contractures, hypogenesia of corpum callosum	Negative	Leigh Syndrome
3.	Hypotonia from birth, inverted nipples, fatigue, mild mental retardation, speech disorders, amenorrhea, myopia, hypothyroidism	Negative	Prader Willi Syndrome
4.	Severe neonatal hypotonia, hepatomegaly, fatigability, hypothermia, passivity drowsiness, psychomotor delay	Negative	Prader Willi Syndrome
5.	Psychomotor delay, hypotonia, ataxia, hepatomegaly, fatigability, general weakness.	Negative	Microdeletion on crs 16 p11.2
6.	Failure to thrive, disliking of fruits, some vegetables and sweets with vomiting, fasting hypoglycemia, hepatomegaly	Positive	Fructose intolerance
7.	Prolonged jaundice, high transaminases, high postprandial lactate, cataracts, hypotonia, hepatomegaly, failure to thrive.	Positive	Galactosemia
8.	Mental retardation, cutis laxa, stereotypic behavior, inverted nipples, dysmyelination on brain MRI	Negative	PKU
9.	Short stature, total alopecia, failure to thrive, feeding difficulty, dsymorphic facial features, lymphedematous hands and foots	Negative	PKU
10	Severe hypoglycemia, hypotonia, psychomotor retardation, seizures, micorcephaly, hepatomegaly, elevated transaminases	Negative	GSD
11	Generalized hypotonia, cutis laxa, hepatomegaly, mild elevated transaminases, osteoporosis, born with multiple congenital anomalies, tetraparesis, developmental delay	Negative	? Mannosidosis

Discussion

CDG are a group of rare disorders with clinical heterogeneity and the suspicions criteria for CDG should be very broad. It is recommended to suspect CDG in any unexplained affected patients, predominantly with neurological manifestations [13]. The variability of clinical symptoms is often a challenge for clinicians and can lead even to the underdiagnosis of this group of pathology that is why the real incidence of CDG is actually unknown. At the same time, the multisystem clinical involvement can mimic other genetic pathologies as: chromosomal aberration, mitochondrial disorders, lysosomal disorders and etc. Usually, clinical phenotype of the CDG, overlaps with mitochondrial disorders because both are multisystem disorders presented from birth with high mortality. The recent statistic data show that about 10% to 20% of individuals with either mitochondrial disease or CDG die in the first 2 to 4 years of life [14]. Searching the primary defect in multisystem affected patients is often unsuccessful, and mitochondrial patients could remain as suspected for CDG ones [15]. In the investigated group a suspected for CDG patient was diagnosed with a mitochondrial disorder – Leigh Syndrome (S-1, tab. 3).

A variety of complex tests is necessary to establish a diagnosis and to describe a subtype of CDG and the "gold standard" to first recognize CDG is the screening through IEFT, first introduced in 1984 by Jaeken J. [16]. This method allows diagnosing predominantly the disorders of N-glycosylation, because transferrin is N-glycosylated. The diagnosis way is followed by many steps based on the biochemical tests, enzyme assay and genetic analysis either by single gene, CDG panel or WES/WGS and clinical glycomics as well [2, 10]. Actually, to define a CDG diagnosis takes more time and it is more expensive than IEFT screening [11]. At the same time, the negative profile of IEFT does not exclude a CDG form and it is necessary to consider other types of glycosylation troubles as O-, GPI-anchor-, lipid- and multiple defects of glycosylation needing to be investigated by other specific tests.

However, the percentage of solved cases constituted about 30%, that is still quite low, which is caused by: either the lack of sequence coverage of the variant, or disease causes outside the coding sequences, or the presence of too many variants of unknown significance [2]. That is why, the important connection of genomics with functional *-omics* methodologies in the diagnosis of metabolic disorders is recognized more and more. For CDG, many cases were unsolved until the inclusion of glycomics into clinical practice, to present the functional defect [2]. These moments, revealed the complexity of diagnosis of CDG and at the same time this process involves a multidisciplinary team, multiple resources, and the implications of various international research groups as well.

Conclusions

Taking into account the fact that CDG is a group of ultrarare disorders with multisystem involvement and heterogeneous phenotype, the screening criteria for suspected CDG should be very broad. Differential diagnosis of this group of disorders should be very detailed and meticulous, because the phenotype most often mimics other genetic disorders. Because CDG is a new exponential group of disorders, it is recommended in the process of diagnoses of CDG to get involved a multidisciplinary researchers around the world and to develop the screening diagnosis in each country.

References

1. Jaeken J, Vanderschueren-Lodeweyckx M, Casaer P, Snoeck L, Corbeel L, Eggermont E, Eeckels R. Familial psychomotor retardation with markedly fluctuating serum proteins, FSH and GH levels, partial TBG deficiency, increased serum arylsulphatase A and increased CSF proteins: a new syndrome? Pediatr Res. 1980;14(2):179. https://doi.org/10.1203/00006450-198002000-00117.
2. Abu Bakar N, Lefeber DJ, van Scherpenzeel M. Clinical glycomics for the diagnosis of congenital disorders of glycosylation. J Inherit Metab Dis. 2018;41(3):499-513. https://doi.org/10.1007/s10545-018-0144-9.
3. Jaeken J, Peanne R. What is new in CDG? J Inherit Metab Dis. 2017;40(4):569-586. doi: 10.1007/s10545-017-0050-6.
4. Francisco R, Marques-da-Silva D, Brasil S, Pascoal C, dos Reis Ferreira V, Morava E, et al. The challenge of CDG diagnosis. Mol Genet Metab. 2019;126(1):1-5. https://doi.org/10.1016/j.ymgme.2018.11.003.
5. Marques-da-Silva D, Dos Reis Ferreira V, Monticelli M, et al. Liver involvement in congenital disorders of glycosylation (CDG). A systematic review of the literature. J Inherit Metab Dis. 2017;40(2):195-207. doi: 10.1007/s10545-016-0012-4.
6. Freeze HH, Eklund EA, Ng BG, Patterson MC. Neurological aspects of human glycosylation disorders. Annu Rev Neurosci. 2015 Jul 8;38:105-25. doi: 10.1146/annurev-neuro-071714-034019.
7. Rymen D, Jaeken J. Skin manifestation in CDG. J Inherit Metab Dis. 2014;37(5):699-708. doi: 10.1007/s10545-014-9678-7.
8. Marques-da-Silva D, Francisco R, Webster D, Dos Reis Ferreira V, Jaeken J, Pulinilkunnil T. Cardiac complications of congenital disorders of glycosylation (CDG): a systematic review of the literature. J Inherit Metab Dis. 2017 Sep;40(5):657-672. doi:10.1007/s10545-017-0066-y.
9. Monticelli M, Ferro T, Jaeken J, Dos Reis Ferreira V, Videira P. Immunological aspects of congenital disorders of glycosylation (CDG): a systematic review of the literature. J Inherit Metab Dis. 2016 Nov;39(6):765-780. doi: 10.1007/s10545-016-9954-9.
10. Paprocka J, Jeleza-Stanek A, Tylki-Szymanska A, Grunewald S. Congenital disorders of glycosylation from neurological perspective. Brain Sci. 2021 Jan 11;11(1):88. doi: 10.3390/brainsci11010088.
11. Van Scherpenzeel M, Willems E, Lefeber DJ. Clinical diagnostics and therapy monitoring in the congenital disorders of glycosylation. Glycoconj J. 2016;33(3):345-358. doi: 10.1007/s10719-015-9639-x.
12. Maratha A, Colhoun HO, Knerr I, Coss KP, Doran P, Treacy EP. Classical galactosaemia and CDG, the N-Glycosylation interface. A Review. JIMD Rep. 2017;34:33-42. doi: 10.1007/8904_2016_5.
13. Jaeken J. Congenital disorders of glycosylation (CDG): it's (nearly) all in it! J Inherit Metab Dis. 2011;34(4):853-858. doi: 10.1007/s10545-011-9299-3.
14. Funke S, Gardeitchik T, Kouwenberg D, et al. Perinatal and early infantile symptoms in congenital disorders of glycosylation. Am J Med Genet A. 2013;161a(3):578-84. https://doi.org/10.1002/ajmg.a.35702.
15. Briones P, Vilaseca MA, Garcia-Silva MT, Artigas J. Congenital disorders of glycosylation (CDG) may be underdiagnosed when mimicking mitochondrial disease. Eur J Paediatr Neurol. 2001;5(3):127-131. doi: 10.1053/ejpn.2001.0483.
16. Jaeken J, Van Eijk HG, van der Heul C, Corbeel L, Eeckels R, Eggermont E. Sialic acid-deficient serum and cerebrospinal fluid transferrin in a newly recognized genetic syndrome. Clin Chim Acta. 1984;144(2-3):245-247. doi: 10.1016/0009-8981(84)90059-7.

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Authors' contributions

DB collected and interpreted the data. DB, CB, DT, VS and NU analysed the result of screening by IEFT. NU revised the manuscript critically. All the authors approved the final version of the manuscript.

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Ethics approval and consent to participate

This research project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 48 of 03.07.2019).

Conflict of interests

No competing interests were disclosed

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Spatial disparities in mortality by causes of death in the Republic of Moldova

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Abstract

Background: Previous studies have shown long-term unfavourable changes in mortality in the Republic of Moldova accompanied by recent improvements. Little is known about the regional mortality differentiation which is an important tool for evidence-based public health policy. The aim of the study is to assess the current geographical disparities of all-cause and cause-specific mortality in Moldova and to identify evidence-based modalities to reduce them.
Material and methods: This cross-sectional study is based on the corrected results of the 2014 census and individual death records for the 2012-2016 period provided by the National Agency for Public Health. Global Moran's index and local indicators of spatial autocorrelation were computed based on contiguity matrix.
Results: All-cause mortality gradient between the northern and central regions was found for males (Moran's index=0.47, p<0.001) and females (Moran's index=0.44, p<0.001). Digestive and cardiovascular diseases for both sexes and external causes of death for males had a statistically significant influence on the inter-regional mortality differentiation. Liver cirrhosis contributed the most to the geographical difference between the North and the Centre (Moran's index=0.59, p<0.001), especially for females.
Conclusions: The results of this study point to the existence of different drinking habits of the Moldovan population between the northern and central regions. The central regions that form the cluster of "high-high" mortality from liver cirrhosis should be considered as primarily targets for antialcohol policies.
Key words: mortality, causes of death, spatial autocorrelation.

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Introduction

The problem of high mortality in the countries of the former Soviet Union (FSU), especially in its European part, is largely discussed in the literature. Studies have shown that an increase in adult mortality in these countries that began from the mid-60s of the last century left them far behind the western countries where life expectancy progress has accelerated since the 1970s due to a massive decline in cardiovascular mortality [1]. At the level of causes of death, the main contributors to this long-term health crisis in Russia [2], Ukraine [3] or the Baltic countries [4] are cardiovascular mortality and external causes of death, a category that includes accidental poisoning, traffic accidents, suicides, homicides. Moreover, it was shown that a harmful alcohol consumption behaviour in these traditionally spirits drinking countries was considered as a major factor of population health deterioration [5]. After several decades of rising mortality and short-term fluctuations, considerable recent improvements in life expectancy were achieved in Estonia from the mid-90s, Russia from 2005 or Ukraine from 2009, which points to the beginning of the convergence trend in life expectancies between the FSU countries and the western countries [6]. Although data quality for Moldova is somewhat more questionable as compared to Russia or Ukraine [7], the long-term overall mortality trends are very similar in these countries. The high level of cardiovascular mortality plays a crucial role for low life expectancy of the Moldovan population disregarding its recent improvements [8]. Further, in Moldova, which is a wine drinking country, the role of chronic alcoholism due to a high consumption of unregistered home-made wine is a particular health issue, especially for female population [9].

Along with mortality studies at the national level, researchers pay more and more attention to the regional mortality trends and patterns in the FSU countries. Thus, several studies have been conducted for Ukraine, Russia, Belarus. Poniakina S. demonstrated the geographical diversity of mortality patterns in Ukraine between the western regions that have lower cardiovascular and external mortality and the eastern (for males) and south-eastern regions (for females) whose population face more serious health problems for these two leading causes of death [10]. Timonin S., Danilova I. et al. showed that the recent life expectancy growth in Russia unfold differently across regions, and the two major cities, Moscow and Saint Petersburg, contribute the most to inter-regional divergence [11]. Grigoriev P. et al.

demonstrated that an increasing inter-regional mortality inequality in Belarus is associated with diverging trends from external causes of death [12]. The availability of cause-specific mortality data at the regional level for more and more countries allows researchers to move from international mortality comparison between countries to large-scale studies on cross-country differences in mortality taking into account the significant disparities within countries. Thus, using spatial autocorrelation technics, it was found that the districts located along the Belarusian–Lithuanian border, especially those on the Belarusian part, suffer enormously from conditions associated with an increased alcohol consumption such as liver cirrhosis and alcohol poisoning [13].

Although there are studies of long-term trends in cause-specific mortality in Moldova, little is known about its regional patterns. The study reported in this paper addresses to the regional mortality differences by causes of death in Moldova. The aim of the study is to assess current geographical disparities of overall and cause-specific mortality in Moldova and to identify evidence-based modalities to reduce them.

Material and methods

Present study relied on the 2014 usual resident population according to the 2014 Census adjusted by the Natural Bureau of Statistics (NBS) based on the post-census survey. As for mortality data, were used the individual death records for the period 2012-2016 codified according to the detailed (4-digit level) 10th revision of the International Classification of Diseases and Causes of Death (ICD-10) provided by the National Agency for Public Health (NAPH), which is responsible for the centralized codification of causes of death. To ensure better robustness of death rates at the regional level, was used the average for the given five-year period. Ill-defined causes of death (R00-R99) that constitute less than 1% for the analyzed period were redistributed proportionally among all causes of death. After that the data were aggregated by causes of death according to two lists: a short list that includes seven broad groups of causes of death and an extended one that consists of 20 items.

The mortality data were aggregated by 35 administrative units, including 2 municipalities (Chisinau, the capital of the country, and Balti). The mean population size of regions is 81 thousand (with Chisinau) and 63 thousand (without Chisinau). The minimum population size is 19 thousand (Basarabeasca) and the maximum one is 676 thousand (Chisinau). In 22 out of 35 regions, the population size is 40-80 thousand; in 8 regions, it is 80 thousand and over; in 5 regions – less than 40 thousand. The official statistical data have not been covering Transnistria and the municipality Bender since 1998.

Life expectancy and 95% confidence limits across 35 administrative units were calculated based, respectively, on Chiang C. L. method [14] and Silcocks et al. method [15]. Contributions by age and cause of death to the difference between life expectancy at birth in Moldova and its every region were estimated by E. M. Andreev's method [16, 17]. Mortality rates by sex and seven broad groups of causes of death were standardized by indirect method. Cause-specific death rates calculated for Moldova for the years 2012-2016, both sexes, were used as reference rates. Confidence limits were calculated based on Byar's or exact CI method [15]. To produce mortality maps, we used shape files from DIVA-GIS [18]. The "Jenks" optimization method of classification was used to produce life expectancy maps that maximize the differences between the categories of observations [19].

To carry out spatial analysis, global Moran's index and local indicators of spatial autocorrelation (LISA) [20] were calculated based on contiguity matrix. To construct the contiguity matrix that defines the spatial neighbourhood structure, was used the first-order queen structure. The LISA were presented using the LISA cluster maps that have five categories according to the type of spatial autocorrelation. The "*high-high*" cluster (*"hot" spots*) belongs to the regions that have high level of mortality and are surrounded by other regions that have above the average level of mortality. The "*low-low*" clusters (*"cold" spots*) belong to the regions with low mortality level surrounded by the regions with below average levels of mortality. Other two categories of spatial autocorrelation represent "high-low" and "low-high" spatial outliers (these two categories were not identified in the study). Finally, the fifth category represents statistically non-significant spatial autocorrelation labeled as *non-significant*. The significance level was estimated using Monte Carlo approach (number of simulations = 9999). Data were analysed in R.

Results

Life expectancy at birth (e_0) at the national level in 2012-16 is 68.4±0.2 in males and 76.4±0.2 in females. Across regions, it varies between 65.2±2.2 in Soldonesti and 71.5±0.5 in Chisinau among males and between 72.2±2.1 in Cimislia and 79.2±0.5 in Chisinau among females. Depending on the population size, confidence limits for e_0 varies from ±0.5 year for both sexes from Chisinau to ±3.2 years in males from Basarabeasca. Life expectancy at birth is higher than that at the national level only in six regions out of 35 for males and in 12 regions for females.

Figure 1 shows the maps of life expectancy at birth by sex. Life expectancy values are categorized into five categories by the "Jenks" optimization method. For both sexes, the highest and high values of life expectancy are observed in some northern regions and in Chisinau that are defined as the leading regions. In males, high values of e_0 are also observed in Anenii Noi. The regions with the lowest and low life expectancy defined as the lagging regions are located mainly in the central part of the country. A few southern regions that are adjacent to the Centre (Leova, Cimislia, Basarabeasca and Cantemir) also belong to the lagging regions. The nearest to the capital regions (first-order neighbours) have low values of life expectancy with two exceptions (Anenii Noi for both sexes and Ialoveni in males), while those of the second order have the lowest values of life expectancy. The regions with the lowest values of life expectancy, the most lagging regions, form *the belt of high mortality*. As one moves to the

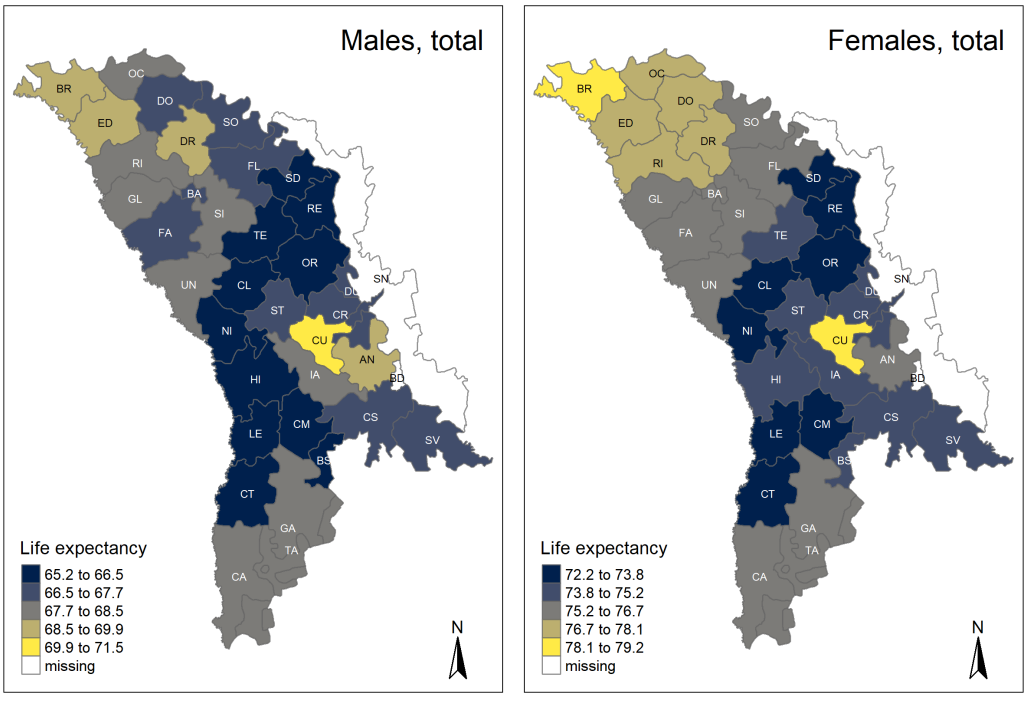

Fig. 1. Life expectancy at birth by regions in the Republic of Moldova, 2012-2016, by sex

Source: author's calculations based on NAPH and NBS data.

Note: "Jenks" optimization method of classification was used.

North or to the South of the country, the situation ameliorates gradually (stagnating regions), and in the most remote northern regions, life expectancy even approaches the level observed in the capital, especially in females. This division of regions into leading (North, capital) and lagging (Centre) can be traced and across age groups, although for older ages (65+) the belt of high mortality becomes less marked (fig. 1).

Contribution of mortality by age groups (less than 1, 1-19, 20-44, 45-64 and 65+) to the differences between e_0 in Moldova and its each region varies by sex. In males from the first three leading regions (Chisinau, Briceni and Edinet), low mortality among young (20-44) and mature (45-64 years old) adults accounts for 60-80% of life expectancy gains. The opposite situation is observed in the lagging regions where high mortality at these two age groups explains the lion's share of life expectancy losses (up to 90% in Cimislia). At the same time, the impact of older age groups among males is of less importance. On the contrary, among females, mortality at mature and older adult age groups contributes the most to the regional mortality differentiation.

At the level of main groups of causes of death (fig. 2), life expectancy differentiation in Moldova is largely explained by diseases of the circulatory system, diseases of the digestive system and external causes of death (in males). More than half of male life expectancy gains in Chisinau as compared to the national level is explained by cardiovascular diseases and external causes of death (1.9 out of 3.2 years). It is interesting to note that the positive contribution of external causes of death is registered only in the capital of the country, while in other regions, even with high life expectancy, it is either close to zero or negative. In females, the positive / negative impact of cardiovascular mortality is more pronounced across leading / lagging regions as compared to males. Females living in the capital gain 2.9 years as compared to the national level mostly due to lower mortality from cardiovascular diseases (60% of the total gain) and digestive diseases (20%). The similar situation for females, even in a lesser extent, is observed in the northern regions like Briceni or Edinet. On the contrary, females from the most lagging regions suffer much more from cardiovascular diseases and diseases of the digestive system. The impact of other main groups of causes of death, including neoplasms that occupy the second place in cause-specific mortality structure at the national level, is of minor importance. The municipality Balti seems to be the only exception concerning neoplasms with an unusually big as compared to other regions negative contribution for both sexes.

Decomposition of the difference in life expectancy at birth between the most lagging regions and the most leading regions by age and causes of death can help to better clarify the most affected population subgroups. The municipality Chisinau was selected as the most leading region where 24% of female and 23% of male population live (e_0 in males is 71.6 years and e_0 in females is 79.2 years). Since the belt of high mortality is more extended for life expectancy at age 25 or 45 than that at birth, the selection of the most lagging regions was based on the maps produced for e_{25}. In eleven regions with the lowest male e_{25} (less than 42.9 years), 21% of male population live. 16% of female population live in nine regions with the lowest female e_{25} (less than 50.2 years).

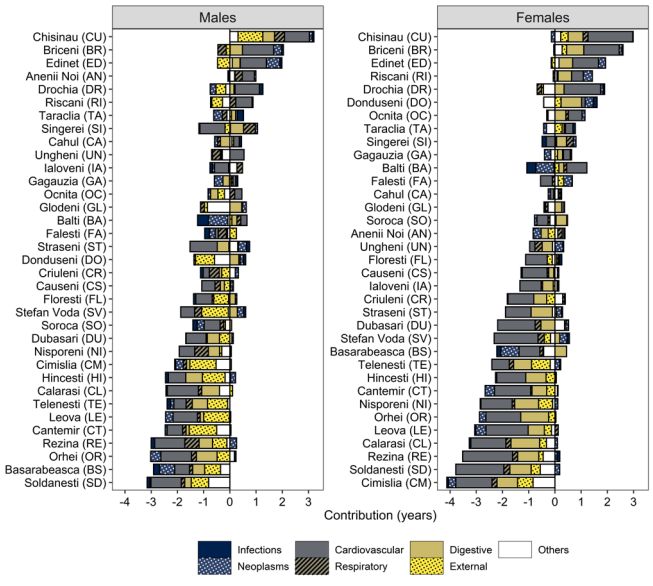

Fig. 2. Contributions by seven causes of death to the differences between life expectancy at birth in Moldova and its 35 administrative units, 2012-2016, by sex

Source: author's calculations based on NAPH and NBS data

Figure 3 shows the contribution by age groups and seven main causes of death to the difference in e_0 between the most lagging regions and the municipality Chisinau. The total difference in life expectancy at birth between these two extreme population subgroups constitutes -5.6 years in males and -5.7 years in females. The biggest losses are registered in mature male adults and older female adults. High mortality from diseases of the circulatory system in the most lagging regions reduced e_0 there by 1.8 years in males and by 3.1 years in females. In males, external causes of death (-1.6 year) and diseases of the digestive system (-0.9 year) are the next two main causes of death that deteriorate population health. In females, diseases of the digestive system constitute the second large group of causes of death that substantially increase the gap between the most lagging regions and Chisinau. It is interesting to note that this category of causes of death in females have almost the same negative impact as external causes of death in males (-1.4 years and -1.6 years, respectfully).

The contribution of mortality among children (0-14 years old) is -0.4 years for both sexes with the highest impact of "Other causes of deaths" mostly presented by perinatal causes of death. The young males (15-39 years old) from the most lagging regions lose 1.3 years (23%) of life expectancy at birth as compared to their counterparts from Chisinau. These losses are explained largely by external causes of death (-0.72 year). Among young female adults, the losses are much less pronounced (-0.6 years or 11%), but like in males they are mostly attributable to external causes of death. In males, the most affected age groups are mature adults (40-64 years old) whose high mortality accounts for 2.5 years or 45% of the overall losses. Although the negative contributions are registered for all causes of death in mature male adults, external causes of death and diseases of the digestive system explain more than half of all the losses. Moldovan females aged 40-64 years old from the most lagging regions lose on average 2.2 years of life expectancy compared to females from Chisinau. The negative impact of diseases of the digestive system for this age and sex group (-1.0 year or 44%) is even larger than that of diseases of the circulatory system (-0.73 year or 32%). At older ages, diseases of the circulatory system play the leading role for both sexes. In females, this age and cause of death group constitutes -2.3 years, which accounts for 90% of the overall losses at older ages (-2.5 years) and for 40% of the overall losses (-5.7 years).

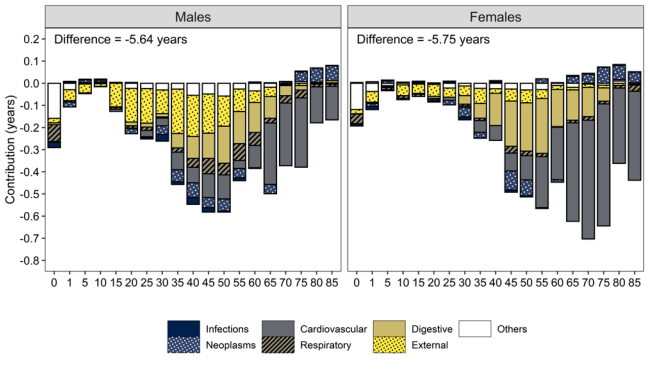

Fig. 3. Contributions by age and seven causes of death to the difference between life expectancy at birth in the most lagging regions and the municipality Chisinau, 2012-2016, by sex

Source: author's calculations based on NAPH and NBS data.

Note: the most lagging regions have life expectancy at age 25 less than 42.9 years in males (11 regions) and less than 50.2 years in females (nine regions)

The results of the global spatial autocorrelation for all-cause and cause-specific mortality are presented in table 1. The Global Moran's index is positive and statistically significant for overall mortality, diseases of the circulatory system,

Table 1. Global Moran's index and significance level for indirectly standardized death rates from seven major causes, by sex, Moldova

No	Cause of death	ICD-10 code	Males	Females
1.	Infections	A00-B99	0.148 (>0.05)	0.081 (>0.05)
2.	Neoplasms	C00-D48	0.037 (>0.05)	0.128 (>0.05)
3.	Diseases of the circulatory system	I00-I99, G45	0.309 (<0.01)	0.308 (<0.01)
4.	Diseases of the respiratory system	J00-J98, U04	0.041 (>0.05)	0.235 (>0.05)
5.	Diseases of the digestive system	K00-K93	0.438 (<0.001)	0.597 (<0.001)
6.	External causes of death	V01-Y98,	0.204 (<0.05)	0.126 (>0.05)
7.	Other causes	D50-G44, G47-H95, L00-Q99	0.05 (>0.05)	0.00 (>0.05)
8.	All causes	A00-Y98	0.473 (<0.001)	0.438 (<0.001)

Source: author's calculations based on NAPH and NBS data

diseases of the digestive system and external causes of death (only in males). It means that in Moldova, there are clusters of regions with the similar mortality patterns, i.e., regions with high mortality are surrounded by regions with high mortality ("hot" spots), while regions with low mortality are surrounded by regions with low mortality ("cold" spots). Diseases of the digestive system mostly presented by liver cirrhosis at the national level have the highest values of the global spatial autocorrelation, especially for females. At the same time, diseases of the circulatory system for both sexes and external causes of death for males have a less strong impact on the inter-regional mortality differentiati.

Figure 4 shows the location of the "high-high" and "low-low" clusters for selected causes of death on the map for both sexes. Depending on the cause of death, the location of these "hot" and "cold" spots on the map may differ. The division between the leading North and the lagging Centre is particularly impressive for liver diseases (Moran's index = 0.59, p<0.001). For diseases of the circulatory system, "low-low" clusters were depicted in the North of the country (Briceni, Ocnita, Edinet and Riscani), while "high-high" clusters were found for a few central regions (Soldanesti, Rezina, Orhei). The similar division remains and for heart diseases (Moran's index=0.29, p<0.05). However, for cerebrovascular diseases the most affected regions are situated in the South rather than in the Centre (Moran's index=0.3, p<0.001). Furthermore, the Moldovan population from the South of the republic seems to suffer much more from "Endocrine, nutritional and metabolic diseases" (Moran's index=0.24, p<0.05) mostly presented by diabetes mellitus as compared to the rest of the country. "Hot" spots for external causes of death were depicted for Hancesti, Leova, Cimislia and Soldonesti and a "cold" spot for the municipality Chisinau. Then, the municipality Chisinau and its first-order neighbours form the cluster of "low-low" mortality for certain conditions originating in the perinatal period, while in Soldanesti and Rezina, the situation is opposite (Moran's index=0.22, p<0.05).

Although the global Moran's index is not statistically significant for the whole category of neoplasms, the spatial autocorrelation analysis at the level of more detailed causes revealed certain specific geographical patterns. Thus, in the South of the country (Basarabeasca, Gagauzia, Cahul, Cantemir, Cimislia, Taraclia), the clusters of "high-high" mortality were detected for malignant neoplasms of stomach (Moran's index=0.19, p<0.05) and urinary organs (Moran's index=0.18, p<0.05). Another interesting finding is that cluster of "high-high" mortality from HIV infection was depicted for the municipality Balti and its first-order neighbours (Riscani, Glodeni, Singerei) (Moran's index=0.26, p<0.001).

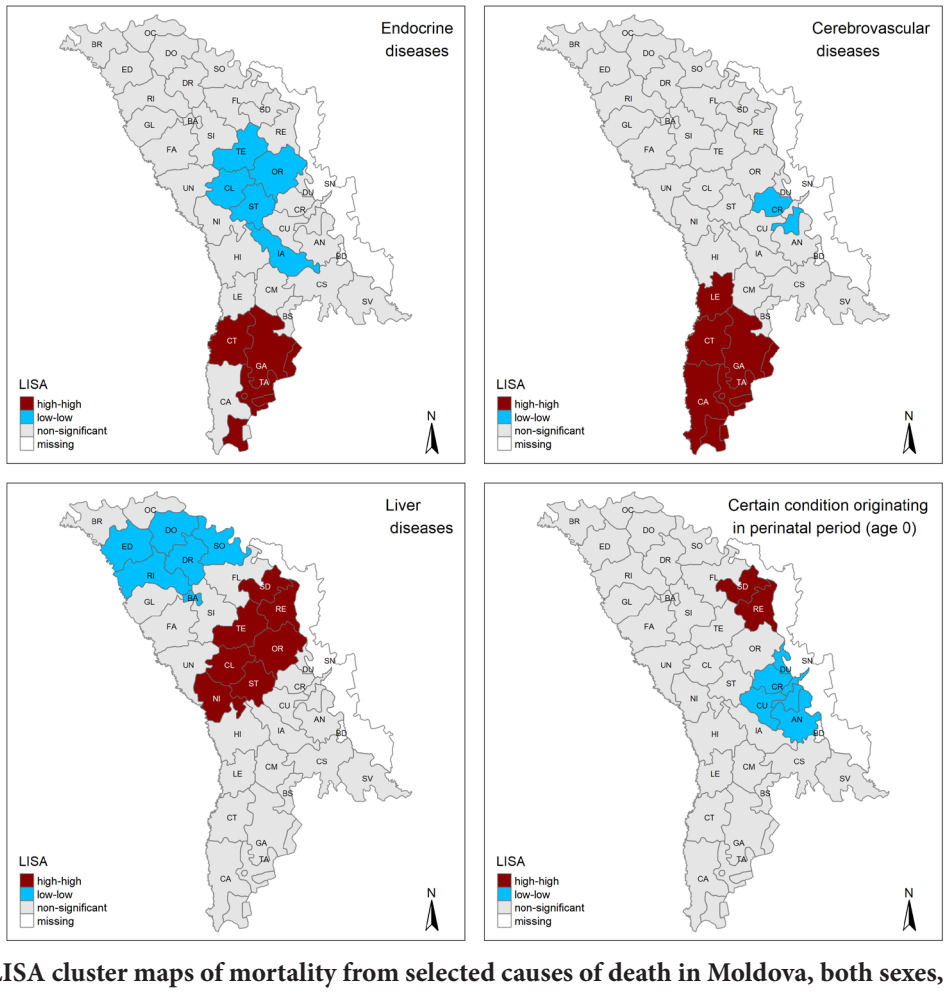

Fig. 4. LISA cluster maps of mortality from selected causes of death in Moldova, both sexes, 2012-2016
Source: author's calculations based on NAPH and NBS data

Discussion

Before discussing the main finding of this study, it is necessary to consider its data quality issues and limitations. Studies on regional data analysis may be complicated by the variation in regional coding practices of causes of death. It is of particular importance for those countries where the system of codification of causes of death is decentralized. Since in Moldova the codification of causes of death is centralized, this problem can be omitted. Then, the statistical continuity of death time series is complicated by periodic revisions of the classification of causes of death and by the changes in coding practices between two classifications. In this study, were used the average mortality data under the 10th revision of ICD-10 for the years 2012-2016. The analysis was performed at the level of the broad groups of causes of death, which is a common measure to avoid discontinuities in time series. The small proportion of ill-defined causes of death (less than 1%) for this period does not affect the results of the study.

The main limitation of the study is the selected time period (2012-2016). To avoid a possible systematic bias that can be induced by intensive internal and international migration flows, was opted the period around the last census conducted in May, 2014. The results of the 2014 census were adjusted by the National Bureau of Statistics due to the incomplete coverage of the population in the municipality Chisinau where it was only 59% [21]. However, since the overall and cause-specific mortality trends in Moldova at the national level have not changed drastically over the last decade, apart from the year 2020 affected by COVID-19 pandemic, it can be assumed that the presented regional mortality patterns correspond and to the period until 2020. The comparative analysis of regional mortality patterns around 2014 census to the period around 2004 census is a future research direction of the study.

In this study, were explored the current regional patterns of all-cause and cause-specific mortality in Moldova. The analysis of all-cause mortality suggests that the inter-regional differences exist between the municipality Chisinau and the North of the country on the one hand and the central regions on the other hand. The regions with the lowest life expectancy values form the belt of high mortality which stretches from Soldanesti to Cantemir. It is interesting to note that most of these regions are the second-order neighbours to the capital of the country. It was surprising to see that in some regions that are the first-order neighbours to Chisinau, the population health is much worse as compared to the capital. Even for such a small country like Moldova, with the total population of 2.6 million, the proximity to the capital does not ameliorate the population's health, but, on the contrary, worsens the situation. On the other hand, the studies on the regional mortality differentiation in the FSU countries have similar results. For example, in Belarus, the inter-regional overall mortality disparities are defined by the differences between the capital and the rest of the country [12].

The spatial analysis of cause-specific mortality patterns revealed that only three broad groups of causes of death play a statistically significant role in the inter-regional mortality differentiation: diseases of the digestive system, diseases of the circulatory system and external causes of death in males. The north-centre gradient in all-cause mortality is largely explained by diseases of the digestive system, liver cirrhosis in particular, both in males and females. The regions involved in the cluster of "high-high" mortality from liver cirrhosis (Soldanesti, Rezina, Telenesti, Orhei, Calarasi, Straseni and Nisporeni) experience serious health problems related to an excessive alcohol consumption. Previous studies showed that chronic consequences of alcohol consumption such as liver cirrhosis had an enormous influence on the Moldovan population's health, especially for females [9]. Unrecorded alcohol consumption dominated by home-made wine plays an important role here. Thus, in Moldova, the consumption of home-made wine constitutes about 30% of the total alcohol consumed, without any significant difference by sex (it is even slightly higher for females). Furthermore, home-made wine consumption is especially popular among Moldovan females aged 45-69 years old, for whom it accounts for about 45% of the total alcohol consumed as compared to 35% for males in the corresponding age group [22].

The results of the spatial analysis suggest that diseases of the circulatory system also contribute to the formation of the north-centre mortality gradient, although to a much less extent as compared to diseases of the digestive system. We explain it by the fact that "high-high" mortality clusters for cerebrovascular diseases were detected in the South of the country, while for heart diseases – in the Centre. Finally, external causes of death account for mortality differentiation between the municipality Chisinau and some central regions.

Conclusions

A statistically significant gradient in all-cause mortality between the northern and central regions was found. At the level of causes of death, liver cirrhosis plays the most crucial role in this inter-regional mortality pattern, especially for female population. The results of the present study point to the existence of different drinking habits of the Moldovan population between the North and the Centre of the country. The central regions that form the cluster of "high-high" mortality from liver cirrhosis should be considered as primary targets for antialcohol policies.

References

1. Vallin J, Meslé F. Convergences and divergences in mortality: a new approach of health transition. Demogr Res. 2004;10:11-44. doi: 10.4054/DemRes.2004.S2.2.
2. Shkolnikov VM, Meslé F, Vallin J. Health crisis in Russia I. Recent trends in life expectancy and causes of death from 1970 to 1993. Popul Engl Sel. 1996;8:123-154.
3. Meslé F, Vallin J. Mortality and causes of death in 20th-century Ukraine. Dordrecht: Springer; 2012. 282 p. doi: 10.1007/978-94-007-2433-4.
4. Jasilionis D, Meslé F, Shkolnikov VM, Vallin J. Recent life expectancy divergence in Baltic countries. Eur J Popul. 2011;27(4):403-431. doi: 10.1007/s10680-011-9243-0.
5. Nemtsov AV, Levchuk NM, Davydov KV. [Alcohol-related mortality in Ukraine and Russia (1980-2007)]. [Narcology]. 2010;(10):26-35. Russian.

6. Meslé F, Vallin J. The end of east-west divergence in European life expectancies? An introduction to the special issue. Eur J Popul. 2017;33(5):615-627. doi: 10.1007/s10680-017-9452-2.
7. Penina O, Meslé F, Vallin J. Correcting of under-estimation of infant mortality in Moldova. Popul Engl Ed. 2010;65(3):499-514. doi: 10.2307/41061787.
8. Raevschi E. Overview of evolution of premature mortality from major cardiovascular diseases in the Republic of Moldova, 2003-2015. Mold Med J. 2017;3(60):46-49.
9. Penina O. Alcohol-related causes of death and drinking patterns in Moldova as compared to Russia and Ukraine. Eur J Popul. 2017;33(5):679-700. doi: 10.1007/s10680-017-9450-4.
10. Poniakina S. Causes and evolution of mortality disparities across regions in Ukraine [dissertation] [Internet]. Paris: Université Paris 1 Panthéon-Sorbonne; 2014 [cited 2021 Apr 12]. Available from: https://tel.archives-ouvertes.fr/tel-02187996/file/2014-12-PONIAKINA-cau.pdf
11. Timonin S, Danilova I, Andreev E, Shkolnikov VM. Recent mortality trend reversal in Russia: Are regions following the same tempo? Eur J Popul. 2017;33(5):733-763. doi: 10.1007/s10680-017-9451-3.
12. Grigoriev P, Doblhammer-Reiter G, Shkolnikov V. Trends, patterns, and determinants of regional mortality in Belarus, 1990–2007. Popul Stud. 2013;67(1):61-81. doi: 10.1080/00324728.2012.724696.
13. Grigoriev P, Jasilionis D, Shkolnikov VM, Meslé F, Vallin J. Spatial variation of male alcohol-related mortality in Belarus and Lithuania. Eur J Public Health. 2016;26(1):95-101. doi: 10.1093/eurpub/ckv060.
14. Chiang CL; World Health Organization. Life table and mortality analysis [Internet]. Geneva: WHO; 1979 [cited 2021 Mar 23]. Available from: https://apps.who.int/iris/bitstream/handle/10665/62916/15736_eng.pdf;sequence=1
15. Altman DG, Machin D, Bryant TN, Gardner MJ, editors. Statistics with confidence: confidence intervals and statistical guidelines. 2nd ed. London: BMJ; 2000.
16. Andreev EM, Shkolnikov VM. An Excel spreadsheet for the decomposition of a difference between two values of an aggregate demographic measure by stepwise replacement running from young to old ages [Internet]. Rostock: Max Planck Institute for Demographic Research; 2012 [cited 2021 Apr 12]. Available from: https://www.demogr.mpg.de/papers/technicalreports/tr-2012-002.pdf. doi: 10.4054/MPIDR-TR-2012-002.
17. Riffe T. Package 'DemoDecomp'. Decompose Demographic Functions [R package version 1.0. 1]. 2018. Avilable from: https://cran.r-project.org/web/packages/DemoDecomp/DemoDecomp.pdf
18. DIVA-GIS [computer program] [Internet]. [cited 2021 Jul 29]. Available from: https://www.diva-gis.org/
19. Lovelace R, Nowosad J, Muenchow J. Geocomputation with R [Internet]. 2021 [cited 2021 Apr 16]. Available from: https://geocompr.robinlovelace.net/
20. Anselin L. Local Indicators of Spatial Association - LISA. Geogr Anal. 1995;27(2):93-115. doi: 10.1111/j.1538-4632.1995.tb00338.x.
21. Valcov V. Principalele rezultate ale Recensământului Populației și al Locuințelor (RPL) 2014 [The main results of the 2014 census] [Internet]. Chisinau, 2017 Mar 31 [cited 2020 Feb 26]. Available from: https://statistica.gov.md/public/files/Recensamint/Recensamint_pop_2014/Rezultate/RPL.pdf
22. World Health Organization. Prevalence of noncommunicable disease risk factors in the Republic of Moldova, STEPS 2013 [Internet]. Copenhagen: WHO Regional Office for Europe; 2014. 221 p. [cited 2020 Feb 10]. Available from: http://www.euro.who.int/en/countries/republic-of-moldova/publications/prevalence-of-noncommunicable-disease-risk-factors-in-the-republic-of-moldova.-steps-2013-2014

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Author's contribution
OP conceptualized the idea, conducted literature review, collected the data, interpreted the data, and wrote the manuscript.

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Ethics approval and consent to participate
No approval was required for this study.

Conflict of Interests
There is no known conflict of interests to declare.

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Clinical and paraclinical manifestations in patients suspected of being infected with COVID-19

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Abstract

Background: It was intended to study the structure of the symptoms in patients suspected of having SARS-CoV-2 virus infection, as well as to find any correlations between the clinical, paraclinical and radiological manifestations in positive versus negative patients, in order to further facilitate the diagnosis and triage of patients.
Material and methods: 101 patients seeking medical attendance at the COVID-19 Triage Center in Chisinau have been examined, presenting various respiratory symptoms. The frequency of symptoms and the results of the paraclinical investigations were evaluated based on the results of the PCR tests for SARS-CoV-2 infection and the assessment of correlations (Pearson).
Results: Out of 101 subjects, 50 tested SARS-CoV-2 positive, and the remaining 51 – negative. The clinical manifestations of SARS-CoV-2 suspects were as follows: fatigue – 72%, sweating – 54%, chills – 52%, fever – 49%, subfebrility – 39%, myalgias and arthralgias – 37%, cough – 35% (sputum – 17% and hemoptysis – 2%), dyspnea – 34%, chest pain – 23%, anosmia – 12%, headache – 11%, dyspeptic syndrome – 8%. Infiltrates on chest radiography were found in 22% of cases.
A weak inverse correlation ($R = -0.22$, $P < 0.05$) between the leukocyte count and SARS-CoV-2 test results was found. An average direct correlation between the presence of fever ($R = 0.36$, $P < 0.05$) and a positive COVID-19 test was also noticed.
Conclusions: Certain symptoms such as anosmia were more commonly seen in patients with positive COVID-19 tests. The absence of pulmonary infiltrates and the presence of dyspnoea have been negative predictive factors for COVID-19. Leukopenia has been noticed only in SARS-CoV-2 positive patients. Subfebrility has not shown a predictive significance of COVID-19.
Key words: COVID-19, dyspnea, leukopenia, anosmia.

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Introduction

COVID-19 is a respiratory condition where pulmonary manifestations are the main clinical presentations of the disease. According to the reported studies, SARS-CoV-2 infection is not limited to the respiratory system, it does affect other organs, as well. Renal dysfunction, gastrointestinal complications, liver failures, cardiac manifestations, neurological abnormalities and hematological manifestations are among the reported extrapulmonary features [1].

Studies report a timeframe of 6 to 41 days from the onset of COVID-19 symptoms until death, with a median of 14 days [2].

The most commonly reported symptoms at the onset of COVID-19 disease are fever, cough, and fatigue, while other symptoms include the presence of sputum, headache, hemoptysis, diarrhea, dyspnea, and lymphopenia [3-5].

Laboratory tests showed leukopenia, of which 70% were neutrophils. Additionaly, increased values of blood C-reactive protein were observed. A high sedimentation rate of erythrocytes and elevated D-dimers have also been found [6].

Studies report the presence of various common symptoms for many viral respiratory diseases such as: fever – 83%, cough – 82%, dyspnea – 31%, myalgia – 11%, confusion – 9%, headache – 8%, sore throat – 5%, rhinorrhea – 4%, chest pain – 2%, diarrhea – 2%, nausea and vomiting – 1% [7].

Therefore, the early diagnosis of SARS-CoV-2 infection has become a challenge for the clinician, and the delayed results of the PCR tests, as well as the presence of false-negative results, have made it even more difficult to establish the diagnosis. Currently there is no test with a specificity and sensitivity of 100% [8].

Thus, it was intended to study the structure of symptoms in patients suspected of having the SARS-CoV-2 virus infection, and to find the correlations between the clinical and paraclinical manifestations in positive versus negative patients, in order to further facilitate the diagnosis and triage of patients.

Material and methods

The current study involved 101 patients (51 men and 50 women), suspected of having the SARS-CoV-2 infection, who sought medical attendance at the Covid-19 Center in Chisinau between April and July 2020, in a state of mild and average severity, presenting various respiratory symptoms. The patients' age varied between 18 and 84 (the average age being 49.3 years). The disease history was collected and a paraclinical examination was performed (full blood count, chest radiography, ECG, SARS-CoV-2 PCR test). The frequency of symptoms was assessed according to the results of the PCR tests for SARS-CoV-2 and the assessment of the correlations (Pearson) between clinical and paraclinical data.

The statistical data was processed using the SPSS program, with a value of „p" less than 0.05 being considered statistically significant.

Results

51 negative and 50 positive tests for SARS-CoV-2 were obtained. The clinical manifestations (in descending order) of SARS-CoV-2 positive patients were as follows: fatigue – 72%, sweating – 54%, chills – 52%, fever – 49%, subfebrility – 39%, myalgias and arthralgias – 37%, cough – 35% (sputum – 17% and hemoptysis – 2%), dyspnea – 34%, chest pain – 23%, anosmia – 12%, headache – 11%, dyspeptic syndrome – 8% (fig. 1). The presence of infiltrates on chest radiography was identified by performing a chest radiography in 21 patients, of which 12 patients were COVID-19 positive and 9 had negative SARS-CoV-2 tests.

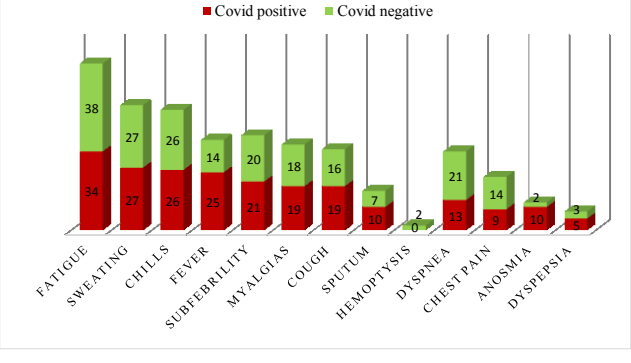

Fig. 1. Structure of symptoms according to the results of the SARS-CoV-2 test

The number of days from the onset of symptoms until testing varied from 1 to 60 days (average length 7.08 days), the results being different depending on the test result. Thus, it was observed that the earlier the onset of symptoms, the greater the chances of testing positive for COVID-19 (fig. 2).

A great number of the suspects presented with multiple comorbidities, making it more difficult to establish the diagnosis, many symptoms being similar for other pathologies (fig. 3).

In 55% of the patients with a negative test, at least one concomitant pathology has been detected. And in those with positive results, multiple morbidity was detected in 58% of cases.

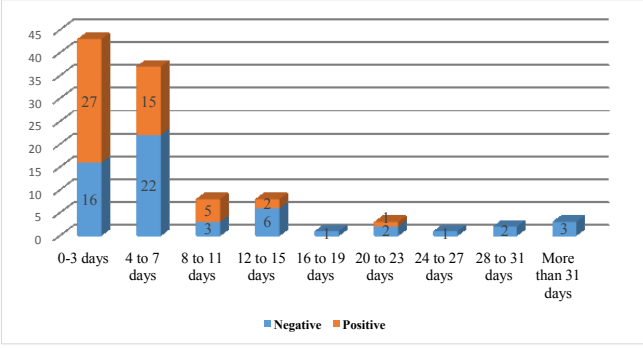

Fig. 2. The number of days from the disease onset

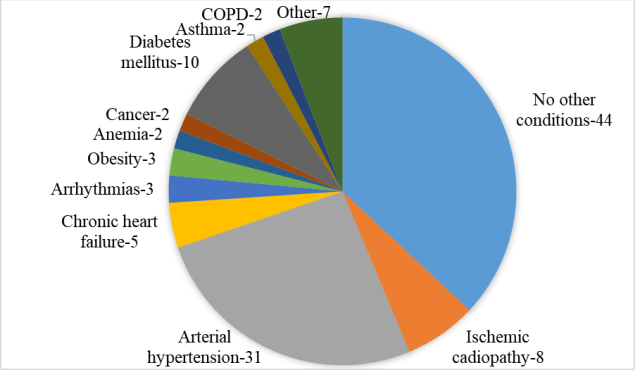

Fig. 3. Structure of multiple morbidities in COVID-19 suspects

The O_2 saturation of the patients was measured on admission, thus values between 83% and 100% were obtained (the average SaO_2 being 96.83%). A weak correlation was measured between the saturation on admision and the SARS-CoV-2 test result, being statistically insignificant (R = 0.06; p> 0.05).

A full blood count was performed in all patients, leukocyte values and erythrocyte sedimentation rate (ESR) were also investigated. A weak, statistically significant inverse correlation was found between the leukocyte numbers and the SARS-CoV-2 test results (R = -0.222; p <0.05), (fig. 4).

Lymphocyte values were also assessed compared to the COVID-19 test results. No correlation was determined between the lymphocyte count and SARS-CoV-2 test results (fig. 5).

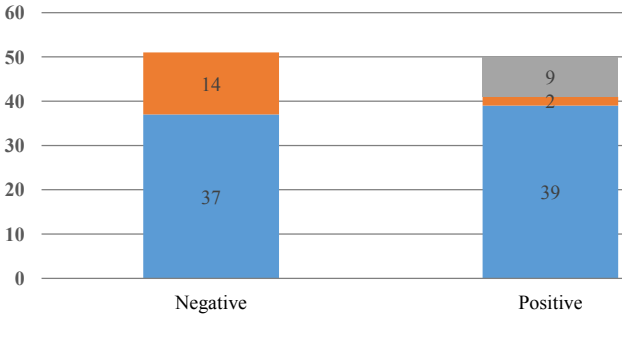

Fig. 4. COVID-19 test results and leukocyte values

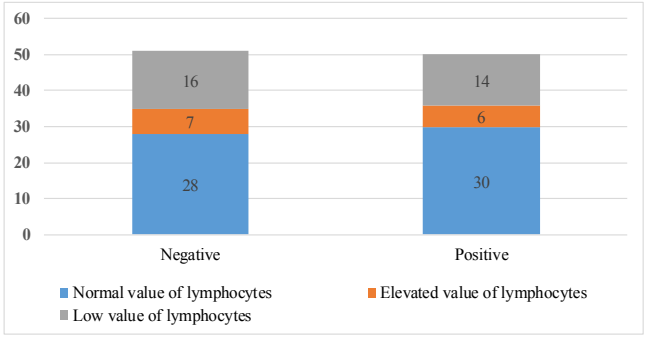

Fig. 5. COVID-19 test results and lymphocyte values

The correlation between the SARS-CoV-2 test results and the ESR values was also measured, obtaining a weak direct correlation, being statistically insignificant (R = 0.04; p> 0.05).

A mean direct correlation (R = 0.41, P <0.05) was found between dyspnea and the presence of pulmonary infiltrates on chest radiography in SARS-CoV-2 positive patients and its absence in patients with negative test results (R = 0.18, P> 0.1).

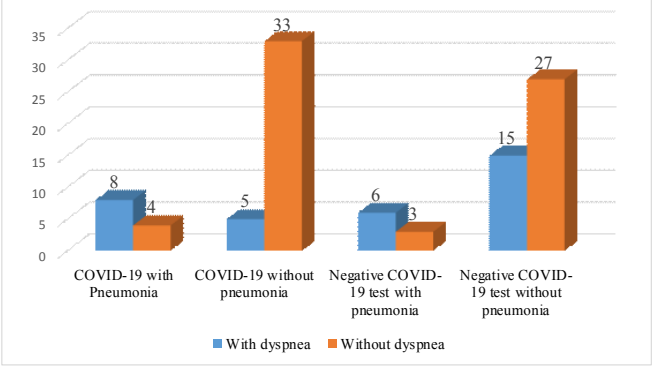

Fig. 6. Correlation between dyspnea and pulmonary infiltrates based on the test results

An average direct correlation was noticed between fever (R = 0.31, P <0.05) and pneumonia in the SARS-CoV-2 positive patients, with its absence in subjects with negative test results. An average direct correlation between fever (R = 0.36, P <0.05) and a positive COVID-19 test result was also observed. Subfebrility was found to be of approximately the same frequency in patients with positive and negative tests for COVID-19.

Discussion

Within the current observational study based on data gained from 101 patients suspected of having a COVID-19 infection, the presence of many symptoms common for respiratory tract infections was noticed, the most common of them being fever, signs of general intoxication and cough. Also, some symptoms such as anosmia have been detected much more frequently in patients with positive SARS-CoV-2 test results. Other studies in the field have shown similar results [9].

Furthermore, dyspnea and chest pain were more common in patients with negative SARS-CoV-2 test results. This phenomenon could be explained by the presence of multiple concomitant pathologies that were also suspected, such as ischemic heart disease, cardiac failure or pulmonary obstructive pathologies [10].

Low leukocyte values in suspect patients could be a strong predictor of SARS-CoV-2 infection, as all 9 patients with leukopenia were diagnosed with COVID-19. Leukocytosis has been found more frequently in patients with negative test results, which could be explained by exacerbation of chronic conditions, such as asthma, chronic obstructive pulmonary disease or some acute conditions such as pyelonephritis.

The values of lymphocytes in suspect patients was of no significance in establishing the presumptive diagnosis of COVID-19, as lymphopenia and lymphocytosis were also present in patients with negative test results. These results refer to patients with a mild and average severity of COVID-19 [11].

Dyspnea was more common in patients with negative SARS-CoV-2 tests, being present in patients who also had radiological imaging findings, but the presence of dyspnea without radiological changes was also a negative predictor of COVID-19. Fever and subfebrility were found in 80% of patients.

Conclusions

1. Anosmia was found 5 times more frequently in test-positive patients, thus being a strong predictor in a suspect case of COVID-19. Other symptoms were found with approximately the same frequency in patients with negative COVID-19 test results.

2. Dyspnea without any lung damage is 3.6 times more common in patients with negative COVID-19 test results.

3. Lung damage was 1.3 times more common in patients with positive versus negative test results.

4. 33% of patients with COVID-19 and pneumonia had no dyspnea.

5. Leukopenia was found in 9% of suspects and only in patients with positive SARS-CoV-2 test results.

6. Fever was 1.8 times more common in patients with positive COVID-19 test results; however, subfebrility was found in a ratio of 1:1 within the study group.

References

1. Behzad S, Aghaghazvini L, Radmard AR, Gholamrezanezhad A. Extrapulmonary manifestations of COVID-19: Radiologic and clinical overview. Clin Imaging. 2020 Oct;66:35-41. doi: 10.1016/j.clinimag.2020.05.013.
2. Wang W, Tang J, Wei F. Updated understanding of the outbreak of 2019 novel coronavirus (2019-nCoV) in Wuhan, China. J Med Virol. 2020;92(4):441-447. doi: 10.1002/jmv.25689.
3. Ren LL, Wang YM, Wu ZQ, Xiang ZC, Guo L, Xu T. Identification of a novel coronavirus causing severe pneumonia in human: a descriptive study. Chinese Med J. 2020;133(9):1015-1024. doi: 10.1097/CM9.0000000000000722.

4. Huang C, Wang Y, Li X, Ren L, Zhao J, Hu Y. Clinical features of patients infected with 2019 novel coronavirus in Wuhan, China. Lancet. 2020;395(10223):497-506. doi: 10.1016/S0140-6736(20)30183-5.
5. Carlos WG, Dela Cruz CS, Cao B, Pasnick S, Jamil S. Novel wuhan (2019-nCoV) coronavirus. Am J Respir Crit Care Med. 2020;201(4):7-8. doi: 10.1164/rccm.2014P7.
6. Lei J, Li J, Li X, Qi X. CT imaging of the 2019 novel coronavirus (2019-nCoV) pneumonia. Radiology. 2020;295(1):18. doi: 10.1148/radiol.2020200236.
7. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, et al. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. Lancet. 2020;395(10223):507-513. doi: 10.1016/S0140-6736(20)30211-7.
8. Kost GJ. The impact of increasing disease prevalence, false omissions, and diagnostic uncertainty on coronavirus disease 2019 (COVID-19) test performance. Arch Pathol Lab Med. 2021;145(7):797-813. doi: 10.5858/arpa.2020-0716-SA.
9. Yang X, Yu Y, Xu J, et al. Clinical course and outcomes of critically ill patients with SARS-CoV-2 pneumonia in Wuhan, China: a single-centered, retrospective, observational study. Lancet Resp Med. 2020 May;8(5):475-481. doi: 10.1016/S2213-2600(20)30079-5.
10. Ejaz H, Alsrhani A, Zafar A, Javed H, Junaid K, Abdalla AE, et al. COVID-19 and comorbidities: deleterious impact on infected patients. J Infect Public Health. 2020;13(12):1833-1839. doi: 10.1016/j.jiph.2020.07.014.
11. Gustine JN, Jones D. Immunopathology of Hyperinflammation in COVID-19. Am J Pathol. 2021;191(1):4-17. doi: 10.1016/j.ajpath.2020.08.009.

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Low-dose anticholinergic therapy causes cognitive impairment in Parkinson's disease patients

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Abstract

Background: Before L-Dopa's discovery, anticholinergic drugs were among the first treatments for Parkinson's disease. Only now trihexyphenidyl (THP) is approved to treat unresponsive L-dopa tremors in young, cognitively unaffected Parkinson's disease patients. However, there are no specific recommendations for disease duration, medication dose, or cognitive status. In low-income countries, THP is still frequently used in Parkinson's disease patients with tremor. The objective of the current study was to evaluate cognitive performance in Parkinson's disease patients receiving a low dose of THP.
Material and methods: The study was performed on nineteen PD patients, nine of whom were on THP. All patients completed MoCA cognitive assessment. The patients were matched depending on their age, disease severity based on UPDRS III and duration of the disease.
Results: The THP patients were taking an average dose of 3.3 mg of THP daily for an average of 1.8 years. There were no statistical differences between THP patients and non-THP patients in age (64.8± 4.8 vs 67.2±6.9, p=0.4), UPDRS III (32.1±8.9 vs 41.5±20.6, p=0.2) and disease duration (6.2±4.9 vs 7.0 ± 4.0, p=0.7). The THP patients had lower cognitive performance, with a total MoCA of 19.22 ± 3.3 vs. non-THP patients 24.2±3.0, p=0.003.
Conclusions: In Parkinson's disease patients, even a low dose of THP causes significant cognitive loss.
Key words: Parkinson's disease, cognition, dementia, anticholinergic.

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Introduction

Parkinson's disease is one of the most common neurodegenerative diseases. Around the world, estimates of Parkinson's disease (PD) incidence range from 5 to 35 new cases per 100000 individuals per year [1]. In recent years, it has become apparent that PD has a wide range of non-motor features, including autonomic dysfunction, sleep disorders, hyposmia, and, as well as cognitive impairment [2-4].

Progressive degeneration of dopaminergic neurons in *substantia nigra pars compacta* is known to be the major neuropathological feature of PD. For the past 50 years, the most effective symptomatic treatment has been the administration of dopaminergic replacement drugs, especially levodopa [5]. However, later was observed that there are many symptoms of PD that do not respond to levodopa, such as axial symptoms, cognitive impairment or dementia [6]. In this context, recent evidence suggests that the degeneration of adrenergic, serotonergic, glutamatergic and cholinergic neurons also plays an important role [7].

According to recent studies, approximately 36% of PD patients develop dementia after 4 years of follow-up, and 83 percent have dementia after 20 years of follow-up, which is thought to be due to cholinergic deficiency [8]. As a result, cholinergic deficiency plays an important role in the pathophysiology and bio-cellular mechanisms of Parkinson's disease [9]. Cholinergic neurons, which are widely distributed throughout the brain parenchyma, play an important role in a variety of brain processes, including cognitive functions [6, 7]. According to a recent study, patients with dementia-free Parkinson's disease had moderate cholinergic dysfunction, whereas those with dementia associated with Parkinson's disease had severe cholinergic deficiency in various cortical regions [10].

One of the first Parkinson's disease treatments were anticholinergic drugs [11]. Currently, anticholinergics are approved for unresponsive L-dopa tremors in young and cognitively unaffected patients and because of a low price it is still widely used in low-income countries [12]. Regardless, a relevant issue arises because people with Parkinson's disease may have a subclinical cholinergic deficiency and the use of anticholinergic drugs may result in faster cognitive deterioration due to cholinergic system suppression, as well as a relatively unknown risk, the risk of long-term drug dependence. The goal of this study was to evaluate cognitive performance in Parkinson's disease patients taking a low

dose of trihexyphenidyl (THP) and patients who did not take any anticholinergic medications.

Material and methods

The study included 19 patients divided into two groups: 9 patients with Parkinson's disease who received trihexyphenidyl daily and 10 patients with Parkinson's disease who did not receive trihexyphenidyl at all. The two groups of patients were similar in terms of age and disease severity. The Unified Parkinson's Disease Rating Scale (UPRDS) part III was used to assess disease severity, and the Montreal Cognitive Assessment Test (MOCA) was used to compare cognitive status.

The statistical analysis was performed using SPSS, version 23.0. Data are expressed by mean and standard deviation in the case of normal distribution and median with IQR for variables that are non-normally distributed. The differences between the two groups of patients were analyzed with the T or Mann-Whitney test, as appropriate. In all analyses, p values <0.05 were considered significant.

The current research is a part of a larger project for research on PD that was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (protocol No 44 of 12.04.2018).

Results

Patients were divided into two groups: 9 Parkinson's disease patients who received trihexyphenidyl (THP group) were compared to 10 Parkinson's disease patients who did not receive trihexyphenidyl (non-THP group). Patients in the THP group received this medication for an average of 1.8 years, at a daily dose of 3.3 mg THP. The patients in the two groups did not differ in terms of age, disease duration, disease severity, as measured by UPDRS III. Patients' cognitive ability, as measured by the MoCA test, was statistically significant between patient groups (tab. 1).

Question number 3 of the MoCA test (attention) showed the largest statistical difference, so that patients in the THP group had an average value of 1.56 ± 0.5 compared to patients in the non-THP group, who had an average value of 2.7 ± 0.5, so the difference is statistically significant, p = 0.001. Among the separate items of UPDRS III only the kinetic tremor of the left upper limb was significantly different between both groups (p = 0.04), patients in the non-THP group being more affected.

Table 1. Demographic and clinical characteristics of both patients' groups

	THP (n=9)	non-THP (n=10)	p
Age (years)	64.8 ± 4.8	67.2 ± 6.9	0.4
Disease duration (years)*	6.2 ± 4.9	7.0 ± 4.0	0.5
UPDRS III	32.1 ± 8.9	41.5 ± 20.6	0.2
MoCA total	20 (5)	25 (6)	0.003

THP – group of patients administering trihexyphenidyl; non-THP – group of patients who did not administer trihexyphenidyl. Data are presented as mean ± standard deviation for all except marked data with *, presented as median (IQR). The value of p was calculated with the student t test, except for the marked data with *, which was calculated with the Mann-Whitney test due to abnormal data distribution. UPDRS III – the motor part of the Parkinson's disease disability scale. MoCA – Montreal cognitive assessment.

Discussion

There's no doubt that PD causes cognitive impairment, which leads to dementia and a higher mortality and morbidity rate. At the ten-year follow-up, 60% of patients are diagnosed with dementia; at the twenty-year over 83% are diagnosed with the disease. Anticholinergic medications diminish attentional function, which is most typically associated with alertness, and causes recent memory problems [8, 13]. Therefore, even if anticholinergics provide a benefit in terms of motor function, there is a high chance that the patients may acquire a cognitive issue earlier in the course of the disease. We found in this study that even a small daily dose of THP leads to a worse cognitive outcome.

In patients with PD, the density of cholinergic neurons is reduced in all cortical areas [14]. Degeneration of cholinergic neurons, however, is only one of the mechanisms underlying cognitive impairment, according to recent studies [7, 9]. Previous clinical observations with anticholinergic drugs, which were the first symptomatic treatment for patients with PD before the discovery of levodopa, led to the hypothesis that both cholinergic and dopaminergic signalling systems must be in balance for the striatum to function normally in movement control [11]. Anticholinergic medicines are thought to attenuate the increased striatal cholinergic tone generated by the loss of dopamine in PD patients, restoring the balance between these two signalling systems to some extent [6]. However, it is still unclear whether and how cholinergic disorders contribute to motor and non-motor symptoms. The broad degradation of cholinergic neurons in most patients with PD causes cognitive deficits at some point in the disease and long-term THP treatment has the potential to cause irreversible alterations in the brain, according to the findings of experimental studies on mice [15].

The prefrontal cortex and the frontal basal nuclei, which are severely affected by cholinergic deficiency, are also implicated in the control of gait. Gait and balance can only be coordinated with cognitive function [16, 17]. As a result, THP could affect independence and quality of life by exacerbating gait and balance problems.

Additionally, THP use is often accompanied by physical symptoms, such as dilated pupils, flushed skin, constipation, as well as rapid heart rate and ataxia [18]. Giving up this drug is extremely difficult, especially if a cognitive deficit has already developed. International guidelines recommend the use of THP only in young PD patients, without cognitive impairment, who have a low risk of developing dementia.

However, younger patients have a higher risk of developing drug abuse with THP.

Conclusions

As a result, it can be concluded that there are no clear data on the age of individuals who would benefit and what amount of THP would be effective for the treatment of PD motor symptoms without causing irreversible effects. No doubt this is a serious public health concern because THP is commonly used in the Republic of Moldova, and chronic THP use causes cognitive deterioration, dependence, and abuse even in a low dose.

References

1. Marek K, Jennings D, Lasch S, et al. The Parkinson Progression Marker Initiative (PPMI). Prog Neurobiol. 2011;95(4):629-635. https://doi.org/10.1016/j.pneurobio.2011.09.005.
2. Bosboom JL, Stoffers D, Wolters EC. Cognitive dysfunction and dementia in Parkinson's disease. J Neural Transm. 2004;111(10-11):1303-1315. https://doi.org/10.1007/s00702-004-0168-1.
3. Poewe W, Seppi K, Tanner CM, Halliday GM, Brundin P, Volkmann J, Schrag AE, Lang AE. Parkinson's disease. Nat Rev Dis Prim. 2017;3:1-21. https://doi.org/10.1038/nrdp.2017.13.
4. Ascherio A, Schwarzschild MA. The epidemiology of Parkinson's disease: risk factors and prevention. Lancet Neurol. 2016;15(12):1257-1272. https://doi.org/10.1016/S1474-4422(16)30230-7.
5. Carranza M, Snyder MR, Davenport Shaw J, Zesiewicz TA. Parkinson's disease: a guide to medical treatment. Torino: SEEd; 2013. 354 p.
6. Hilker R, Thomas AV, Klein JC, Weisenbach S, Kalbe E, Burghaus L, et al. Dementia in Parkinson's disease: functional imaging of cholinergic and dopaminergic pathways. Neurology. 2005;65(11). https://doi.org/10.1212/01.wnl.0000191154.78131.f6.
7. Ztaou S, Amalric M. Contribution of cholinergic interneurons to striatal pathophysiology in Parkinson's disease. Neurochem Int. 2019;126:1-10. https://doi.org/10.1016/j.neuint.2019.02.019.
8. Williams-Gray CH, Evans JR, Goris A, Foltynie T, Ban M, Robbins TW, Brayne C, Kolachana BS, Weinberger DR, Sawcer SJ, Barker RA. The distinct cognitive syndromes of Parkinson's disease: 5 year follow-up of the CamPaIGN cohort. Brain. 2009;132(Pt 11):2958-2969. https://doi.org/10.1093/brain/awp245.
9. Tanimura A, Pancani T, Lim SAO, Tubert C, Melendes AE, Shen W, Surmeier DJ. Striatal cholinergic interneurons and Parkinson's disease. Eur J Neurosci. 2018 May;47(10):1148-1158. doi: 10.1111/ejn.13638.
10. Gomperts SN. Lewy body dementias: dementia with Lewy bodies and Parkinson's disease dementia. Continuum (Minneap Minn). 2016;22:435-463. doi: 10.1212/CON.0000000000000309.
11. Pillon B, Dubois B, Duchesne N, Masson H. Acute and long-term administration of anticholinergics in Parkinson's disease: specific effects on the subcortico-frontal syndrome. Brain Cogn. 1999;40(2):289-313. doi: 10.1006/brcg.1999.1083.
12. Popil L, Gavriliuc O, Rotaru L, Gavriliuc M. Efectele tratamentului cu Trihexifenidil asupra funcției cognitive la pacienții cu boala Parkinson [Effects of trihexyphenidil treatment on cognitive function in patients with Parkinson's disease: review article]. [Bull Acad Sci Moldova]. 2020;(3):27-33. Romanian.
13. Sasikumar S, Strafella AP. Imaging mild cognitive impairment and dementia in Parkinson's disease. Front Neurol. 2020;11:1-8. https://doi.org/10.3389/fneur.2020.00047.
14. Deffains M, Adler A, Joshua M, Goldberg JA. Basal ganglia : acetylcholine interactions and behavior. In: Reference module in neuroscience and biobehavioral psychology. Elsevier; 2017. https://doi.org/10.1016/B978-0-12-809324-5.02520-7.
15. Tanda G, Ebbs AL, Kopajtic TA, Elias LM, Campbell BL, Newman AH, Katz JL. Effects of muscarinic M1 receptor blockade on cocaine- induced elevations of brain dopamine levels and locomotor behavior in rats. J Pharmacol Exp Ther. 2007;321(1):334-344. https://doi.org/10.1124/jpet.106.118067.
16. Gavriliuc O, Andrusca A, Gavriliuc M. Clinical indicators of gait freezing in Parkinson' s disease. Mold Med J. 2020;63(2):31-33. https://doi.org/10.5281/zenodo.3866002.
17. Morris R, Martini DN, Madhyastha T, Kelly VE, Grabowski TJ, Nutt J, Horak F. Overview of the cholinergic contribution to gait, balance and falls in Parkinson's disease. Parkinsonism Relat Disord. 2019;63:20-30. https://doi.org/10.1016/j.parkreldis.2019.02.017.
18. Torrents R, Ferré JF, Konareff A, Hemery P, Sherwin K, Lassalle C, Simon N, Scerra S. Misuse of Trihexyphenidyl (Artane) on Réunion Island. J Clin Psychopharmacol. 2018;38(3):250-253. https://doi.org/10.1097/JCP.0000000000000882.

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OG and AA drafted the first manuscript and analyzed the data, LP collected the data, MG designed the trial and revised the manuscript critically. All the authors revised and approved the final version of the manuscript.

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The authors have no conflict of interests to declare.

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Environmental toxic factors and clinical pattern of Parkinson's disease

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Abstract

Background: Parkinson's disease (PD) – the most common neuro-degenerative movement disorder – is considered a result of a multifactorial pathogenic process modulated by cumulative and interactive effects of genes and exposures. An environmental exposure could enhance or create dopaminergic neurons vulnerability and increase PD risk. The purpose of the study was to find if excessive exposure to toxic environmental factors may influence clinical pattern of PD.
Material and methods: The study was conducted on 111 patients diagnosed with PD, study group being defined as PD exposed to toxins (33 patients), control group including PD patients without toxin exposure (78 patients). General epidemiological data and clinical data were recorded.
Results: Toxin exposure was found in 33 patients (29.73%), more of them – men and rural residents. Toxin exposed PD patients had an insignificantly younger age. The most common disease phenotype in the study group was the akinetic-rigid phenotype (64.7%, p = 0.040), bradykinesia being the most common sign at the disease onset (57.6%, p = 0.008). Levodopa equivalent daily dose also was higher in the study group (659.02 ± 232.46, p = 0.042).
Conclusions: Excessive exposure to toxic environmental factors may influence the clinical pattern of PD. In this study the akinetic-rigid type was the predominant disease phenotype associated with toxin exposure. Doses needed for treatment were higher in PD patients exposed to toxins, as an indicator of a more severe motor impairment in this group.
Key words: Parkinson's disease, toxic environmental factors.

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Introduction

Parkinson's disease is the most common neuro-degenerative movement disorder with a prevalence of about 2% in the population over the age of 65 years [1].

PD has no known cause. The exposure of the human population to environmental contaminants is recognized as a significant contributing factor for the development of Parkinson's disease and other forms of parkinsonism. Evidence exists to suggest that age and gender and some environmental factors (pesticide exposure, occupation) are associated with the development of PD. Instead, tobacco use, and caffeine consumption are believed to be inversely associated to the development of PD, tobacco and black tea having a protective effect on age at onset in genetic PD – LRRK2 [2, 3].

PD varies in age of symptom onset, rate of progression, severity of motor and non- motor symptoms, extent of central and peripheral inflammation, maybe, because genetic and environmental factors act synergistically in PD pathogenesis.

A lot of recent research has focused particularly on genetic causes of PD. Although several genes have been implicated as monogenic causes of the disease, these genetic mutations are only responsible for approximately 10% of cases, indicating that the majority of PD is the result of a multifactorial pathogenic process [4] and environmental causes also may play a role in developing the disease. Rare (causative) and common (risk) variants associated with PD have been identified, including SNCA and LRRK2.

Some authors propose that environmental factors (pesticides and infections) increase the risk for PD via the immune system [5], because several of the genes associated with PD risk, function in the immune system. Peripheral immune activation and neuroinflammation in the brain contribute to neuropathology and neurodegeneration [6]. An environmental exposure that increases α-synuclein expression and/or inflammatory cytokine secretion could create a state in which dopaminergic neurons are vulnerable to immune-driven stress [5]. Increased kinase activity associated with the G2019S LRRK2 mutation may contribute to shifts in immune cell population frequencies and function [7]. In response to an exposure or experience, physiological and epigenetic regulation can occur, and the resulting cell-signaling cascades could influence disease pathogenesis [8]. The immune system's response to exposure may depend on the genetic variations or mutations of SNCA, LRRK2,

PINK1, and MHCII. There is a gene-by-environment-by-immune- system triangle in PD pathogenesis [5].

So, PD risk can be modulated by cumulative and interactive effects of genes and exposures [4]. One study showed that gene-environmental interactions increased the OR for PD from about 1.6 at the individual level up to OR 12.6 for some combinations [4].

Environmental factors such as drinking well water, rural living, farming, exposure to agricultural chemicals, farm and industrial compounds, exposure to different metals and industrial compounds as manganese, lead, copper, iron, zinc, aluminium or amalgam have been reported to be associated with the risk of developing PD [9-11].

So far, is known that toxin exposure can promote PD by several mechanisms: oxidative stress, dopamine homeostasis, calcium homeostasis, alpha-synuclein fibrillization, mitochondrial dysfunction neuroinflammation [12].

As toxic exposures to these compounds can result in a spectrum of PD and related disorders, it is imperative to identify, not only their mechanisms of action, but also shared clinical patterns to further delineate the resultant disorders for improving diagnosis, preventive strategies and therapeutic interventions [12].

Material and methods

These are preliminary data of a cohort study of Moldovan patients with incident of Parkinson's disease. Diagnosis of PD was based on widely acknowledged criteria [13]. Structured interview on medical and drug history, family history of neurological and psychiatric diseases, years of education, all previous and current occupations, previous and current lifestyle habits, details regarding exposure to pesticides, and other toxins were recorded and a general neurological and medical examination conducted. Severity of parkinsonism and disability were assessed by the Modified Unified Parkinson's Disease Rating Scale (MDS-UPDRS) [14]. Patients were subclassified at baseline into three groups according to Jankovic method: tremor dominant (TD), akinetic-rigid (AR) or intermediate (IND) [15]. The groups were defined as: (1) Toxic substances contact present (*ToxSC+*) – study group; and (2) absent (*ToxSC-*) – control group. The data analysis was performed via statistical program StatDirect, using descriptive, variation, and correlational analysis. Student's t tests or Mann-Whitney tests were used as appropriate. P values less than 0.05 were considered statistically significant.

Results and discussion

These are preliminary data of a cohort study of Moldovan patients with incident Parkinson's Disease (PD). The study group consisted of 111 consecutive PD patients. The mean age in the cohort was 64.87 ± 7.69 years. By sexes, PD patients, were distributed as follows: 48 were women (43.2%) and 63 were men (56.8%)).

Contact with toxic substances (*ToxSC)*, was recorded by history taking about previous and current occupations, previous and current lifestyle habits, exposure to pesticides, and other toxins; and was found in 33 patients (29.73%). In this study, excessive exposure to toxic environmental factors was more common in men and rural residents. Among *ToxSC+* patients, 30 patients (90.9%) were men and 3 (9.1%) patients were women (p=0.000). Nineteen of the toxin exposed patients (57.6%, p=0.000) were rural residents. Similarly, in a meta-analysis of PD risk, RR was statistically significant for: rural living (RR = 1.43; 95% CI = 1.22–1.69), farming (RR = 1.24; 95% CI = 1.12–1.37) and well-water consumption (RR = 1.30; 95% CI = 1.12–1.51); the association between pesticide use and PD was statistically significant for all studies combined (RR = 1.22; 95% Cl = 1.18–1.27); and use of herbicides (RR = 1.20; 95% CI = 1.06–1.36) or insecticides (RR = 1.32; 95% CI = 1.14–1.52) was associated with statistically significantly increased PD risk [8]. Also, in Norwegian ParkWest study, agricultural work was associated with a higher risk of PD (OR 1.75 (1.03–3.0) P 5 0.009): PD patients were more often agricultural workers than controls (23% PD vs 14.4% of controls, P 5 0.026 [$\chi 2$ test], odds ratio [OR] 1.75 [1.03–3.0]); whether patients or controls were born and raised on a farm did not affect the risk of PD [9].

Different toxic exposure was found in the study group: petrol intake (10 patients (9%)), diesel intake (4 patients (3.6%)), petrol + diesel intake (6 patients (5.4%)), exposure to pesticides (7 patients (6.3%)), to solvents – 3 patients (2.7%), to reinforced concrete (polystyrene) – 1 patient (0.9%), to welding gas – 1 patient (0.9%), to freon – 1 patient (0.9%).

In a study, prevalence of parkinsonism among active male welders age 40 to 69 statewide was 977 to 1336 cases/100000 population. The prevalence of Parkinsonism was higher among welders vs age-standardized data for the general population (prevalence ratio – 10.19, 95% CI 4.43 to 23.43). The authors concluded that the estimated prevalence of parkinsonism was higher within a sample of male welders vs the general population of males [16]. By contrast, the results of Danish Cohort Study (5867 Danish welders and 1735 non- welding metal workers exposed to welding fume from general Danish population in 1987–2008) do not support the hypothesis that welders are at increased risk for Parkinson's disease [17]. It was established that parkinsonism can occur after chronic exposure to high levels of manganese, usually above the permissible exposure limit ceiling at 5 mg/m3 total dust set by the Occupational Safety and Health Administration [18].

In the present study was found that in *ToxSC+* patients, the disease began at an insignificantly younger age than in *ToxSC-* patients (59.24 ± 6.93 vs 60.95 ± 8.86 years, p <0.005). By history taking, was established, that bradykinesia was the most often PD onset symptom in *ToxSC+* patients (57.6%, p = 0.008). Applying the Jankovic method of defining PD motor phenotype, the more frequent PD phenotype in *ToxSC+* patients was the akinetic-rigid one (64.7%, p = 0.040).

There are interesting clinical differences between subgroups of PD patients (tremor dominant (TD) vs postural

instability gait difficulties PIGD), provided by literature. In a study, a protective association of alcohol and smoking was only seen in postural instability gait difficulties (PIGD) – akinetic-rigid PD and not in tremor dominant (TD) PD [9], may be because the underlying pathogenic mechanisms are heterogeneous, including environmental exposure.

Levodopa equivalent daily dose needed to compensate motor impairment in *ToxSC+* patients was significantly higher than in the control group (659.02 ± 232.46 vs 483.77 ± 355.41, p = 0.042), as an indicator of a more severe motor disability in PD patients exposed to environmental toxic factors.

Overall, studies suggest that environmental insults may play an important role in the appearance and progression of PD pathology [19], PD onset and its clinical presentation may be due to a combination of external aggressors and individual genetic susceptibility to this aggression; and low incidence of PD suggests that gene-environment interactions play an important role in the process. According to our results excessive exposure to toxic environmental factors was more common associated with the akinetic-rigid type of Parkinson's disease – a phenotype with more severe motor impairment. And *ToxSC+* patients needed higher doses of dopaminergic drugs – an indicator of a more motor impairment in this category.

Conclusions

Environmental factors may play an important role in the appearance, progression and clinical presentation of Parkinson's disease. This study, replicated that excessive exposure to toxic environmental factors is more commonly found in men and rural residents. According to the received results, toxin exposure was more frequently associated with the akinetic-rigid type of Parkinson's disease and with higher doses of dopaminergic drugs needed for motor symptoms control, indicating a higher severity of motor impairment in toxin exposed PD patients.

References

1. Von Campenhausen S, Bornschein B, Wick R, et al. Prevalence and incidence of Parkinson's disease in Europe. Eur Neuropsychopharmacol. 2005;15(4):473-490. doi: 10.1016/j.euroneuro.2005.04.007.
2. Lüth T, König IR, Grünewald A, et al. Age at onset of LRRK2 p.Gly2019Ser is related to environmental and lifestyle factors. Mov Disord. 2020;35(10):1854-1858. doi: 10.1002/mds.28238.
3. Galanaud JP, Elbaz A, Clavel J, et al. Cigarette smoking and Parkinson's disease: a case-control study in a population characterized by a high prevalence of pesticide exposure. Mov Disord. 2005;20(2):181-189. doi: 10.1002/mds.20307.
4. McCulloch CC, Kay DM, Factor SA, et al. Exploring gene-environment interactions in Parkinson's disease. Hum Genet. 2008;123(3):257-265. doi: 10.1007/s00439-008-0466-z.
5. Kline EM, Houser MC, Herrick MK, et al. Genetic and environmental factors in Parkinson's disease converge on immune function and inflammation. Mov Disord. 2021;36(1):25-36. doi: 10.1002/mds.28411.
6. Hirsch EC, Standaert DG. Ten unsolved questions about neuroinflammation in Parkinson's disease. Mov Disord. 2021;36(1):16-24. doi: 10.1002/mds.28075.
7. Wallings RL, Herrick MK, Tansey MG. LRRK2 at the interface between peripheral and central immune function in Parkinson's disease. Front Neurosci. 2020;14:1-14. doi: 10.3389/fnins.2020.00443.
8. Breckenridge CB, Berry C, Chang ET, et al. Association between Parkinson's disease and cigarette smoking, rural living, well-water consumption, farming and pesticide use: systematic review and meta-analysis. PLoS One. 2016;11(4):1-42. doi: 10.1371/journal.pone.0151841.
9. Skeie GO, Muller B, Haugarvoll K, et al. Differential effect of environmental risk factors on postural instability gait difficulties and tremor dominant Parkinson's disease. Mov Disord. 2010;25(12):1847-1852. doi: 10.1002/mds.23178.
10. Pan-Montojo F, Reichmann H. Considerations on the role of environmental toxins in idiopathic Parkinson's disease pathophysiology. Transl Neurodegener. 2014;3:1-13. doi: 10.1186/2047-9158-3-10.
11. Tanner CM, Kame F, Ross GW, et al. Rotenone, paraquat, and Parkinson's disease. Environ Health Perspect. 2011;119(6):866-872. doi: 10.1289/ehp.1002839.
12. Caudle WM, Guillot TS, Lazo CR, et al. Industrial toxicants and Parkinson's disease. Neurotoxicology. 2012;33(2):178-188. doi: 10.1016/j.neuro.2012.01.010.
13. Postuma RB, Poewe W, Litvan I, et al. Validation of the MDS clinical diagnostic criteria for Parkinson's disease. Mov Disord. 2018;33(10):1601-1608. doi: 10.1002/mds.27362.
14. [Goetz CG, Tilley BC, Shaftman SR, et al. Movement Disorder Society-Sponsored Revision of the Unified Parkinson's Disease Rating Scale (MDS-UPDRS): Scale presentation and clinimetric testing results. Mov Disord. 2008;23(15):2129-2170. doi: 10.1002/mds.22340.
15. Mazakis A. Variable Expressions. I was waiting to see what you would do first. Fayetteville: University of Arkansas Press; 2020. p. 46-47.
16. Cox LA. Prevalence of parkinsonism and relationship to exposure in a large sample of Alabama welders. Neurology. 2006;66(4):616-617. doi: 10.1212/01.wnl.0000216107.19411.e8.
17. Kenborg L, Funch Lassen C, Hansen J, et al. Parkinson's disease and other neurodegenerative disorders among welders: a Danish cohort study. Mov Disord. 2012;27(10):1283-1289. doi: 10.1002/mds.25125.
18. Brenner SR. Searching for a relationship between manganese and welding and Parkinson's disease. Neurology. 2006;66(3):458-459. doi: 10.1212/01.wnl.0000209200.33974.b4.
19. Ball N, Teo WP, Chandra S, et al. Parkinson's disease and the environment. Front Neurol. 2019;10:218. doi: 10.3389/fneur.2019.00218.

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Drug-resistant epilepsy: modern concepts, integrative mechanisms, and therapeutic advances

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Abstract

Background: Drug-resistant epilepsy is the cause of severe disability. Multiple questions remain unanswered both in terms of pathogenesis and therapeutic management. For this narrative review, PubMed database and Infomedica library were searched by using "drug-resistance in epilepsy" and "treatment of drug-resistant epilepsy" as key words. The following filters were applied: "Clinical Trial", "Meta-analysis", "Multicenter Study", and "Randomized Controlled Trial", covering the period of 01.01.2005–06.01.2021.Several hypotheses have been proposed, i.e., pharmacokinetic, intrinsic severity, gene, target, transporter, and neural network hypotheses. Many controlled trials showed different results in terms of seizure control after combined methods of therapies. Immunotherapy, palliative epilepsy surgery alone or associated with neurostimulation procedures including vagus nerve, trigeminal nerve, or deep brain stimulation may be efficient, however, seizure freedom is not always achieved. Genetic epilepsies might benefit from gene and exosome therapy; however, further studies are needed to verify their safety.
Conclusions: Neuroscience of drug-resistant epilepsy faces many challenges. Inflammatory mediators, biomarkers, and genes might allow the identification of new treatment targets, contribute to an earlier diagnosis, and assess the clinical outcomes.
Key words: drug-resistant epilepsy, hypotheses of drug-resistance, therapeutic advances.

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Introduction

Epilepsy is a relatively common neurological disease defined by two unprovoked seizures (or one unprovoked seizure with the likelihood of more) that were not caused by some known and reversible medical condition [1]. It affects about 0.5–1% of the population worldwide or absolutely about 50 million of population, around half of them (24 millions) diagnosed with active idiopathic epilepsy [2]. Approximately 10% of world population will experience a seizure at some point during their lifetime [3].The incidence of epilepsy is about 61 per 100000 persons-years and was higher in low/middle-income countries than in high-income countries, 139.0 vs 48.9 [3]. Epilepsy is still an important cause of disability (25% of people with epilepsy have intellectual disability [4]), mortality, and is surrounded by prejudice and stigma – a well-documented barrier to health seeking behavior, engagement in care and adherence to treatment across a range of health conditions globally [5], perceived by cohabiting relatives of people with epilepsy and surrounded people. Higher perceived stigma is significantly related to generalized seizures, longer disease duration [6], and higher perceptions of stigma are associated with worse quality of life in people with epilepsy. SUDEP is a life-threatening condition with an estimated incidence of 0.58 to 9.0 per 1000 persons-years with a 35% of lifetime cumulative risk in refractory epilepsy patients [7] that is playing an important role in the quality of life of these patients. People with epilepsy have a higher risk of sudden death, with reported annual incidences of 1 per 1000. For those with uncontrolled epilepsy, the incidence is higher as 1 per 200 [8], but the lowest incidence is in children and consists 0–0.2 per 1000 [9]. The risk of sudden death is greater in women, representing 1.45 per 1000 than in men 0.98 per 1000 [10]. The epilepsy treatment with antiepileptic drugs has limited ef-

fectiveness. In case of correct diagnosis, 70% of patients with epilepsy are drug sensitive and seizure free, 50% of them after initial monotherapy [11], 10% in context of alternative monotherapy and 10% with first add-on or polytherapy. The remaining 30% are drug resistant. These data may fluctuate with 25% range of drug-resistance [12].

The modern definition of drug resistance

The concept drug-resistant epilepsy means pharmacoresistant, drug resistant, refractory or medically intractable epilepsy [13]. The definition evaluated during the last years as: a) seizures which have not been completely controlled with AEDs 1 year after onset despite accurate diagnosis and carefully monitored treatment [14]; b) seizures of sufficient frequency and severity after 2 years of AED treatment [15]; c) the patient is not seizure free after 1 year treatment with 2–3 AED [16]. The ILAE consensus concerning definition was obtained in 2009 and it is: failure of adequate trials of two tolerated and appropriately chosen and used AEDs (whether as monotherapies or in combination) to achieve sustained seizure freedom [13]. Discussing about pharmacoresistant epilepsy is very important to rule out cases of nonadherence in epilepsy and cases of pseudorefractory epilepsy [17]The approach of patient with pharmacoresistant epilepsy is difficult and the first step would be the diagnosis reconsideration. The surgery is the superior option in treatment of these patients, but among the 30% of patients with confirmed diagnosis of pharmacoresistant epilepsy only 10–15% become candidates for epilepsy surgery [18].

Mechanisms of drug resistance

Nowadays understanding the multifactorial mechanisms underlying drug-resistant epilepsy has the potential to contribute to more effective development of treatment options for patients with epilepsy. The combination of multiple mechanisms expressed in each individual patient represents the most popular hypothesis. A key limitation of the entire research is the difficulty of demonstrating whether the changes associated with drug-resistant epilepsy are an epiphenomenon of epileptogenesis [19].

Intrinsic severity hypothesis. Rogawski and Johnson proposed the hypothesis that the resistance of antiepileptic drugs is not due to specific drug resistance factors, but due to the degree of severity of epilepsy that is directly related to the response to treatment. This "intrinsic severity hypothesis" was later updated by Rogawski, who postulates that drug resistance is an inherent property of epilepsy that is directly related to the severity of the disease. The increased frequency or density of seizures that precede the onset of antiepileptic therapy is the most important factor associated with a reduced chance of long-term remission of the disease [20]. Although the high frequency of seizures is a predictor of drug resistance, but it is clearly not the only one. The intrinsic severity hypothesis says that common neurobiological factors contribute to both the severity of epilepsy and pharmaceutical co-resistance. Other features of the severity of the pathological condition, such as the extent of structural damage or behavioral phenotype, are also predictors of resistance to antiepileptic drugs. Although the intrinsic severity hypothesis seems biologically possible, it was not applied to disease with a fluctuating or evolving pattern of resistance. In addition, there is very limited evidence to support a direct link between the severity of epilepsy and response to treatment [21]. In this regard, data from studies supporting the hypothesis of intrinsic severity suggest that the high frequency of pre-treatment seizures is an important predisposing factor for refractory epilepsy [22]. A similar conclusion was drawn from a study on children diagnosed with epilepsy, which showed that administration of anticonvulsant therapy during the period of first ten seizures had no aggravating or early remission effect [23]. In another randomized study of 1847 patients with epilepsy, the authors compared the effectiveness of immediate treatment with that of the delayed treatment and found that immediate treatment was associated with reduced seizure frequency in the first 1–2 years, but long-term remission rates did have no difference between the two groups. Therefore, it has been suggested that the theory of intrinsic severity does not sufficiently explain the mechanism of drug resistance in epilepsy [21].

Target hypothesis. Among the various mechanisms of drug resistance that have been proposed, the target hypothesis postulates that acquired (epilepsy-induced) alterations to the structure and/or functionality of brain targets of antiseizure drugs (ASDs) lead to a reduction in their sensitivity to treatment and thus lead to refractoriness [24]. To exhibit antiseizure activity, a drug must act on one or more target molecules in the brain, and these targets include voltage-dependent ion channels, neurotransmitter receptors, and transporters or metabolic enzymes involved in the release, uptake, and metabolism of neurotransmitters [25].

The target hypothesis is primarily based on studies with carbamazepine on voltage-gated sodium channels in hippocampal neurons (CA1 and dentate granule cells). Remy et al. [26] showed that the use dependent block of voltage-dependent Na+ channels of dentate granule cells by carbamazepine is completely lost in patients with carbamazepine-resistant temporal lobe epilepsy (TLE), and the fast recovery from inactivation of the fast Na+ current was carbamazepine-insensitive in pharmacoresistant patients [27]. Also, the authors suggested that a loss of Na+ channel drug sensitivity could explain the development of drug-resistant epilepsy (DRE). In another study, Remy et al. [28] demonstrated that the effects of phenytoin on fast recovery from inactivation of Na+ channels of hippocampal granule neurons were significantly reduced, though not as pronounced as observed with carbamazepine, and that lamotrigine slowed the time course of recovery from fast inactivation. Thus, these results suggested that target mechanisms of drug resistance are cell type- and ASD-specific. Newly, Doeser et al. [29] reported that eslicarbazepine may possess advantages over conventional Na+ channel modulators, because it maintained activity in chronically epileptic tissue. One possibility for altered

sensitivity of Na+ channels in CA1 or dentate granule cells in epileptic tissue is that the subunit composition of these channels is altered, resulting in channels with lower ASD sensitivity [24].

Other drug targets, such as $GABA_A$ receptors, may be altered in patients and animal models with intractable epilepsy [27]. Profound alterations in $GABA_A$ receptor subtype expression have also been reported in adult patients with ASD-resistant TLE and pediatric epilepsy patients undergoing epilepsy surgery. Brooks-Kayal et al. [30], using the rat pilocarpine model of TLE, demonstrated that expression of $GABA_A$ receptor subunit mRNAs is substantially altered in hippocampal dentate granule cells of pilocarpine treated rats versus controls. These changes in $GABA_A$ receptor subunit expression correlated with profound alterations in receptor function and pharmacology. In addition to the enhanced zinc sensitivity, $GABA_A$ receptors from the epileptic hippocampus lose their sensitivity to augmentation by the benzodiazepine site modulator zolpidem. However, none of these studies examined whether ASD-resistant epileptic rats differ from responsive rats in these changes in $GABA_A$ receptor function [27].

Although the target hypothesis is a biologically plausible theory to explain drug resistance, the fact that most drug-resistant patients are resistant to several ASDs acting on different therapeutic targets undermines the general utility of the target hypothesis and instead supports the existence of a mechanism nonspecific to individual ASDs [31].

Transporter hypothesis. The transporter hypothesis is based on two assumptions: (1) overexpression of efflux transporters correlates with pharmacoresistance in epilepsy and (2) antiepileptic drugs (AEDs) are subject to active transport by efflux transporters [31].

Overexpression of efflux transporters at the blood–brain barrier is discussed as one factor that might limit brain penetration and efficacy of AEDs. The best understood efflux transporters are members of the ABC (ATP-binding cassette) superfamily subfamilies B, C, and G, specifically *P-glycoprotein* (ABCB1 or MDR1), the *multidrug resistance-associated proteins* (MRP1, ABCC1; MRP2, ABCC2), and *breast cancer resistance protein* (BCRP, ABCG2) [31].

P-glycoprotein (P-gp), also known as MDR1 or ATP-binding cassette subfamily B member 1 (ABCB1), actively exports hydrophobic and amphipathic molecules from the inside of cells or membranes to the outside, as a critical defense mechanism. Its overexpression in epileptogenic brain tissue in patients with refractory epilepsy has been documented in numerous studies. For the first time overexpression of *MDR1* mRNA was demonstrated in 11 out of 19 resected brain specimens from patients with refractory focal epilepsy. P-gp overexpression was also detected in astrocytes and/or dysplastic neurons in common pathological causes of refractory epilepsy, including dysembryoplastic neuroepithelial tumors (DNT), hippocampal sclerosis (HS), and focal cortical dysplasia (FCD) [32].

The overexpression of a multidrug resistance-associated protein (MRP1) in astrocytes and/or dysplastic neurons in HS, DNT, and FCD has also been described [32]. The results confirm that MRP1 protein expression levels in astrocytes and neurons from brain tissue of epilepsy patients are significantly increased compared to brain tissue from healthy individuals, while endothelial MRP1 expression did not differ between the two [33]. It was reported increased *MRP2* and *MRP5* mRNA levels in endothelial cells isolated from epileptic brain tissue of patients with refractory epilepsy compared to control endothelial cells from human umbilical vein and aneurysm domes. Aronica et al. [34] reported MRP2 protein overexpression in endothelial cells and astrocytes in HS tissue specimens of adult patients with temporal lobe epilepsy (TLE).

Similar to P-gp, BCRP (breast cancer resistance protein) transports a wide variety of substrates, and its tissue distribution contributes to its important roles in restricting absorption and facilitating elimination of drugs and xenobiotics, but due to the lack of evidence on BCRP overexpression in human epileptic brain tissue, BCRP is unlikely a major player in AED resistance as proposed by the transporter hypothesis [31].

Although increased mRNA and protein expression levels of P-gp and MRPs have been demonstrated in resected brain tissue from patients with AED-resistant epilepsy, previous studies did not include proper controls, as it is generally difficult to obtain brain tissue from either patient with drug-responsive epilepsy or from healthy subjects without brain disease. Therefore, it is still unclear if overexpression of efflux transporters correlates with and potentially causes AED resistance, or if it is an epiphenomenon of epilepsy in humans that is unrelated to AED resistance [35].

Conclusive evidence that AEDs are transported by efflux transporters at therapeutic concentrations is considered the weak link in the transporter hypothesis. Early studies suggested that several AEDs may be substrates for P-gp and/or MRPs. Researchers who attempted to identify AEDs as substrates of P-gp, MRPs, and/or BCRP mainly used three approaches: transporter-overexpressing cell lines, transporter inhibition in cell lines and/or in animals, and transporter gene knockout mice [36]. Each of these approaches has its own strengths and weaknesses. For example, transporter-overexpressing cell lines only allow *in vitro* analysis. Transporter inhibitors may lack specificity and interact with more than one transporter, and knockout mice may show potential compensatory upregulation of other transporters, which may complicate the situation. Therefore, all three approaches may need to be used together in one thorough study to obtain conclusive data [32]. In addition, compared to chemotherapeutic drugs that are usually high-affinity substrates for P-gp and MRPs, AEDs are weak substrates for the efflux transporters and more easily cross the blood-brain barrier under physiological conditions [37].

AED Transport by P-gp. P-glycoprotein transports primarily hydrophobic and amphipathic compounds [7].

Most AEDs are planar lipophilic molecules, and therefore, theoretically many AEDs should be P-gp substrates [19]. The first report of P-gp-mediated transport of an AED came from Tishler et al. [38], who reported lower steady-state intracellular phenytoin concentrations in MDR1-expressing neuroectodermal cells as compared to MDR1-negative cells. Phenobarbital, lamotrigine, felbamate, and oxcarbazepine were shown to be transported by P-gp in rat brain microdialysis studies using verapamil as a P-gp inhibitor [39]. Owen et al. [40] concluded that carbamazepine was not a substrate for P-gp. In contrast, other studies, supported that P-gp transports carbamazepine. Data from another microdialysis study in rat suggest that P-gp does not transport levetiracetam and valproic acid [41].

Luna-Tortós et al. [42] pointed out that conventional bidirectional transport assays may not be suitable to identify AEDs as P-gp substrates due to the highly permeable nature of most AEDs. Using a modified transport assay (concentration equilibrium transport assay; CETA) which allows evaluating active transport separately from passive permeability, it was detected P-gp transport of phenytoin, phenobarbital, lamotrigine, levetiracetam, and topiramate, but not carbamazepine in MDR1-transfected LLC-PK1 cells. Zhang et al. [36] used both the cell monolayer bidirectional assay and CETA in MDR1-transfected MDCKII and LLC-PK1 cells to test if phenytoin, phenobarbital, or ethosuximide were transported by P-gp. Results from the CETA experiments suggested concentration-dependent P-gp transport of phenytoin in both MDCKII-MDR1 and LLC-PK1-MDR1 cells and transport of phenobarbital only in MDCKII-MDR1 cells.

The only clinical evidence linking overexpression of blood-brain barrier P-gp to reduced AED brain levels came from a pilot study by Marchi et al. [43], who demonstrated an inverse correlation between the brain-plasma concentration ratio of the major active metabolite of oxcarbazepine, 10,11-dihydro-10-hydroxy-5*H*-dibenzo(b,f)azepine-5-carboxamide(10,11-dihydro-10-hydroxycarbama-zepine), and the *MDR1* mRNA brain expression levels in resected epileptic tissue from patients with refractory epilepsy.

Since different models yield different results, both *in vivo* and *in vitro* data seem to be needed to identify which AEDs are substrates for which transporter [31]. In this regard, by combining the available evidence, Zhang et al. [35] suggested that lamotrigine, oxcarbazepine, phenobarbital, and phenytoin are considered definite P-gp substrates, because P-gp-mediated transport of these AEDs has been supported by both *in vivo* and *in vitro* evidence.

AED Transport by MRPs. MRPs transport neutral organic drugs and amphiphilic organic anions including drugs conjugated to glutathione, sulfate, glucuronate, and phosphate. Thus, it is possible that MRPs transport a number of AEDs and/or their metabolites and limit their access to the brain. Phenytoin transport by MRP1 and/or MRP2 was shown *in vivo* in normal rats using brain microdialysis with the MRP1/2 inhibitor probenecid. Carbamazepine and oxcarbazepine were shown to be substrates of MRP1 and/or MRP2 in microdialysis *in vivo* studies with probenecid [39]. Valproic acid was the first AED found to be a substrate for MRPs in brain endothelial cells. Potschka et al. [44] showed that levetiracetam was not transported by MRP1/2. Baltes et al. [45] conducted bidirectional transport assays in monolayers of MRP2-transfected MDCKII kidney cells, and none of the AEDs tested (phenytoin, levetiracetam, carbamazepine) was found to be transported by MRP2.

Using CETA in MDCKII kidney cells transfected with human MRP1, MRP2, or MRP5, Luna-Tortós et al. reported that none of the AEDs tested (topiramate, valproate, carbamazepine, phenytoin, levetiracetam, lamotrigine, and phenobarbital) was transported by any of those MRPs [46].

In vivo studies may be needed to confirm the findings from *in vitro* experiments, but few clinical studies have focused on studying the relationship between AEDs and MRPs.

AED Transport by BCRP. Substrate specificity of BCRP significantly overlaps with that of P-gp. However, the role of BCRP in AED resistance is less well studied in comparison to P-gp or the MRPs [31]. Using BCRP-transfected MDCKII cells, Cerveny et al. [47] reported that none of the tested AEDs (phenobarbital, phenytoin, ethosuximide, primidone, valproate, carbamazepine, clonazepam, and lamotrigine) was transported by BCRP. However, Nakanishi et al. [48] reported that the brain-to-plasma concentration ratio values of phenobarbital, clobazam, zonisamide, gabapentin, tiagabine, and levetiracetam were higher in *mdr1a/b/Bcrp* triple knockout mice than those in *mdr1a/b* double knockout mice, suggesting the involvement of BCRP in the transport of these AEDs.

Current evidence suggests that most AEDs are not transported by BCRP, though discrepancies exist between *in vitro* and *in vivo* findings [49].

Efflux Transporter Upregulation Mechanism in Epilepsy. An important question that stems from the transporter hypothesis is whether overexpression of efflux transporters at the blood-brain barrier observed in epilepsy is acquired or constitutive. Current evidence suggests that seizures, genetic factors, or a combination of both are likely to be the major contributors to efflux transporter overexpression at the blood-brain barrier in epilepsy [31]. Experimental data mostly from animal studies support that P-gp upregulation in epileptic regions of the brain occurs mainly as a result of seizure activity. Van Vliet et al. [50] also reported increased MRP1, MRP2, and BCRP protein expression levels in rat astrocytes and cerebral blood vessels after acute status epilepticus and in chronic epilepsy. Similar to the finding with P-gp, overexpression of these transporters was greater in chronic epileptic rats that demonstrated progression of epilepsy.

Recent research in the field has postulated two main mechanisms leading to efflux transporter overexpression in the brain in epilepsy: (1) AED-mediated induction of efflux transporters *via* nuclear receptors and (2) seizure-induced signaling causing efflux transporter overexpression [31]. Regarding the first mechanism, studies on whether AEDs

induce efflux transporter overexpression have yielded inconsistent results. However, studies have shown that seizures induce brain capillary P-gp expression levels. If P-gp levels were already maximally induced in the study of Wang-Tilz et al. [51], one would not expect to see additional increases in P-gp expression levels by AEDs. Consistent with this, Wen et al. reported due to AED activation of the ligand-activated transcription factors pregnant X receptor and/or constitutive androstane receptor [52]. It is important to note that AED-mediated upregulation of drug efflux transporters at the blood-brain barrier and in other tissues does not explain why some patients are resistant to the very first AED they are given. While this speaks against the theory that AEDs are the main cause for drug resistance due to transporter upregulation, it is possible that AEDs are one contributor, among others, to refractory epilepsy [31].

The second mechanism that has been shown to result in increased efflux transporter expression levels is through recurring seizures. In this regard, Lazarowski et al. [53] showed that daily induced seizures result in a progressive increase of P-gp protein expression at the blood-brain barrier, and further will cause resistance. Importantly, resistance to drugs that are not P-gp substrates, such as carbamazepine, diazepam, or levetiracetam was not observed. Therefore, this new model could be useful for screening novel AEDs that are P-gp substrates and have the potential to control seizures in pharmacoresistant epilepsy.

The molecular signaling mechanism underlying increased efflux transporter expression levels in epilepsy has been studied. In this regard, evidence from *in vitro* and *in vivo* rodent studies suggests that targeting this pathway could control P-gp expression and activity levels, and thus, help increase AED brain penetration and improve AED efficacy to control seizures in drug-resistant epilepsy. To fully assess if P-gp upregulation has any relevant consequences on pharmacoresistance, studying P-gp expression in brain tissue from both AED-responsive and AED-resistant patients and/or conducting PET imaging using P-gp substrates or inhibitors in patients would be critical. At present, aspects of the transporter hypothesis are still controversial, and further research is needed to determine the clinical relevance of efflux transporter overexpression at the blood-brain barrier [31].

Pharmacokinetic hypothesis. The pharmacokinetic hypothesis proposes that overexpression of efflux transporters in peripheral organs, such as intestine, liver, and kidney decreases ASD plasma levels in refractory epilepsy patients, thereby reducing the amount of ASD available to cross the blood-brain barrier and reach the epileptic focus in the brain [54].

Alterations in expression and functionality of multidrug transporters in patients with intractable epilepsy need not necessarily be restricted to the brain but could also occur in other tissues, such as the small intestine, where P-glycoprotein is thought to form a barrier against entrance of drugs from the intestinal lumen into the bloodstream, thereby limiting their oral bioavailability. In support of this theory, several studies have reported persistent subtherapeutic plasma levels of anticonvulsants (including phenytoin and phenobarbital) despite aggressive and chronic administration of anticonvulsants in patients with drug-resistant epilepsy that has been associated with overexpression of *MDR1* [32]. Support for the pharmacokinetic hypothesis comes from studies showing persistently low ASD levels in patients with drug-resistant epilepsy, which, however, relate to drug metabolizing enzymes rather than to efflux transporters such as P-glycoprotein [31]. The metabolism of ASDs is mainly mediated by liver cytochrome P450. Some of the cytochromes of this group have allelic types encoding isoforms which have different activity and, in turn, can affect the concentration of many drugs, including ASDs, in the blood serum. Cytochrome P450 metabolic enzymes not only occur in the periphery, but also in the brain parenchyma and endothelial cells of the blood-brain barrier, thus adding to the barrier function. Changes in the cerebrovascular hemodynamic conditions can affect expression of cytochrome P450 enzymes and multidrug-resistance transporters, leading to a synergistic role in drug resistance [55].

The liver is involved in potential pharmacokinetic changes by overexpression of P-gp, 99mTc-hexakis-2-methoxyisobutylisonitrile, what increased hepatic clearance that could contribute to ASD resistance [56]. The animal studies do not support the pharmacokinetic hypothesis [27]. In addition, data from clinical studies show that ASD-responsive and ASD-resistant patients display adverse events to the same extent, suggesting similar plasma ASD levels in the two groups of patients [32]. One explanation for this observation is that efflux transporter overexpression is restricted to the epileptic focus. This observation also suggests that the same plasma ASD concentrations are due to the same enzyme and transporter expression levels in peripheral organs. While both explanations are plausible, one does not necessarily lead to the other.

Together, the pharmacokinetic hypothesis of refractory epilepsy as a stand-alone theory is difficult to validate. One can argue that because abnormalities in ASD plasma concentrations can be readily captured by therapeutic drug monitoring, pharmacokinetic variability is probably not a major contributor to pharmacoresistance in situations where ASD doses are adjusted accordingly. This argument, however, is further complicated because therapeutic ASD plasma concentrations vary among patients, and no one specific therapeutic ASD concentration range is applicable to all patients.

Neural network hypothesis. Growing body of electrophysiological, neuroimaging, and clinical evidence suggests that epilepsy, including drug-resistant epilepsy, is a network disorder. Recent observations led to the emergence of the neural network hypothesis that might mechanistically explain the development of drug-resistant epilepsy [31]. According to this hypothesis, epilepsy-associated structural and functional alterations lead to an abnormal and maladaptive remodelling of neural networks that become resistant to antiepileptic medication. The underlying substrates of a maladaptive network architecture range from axonal sprouting and synaptic

reorganization to neurogenesis and gliosis [31]. Structural alterations in both drug-resistant lesional and non-lesional epilepsy are widespread and extend to regions beyond the borders of the epileptogenic focus. Thus, patients with temporal lobe epilepsy show significantly reduced cortical thickness in the supramarginal, middle and upper temporal gyri, temporal pole, insula, cuneus, superior frontal, precentral, posterior cingulate, operculum, lateral orbitofrontal, lingual, upper parietal, postcentral, lower parietal, lateral occipital, paracentral and isthmus cingulate of the right hemisphere [57]. In the left hemisphere, cortical thinning was identified in the supramarginal, precentral, and middle frontal regions. Like structural connectivity, functional connectivity displays distinct and frequency-dependent changes in the interregional neural oscillations. Thus, in theta frequency, connectivity from the temporal and frontal lobes to thalamus initially shows a continuous decrease in the connectivity intensity, later followed by a continuous increase before the spike generation [57]. In contrast, connectivity from the thalamus to the frontal lobe shows an inverted pattern – initial continuous increase in connectivity intensity, followed by a decrease in connectivity [57]. Several factors modulating the topology of neural networks in patients with drug-resistant epilepsy have been described. Thus, patients with sleep- and awake-related seizures display different patterns of structural alterations and network organization. Patients with sleep-related seizures compared to those with awake-related seizures have larger volumes of bilateral insula, superior temporal, and orbitofrontal cortices [58]. Volumes of hippocampus, amygdala, caudate, pallidum, and putamen are larger in patients with sleep-related seizures than in patients with awake-related seizures [58].

Summing up, patterns of brain network reorganization in drug-resistant epilepsy are characterized by an increased segregability, low integrability, and reduced resilience [59]. However, one of the substantial limitations of this hypothesis is that neural network alterations lead to treatment refractoriness not in all epilepsy patients, and therefore, further studies are required to establish the clear-cut network alterations related to drug resistance.

The gene variant hypothesis. In recent years, numerous studies have demonstrated that genetic variation is involved in the drug resistance of epilepsy [60]. Gene variants in transporters, targets as well as metabolizing enzymes, are hypothesized to contribute to drug resistance mechanisms, especially genetic variations found in drug resistance-related genes, including the voltage-dependent sodium and potassium channels genes, and the metabolizer of endogenous and xenobiotic substances genes. Advances in genomic technologies have facilitated the genome-wide discovery of common and rare variants and have increased our understanding of genetics in epilepsy; however, the mechanisms underlying pharmacological resistance have not been fully elucidated. The most frequently studied polymorphisms are those associated with multidrug resistance genes (MDR): ATP-binding cassette subfamily B member 1 (ABCB1 or MDR1) and ATP-binding cassette subfamily C member 2 (ABCC2 or MRP2); SCN α subunits 1, 2 and 3 (SCN1, SCN2 and SCN3); and metabolizers of endogenous and xenobiotic substances, cytochromes P450 families 2 and 3 (CYP2 and CYP3) [61]. For example, a meta-analysis on the ABCB1 C3435T polymorphism showed association of CC genotype with drug-resistant epilepsy in Caucasians only, while a more recent systematic review showed the TT genotype polymorphism to be correlated [62]. Also, in intractable epilepsy and other mental disabilities, whole-exome sequencing (WES) identified *de novo* variants in the Bernardinelli-Seip congenital lipodystrophy 2 (*BSCL2*) gene in two patients [63], or the case of recently reported changes in the *KCNQ2* gene which present with both benign seizure disorders and early onset epileptic encephalopathies (EOEE), the latter including patients who present refractory seizures following standard AED treatment and development delay [64].

It is hypothesized that genetic variants may also contribute to the efficacy of drug treatments for epilepsy; for example, adverse or toxic reactions, teratogenic risk in pregnancy, as well as long-term outcomes have been observed among PWE [65]. In recent studies, the association between genetic polymorphisms, treatment responses in epilepsy and antiepileptic drugs (AED) reactions (toxic, adverse or those related with its efficacy) have been investigated.

It was reported that polymorphisms in the human leukocyte antigen (*HLA*) gene were associated with severe cutaneous adverse AED reactions [66], and polymorphisms in a number of other genes, including *ABCB1, ABCC2, GABRA6, GABRG2, CYP2C9, CYP3A4*, UDP-glucuronosyltransferase (*UGT)1A1, UGT1A4, UGT1A6, UGT2B7, SCN2A* and *SCN1A*, have been associated with the concentration, response and efficacy of some of the most commonly used AEDs in clinical practice, including carbamazepine, oxcarbazepine, phenytoin, lamotrigine and valproic acid. Esmaeilzadeh et al. reported an association between HLA polymorphisms and severe cutaneous adverse reactions (SCARs) induced by drugs and it was found that the hypersensitivity to different AEDs, including phenytoin, carbamazepine, valproic acid, topiramate and lamotrigine, was associated with *HLA-A* gene polymorphisms [67]. Although this hypothesis proposes an inherent resistance as the cause of intractable seizures, several underlying processes are also involved in epileptic seizures including microglial activation, glutamate-induced excitotoxicity, mitochondrial dysfunction, oxidative stress, and the formation of reactive oxygen species [60]. Ultimately, this requires simultaneous malfunction of several enzymes, proteins, channels, and receptors [68].

Consequently, it is difficult to believe that an individual is born with such a vast number of gene polymorphisms to the extent that both pharmacokinetic and pharmacodynamic mechanisms are hindered. Overall drug resistant patients are a highly heterogeneous group and no theory in isolation can explain multi-drug inefficacy in every patient [69].

The epigenetic hypothesis. The genome is one source of endogenous variation, contributing to different disease risks between different people. There are, however, other sources of variation, such as "omes" beyond the genome: the

epigenome, transcriptome, proteome, microbiome, and so on. Some of these "omes" have been interrogated for their role in drug resistance in epilepsy, but it must be acknowledged at this point that the data available are even more sparse than for most genome-based studies and that, currently, none of these "omes" have been proven to influence drug resistance [27]. Studying epigenomic contribution to drug resistance in epilepsy [70], which is likely to be due to processes in the brain, is very challenging.

Epigenetic mechanisms have also been proposed to explain the development of pharmacoresistance in epilepsy patients, influencing the sustained patterns of gene expression that regulate AED uptake and mechanism of action. In the future, drugs inhibiting DNA methyltransferases (DNMTs) and histone lysine deacetylases (KDACs) could provide new treatments for patients unaffected by currently available anti-epilepsy medications. Not only are drugs acting on epigenetic processes currently available and in development for a variety of human diseases, but there is some evidence that one or more commonly used AEDs may act, in part, through epigenetic mechanisms. Most of the work identifying epigenetic changes in epilepsy has focused on DNA methylation. Recently, there has been exceptional interest in the role of microRNAs, especially where attention has also focused on their potential for use as biomarkers to support early diagnosis and prognosis in the clinic. In contrast, other aspects, such as histone modifications and long noncoding RNAs, have been less studied [71].

Among classes of molecules constituting the epigenome are histones and noncoding RNAs, both long and shorter, including microRNAs. The latter contribute to RNA silencing and post-transcriptional regulation of gene expression, altering expression levels of multiple proteins. A central problem in studying the epigenome in humans is to disentangle cause from effect and relevance either way from epiphenomena [27]. The mechanisms of action of ketogenic diet in epilepsy have been revealed recently, such as epigenetic mechanism for increase the adenosine level in the brain and inhibition of DNA methylation. Thus, although a series of microRNAs have been shown to associate with human TLE [72], the studied tissue had been surgically resected from people with drug resistance, and cause and effect (for either disease susceptibility or drug resistance) could not be distinguished. In animal models, manipulation of specific microRNAs can influence seizures and disease [73], though some data are less supportive, however, whether this would be the case in human epilepsy, and specifically whether this approach would counter drug resistance, remains unknown.

Neuroinflammatory hypothesis. Accumulating evidence over the past decade indicates an important pathophysiological role of brain inflammation in pharmacoresistant epilepsy. Different inflammatory molecules and pathways have been shown to significantly contribute to the mechanisms of seizure generation and progression in different experimental models [74]. Inflammation refers to the complex biological response of tissues against infections or sterile (non-infectious) injuries, it is closely associated with the activation of both innate and adaptive immune cells. It represents a key homeostatic mechanism of the body's defense, which is crucial for activating mechanisms for tissue repair, via the production of a large array of inflammatory cytokines and related effector molecules. A novel emerging concept in inflammation is the specific interaction between the innate immune system and injured brain tissue, known as neuroinflammation. Although neuroinflammation currently lacks a consensus definition, in general it can be seen as the biosynthesis and release of molecules with inflammatory properties by resident cells of the brain, including activated microglia and astrocytes, neurons, endothelial cells of the blood-brain barrier, and blood-born macrophages.

A notable finding is that the inflammatory mediators released by the resident brain cells during epileptic activity (i.e. cytokines, chemokines, alarmins/danger signals, prostaglandins, complement factors) are not only effectors molecules of the immune system promoting local inflammation, but also function as neuromodulators directly affecting neuronal function and excitability [75]. Prominent changes in several immune/inflammatory pathways, such as IL-1R1/TLR4, COX-2, TNF-a, complement and chemokines have been reported within epileptogenic lesions in preclinical and clinical studies. These pathways seem critically involved in ictogenesis and epileptogenesis. A more detailed assessment of the use of these inflammatory pathways is needed as potential biomarkers which contribute to the development of epilepsy or to measure the effectiveness of therapeutic interventions.

Gut-brain axis. The "gut microbiota-brain axis" is the interaction between gut microbiome and brain. This bidirectional communication is provided by the nervous, endocrine, immune, circulatory, and metabolic pathways [76]. Ketogenic Diet (KD) may be considered another alternative treatment in drug-resistant epilepsy (DRE). This type of diet was used since 1921, in the treatment of intractable epilepsy in children.

Animal models. Olson et al., demonstrated in two-seizure mouse models, KD changes microbiota and leads to anti-seizure protection. KD fed-mice have demonstrated a decreasing in seizure duration and frequency. Also, it was measured the level of GABA and glutamate. GABA levels were higher in KD fed-mice. The direct association between chronic stress-induced epilepsy and intestinal dysbiosis in Sprague-Dawley rats was reported in a study [77].

KD, microbiome and epilepsy. KD is a high-fat, and very low-carbohydrate diet. KD in children with retractable epilepsy was evaluated in randomized controlled prospective study. Patients had a relevant decreasing in seizure severity. Seizures were reduced to at least 50% in children who received KD. The effect of the KD on gut microbiome in children with DRE, due to GLUT-1 deficiency was investigated for 90 days. Compared with baseline increasing in *Desulfovibrio* species was established after KD. There were no significant differences in *Bacteroidetes* and *Firmicutes* levels. Xie et al., used KD (4:1 ratio) for a week. They noticed that 64% of children with DRE had ≥50% decreasing in seizure frequen-

cy. Compared with baseline, while *Bacteroides* increased markedly, *Bifidobacterium* increased less significantly after KD therapy. *Cronobacter*, which was at high levels before KD, after therapy presented similar levels with the control group [78]. KD (4:1 ratio) was used in children (five patients had Dravet syndrome, three each had West syndrome and Lennox-Gastaut syndrome, and the remainder couldn't be classified) for six months. Reduction of *Firmicutes* and increasing *Bacteroidetes* levels were reported after therapy. KD-non-responder group demonstrated increased levels of *Clostridiales*, *Ruminococcaceae*, *Rikenellaceae*, *Lachnospiraceae*, and *Alistipes*, comparative with responder group [79]. Lindefeldt et al., evaluated the effect of KD on eleven children with DRE (due to perinatal asphyxia, encephalitis, cortical dysplasia, tuberous sclerosis, and unknown cause) and one with pyruvate dehydrogenase deficiency. Relative abundance of *Escherichia coli* increasing was observed on KD. Levels of *Bifidobacteria* as well as *Eubacterium rectale* and *Dialister* were decreased during KD therapy [80].

Gut dysbiosis in epilepsy patients. Probiotics show beneficial effect on seizures in patients with DRE. In a pilot study was shown seizures reduction and higher quality of life in 28.9% of adult patients with DRE, who were treated with probiotic cocktail [81]. Another study demonstrated that rotavirus infection manifests a risk factor for neonatal seizures. Probiotic administration after birth was linked with reduced risk of seizures [82]. Peng et al, established that *Bacteroidetes phylum* is the largest one (56.7 % in Drug Sensitive (DS) and 57.2% in Healthy Control (HC)); *Firmicutes phylum* is the second dominant (38.2% in DS and 37.5% in HC). The while, *Bacteroidetes* was relatively lower (45.7%), and the *Firmicutes* was the largest phylum (46.9%) in patients with DRE. Also, it was shown an increasing of other rare phyla in the DRE group; *Verrucomicrobia* was more abundant in the DRE group (0.32%) than (0.03% in DS) and (0.09% in HC) groups. Patients with DRE have altered composition of gut microbiota; so, dysbiosis may be implicated in the pathophysiology of DRE.

Biomarkers of drug-resistant epilepsy

Biomarkers may play a role in individualized epileptic treatment, based on the patients' biomarker profile. Molecular biomarkers, inflammatory markers can indicate not only the presence, type, and severity of neuropathologically damaged tissue with epileptogenic potential, but may also have the potential for localizing epileptogenic zone (EZ) [83].

Metabolites, proteins, messenger RNAs (mRNAs) and microRNAs (miRNAs) play a critical role in the regulation of neuronal biological processes, as well they might have importance in early disease diagnosis, effective prognostic monitoring and can be clubbed with existing techniques to preoperatively localize the EZ. Thus, the challenge is to find a biomarker which one can help accurately localize the EZ and offering new therapeutic approach strategies. Several microarray and target studies have reported a differential expression of more than 100 miRNAs in epilepsy. From those, miR-146a, miR-155 and miR-132 have a key role in epilepsy-related biological processes, such as neuroinflammation, neuronal growth, neuroprotection and neurodegeneration [84]. MiRNA-155 has been shown, in experimental studies, to be much higher in patients with generalized genetic epilepsy compared to control, and miRNA-4521 could serve as a potential biomarker in refractory epilepsy. Changes in the mRNA levels of various glutamate and gamma-aminobutyric acid (GABA) receptor subunits modulating of glutamatergic and GABAergic synaptic transmission, also mRNA polyadenylation profile can be utilized as a biomarker in generalized, and focal epilepsies [85]. Inflammatory molecules have been identified in experimental models of epilepsy and surgically resected brain tissue from treatment-resistant epilepsy. Different components especially, cyclooxygenase-2 (COX-2) play an important role in the post seizure inflammation, possibly contributing to secondary damage in the brain and the increased likelihood of repetitive seizures. High mobility group box-1 (HMGB1), that takes part in the immune response via activating macrophages and endothelial cells, leading to the release of tumor necrosis factor-a (TNF-a), interleukin-1 (IL-1), interleukin-6 (IL-6) has been indicated as a potential therapeutic agent in epilepsy and as a non-invasive biomarker, which could identify patients with high risk of epilepsy. The level of HMGB1 has been shown to increase within 3–4 h after seizure, proving HMGB1 to be a promising marker. Moreover, it is known that in drug-resistant epilepsy (DRE) resulting from Rasmussen's encephalitis, some patients showed seizure improvement following adalimumab administration, an anti-TNF-a therapy [86].

In sum, biomarker discovery and validation efforts need to balance between curation and control on the one hand, while allowing for broader coverage and generalizability on the other [87]. In time, this will lead to the development of effective strategies for the early screening, clinical diagnosis, treatment, and prevention of refractory epilepsy.

Advances in pharmacological treatment

Several antiepileptic drugs are currently in clinical trials whose mode of action is either historical, novel or adapted from previous AEDs. Everolimus, a compound which had been in use primarily for other clinical indications (renal cancer, organ transplant immunosuppression, metastatic neuroendocrine tumors) was approved in 2018 and has shown promise as an antiepileptogenic agent in patients with tuberous sclerosis. It works as an inhibitor of an overactive and dysregulated mTOR pathway. EXIST-3 trial [88] showed response rate of 28.2% and 40.0% for low-exposure and high-exposure everolimus, respectively, compared to placebo of 15.1%. Other compounds already in use for other medical indications and being tested in epilepsy include melatonin, biperiden, fenfluramine, bumetanide and verapamil [89]. Apart from these clinical in-use medications, drugs specifically designed for epilepsy are under investigation.

Clinical studies of cenobamate, which was recently approved for the treatment of partial-onset seizures in adults, showed approximately 20% of patients experienced seizure freedom, which is very impressive compared with previous add-on clinical trials with various other novel AEDs in pa-

tients with DRE [90]. A similar impressive antiseizure effect has been observed with the novel ASD fenfluramine in Dravet syndrome, in which approximately 25% of patients had long-term seizure freedom, suggesting that the long hoped-for breakthrough is a feasible goal [91].

Other compounds undergoing recruitment in phase 2/3 studies (registered on Clinicaltrials.gov) include padsevonil, neurosteroids (ganoxolone), XEN1101, E2082 and vorinostat. Several other drugs appear to be suspended due to failed early stage efficacy studies and others will need further preliminary data to assess their potential [89]. Cannabidiol studies have had a surge of interest after successes in recent trials of patients with Dravet and Lennox-Gastaut (LGS) syndromes and further open-label study has shown good retention rate, efficacy and safety data [92].

Epidiolex is the first canabidiol oral solution to be FDA (Food and Drug Administration) approved. Double-blind controlled studies showed significant seizure reduction in the range of 17%–23% compared to placebo for monthly convulsive seizures in Dravet syndrome and monthly total seizure reduction in the two pivotal LGS trials [93]. Overall, most of the drugs in the pipeline have similar mechanism of actions to traditional drugs. Clinical benefits over existing drugs are, therefore, unlikely in the near future. Alongside the novelty in drug therapy explored by pharmaceutical companies, viral-vector mediated gene therapy has demonstrated success in animal models of focal neocortical epilepsy using chemical-genetics and optogenetics, but no human trials are yet in progress.

Gene therapy. The use of novel therapeutic approaches in the management of epilepsy is steadily progressing. Neurological disorders are sometimes caused by inherited or acquired genetic changes that lead to abnormal nervous system development, neurodegeneration, or impaired neuronal function. About 30% of the epilepsies have been thought to have a genetic origin [94]. Gene therapy, as an emerging and novel therapeutic approach has curative potentials of the most common neurological disorders including DRE. It had been formerly defined as a method to replace the defective copy of a gene with a normal copy which acts correctly in the cells [95]. There are various methods to transfer and express a gene in a particular region of the brain that include cell transplantation, liposomes, non-viral and viral vector delivery, (adenovirus, adeno-associated virus, herpes simplex virus, lentivirus, and retrovirus). Gene therapy for DRE treatment aims to induce the local release of anticonvulsant or antiepileptogenic properties to counterbalance between excitation and inhibition in the brain. It offers the possibility of targeting therapeutic genes expressing in the seizure generating area without needing tissue ablation. These results are very promising; however, it is important to note that only infrequent types of epilepsy are caused by a single mutant gene; while they are commonly caused by inheritance of two or more susceptibility genes that usually influence a large part of the brain. Therefore, an extensive gene transfer is needed; however, the presently available gene therapies provide only local effects [94]. Improvement in the gene therapy-treating epilepsy can be possible through progress in understanding the disease mechanisms, designing suitable gene vectors, selecting suitable genes, and choosing the right delivery methods. Taken together, gene therapy can be considered as a therapeutic approach in the management of epilepsy; but further studies are needed to verify the safety and efficacy of this method in human. Gene therapy is a more challenging and complicated process than the simple concept of gene replacement. Techniques for transferring of exogenous genes into the desired sequence of target cells have been remarkably improved. Clustered regularly interspaced short palindromic repeats (CRISPR) are types of nucleic acid sequences that function in harmony with CRISPR-associated (Cas) proteins to provide immunity in bacteria and archaea against foreign invasion of nucleotides, such as viruses, plasmid, and phages. CRISPR-Cas9 system has recently attracted increasing attention for therapeutic applications [96]. This system has presented a novel approach in repairing gene defects for treating various types of disorders including neurological diseases. Due to its potential in targeted gene editing and repairing of genetic mutations, CRISPR-Cas9 can also be considered for applying as a possible therapeutic approach in treating DRE with genetic origins. Although it is the most powerful and useful technique for the multiplexed genome manipulating, there are still some challenges regarding efficiency and accuracy concerns, and further studies are needed to verify its safety before clinical applications. Several studies with the aim of epilepsy treating have applied various cell types, such as neural stem cells, mesenchymal stem cells, hippocampal precursor cells, GABAergic precursor cells, and bone marrow-derived mononuclear cells. They all aim to diminish seizure severity and frequency in the brain through different mechanisms of action. Although the results are encouraging, to use cell therapy in clinical application, additional and vigorous studies are necessary to test safety and efficacy of this approach [97].

Exosome therapy. The exosome is a kind of extracellular vesicles which have initially been recognized in the 1980s. Exosomes are characterized by homogeneous shaped nano (40–100 nm) membranous vesicles with a density of 1.13–1.19 g/cm^2. They can be secreted by several body cell types and have been detected in the various biological fluids including blood, urine, saliva, CSF, breast milk, amniotic fluid, malignant ascites, bronchoalveolar fluid, and synovial fluid. Depending on their source cells, exosomes can contain a variety of lipids, proteins, and genetic elements, such as DNA, non-coding RNAs, mRNAs, and microRNAs. These agents which are also known as "cargo" can be delivered to the surrounding cells or transferred to other distal cells and alter the recipient cell function. Therefore, exosomes can be considered as a novel form of intercellular communication. Interestingly, it has been shown that many cells of the nervous system, such as neurons, microglia, astrocytes, oligodendrocytes, and neural stem cells can release exosomes. Thus, exosomes may have a role in the function, development, and pathologies of the nervous system [98]. Emerging pieces of evidence have suggested that exosomes can be

used to rescue neuronal pathologies and alterations. It has been revealed that these vesicles have crucial roles in the regeneration process and repair of the nervous system. Their simple structure, low immunogenicity, and ability to cross the blood-brain barrier (BBB) have made a great opportunity to engineer and apply them as vehicles for delivering microRNAs, drugs, proteins, and other active agents to the brain. MicroRNAs have been known as master regulators of gene expression. In the nervous system, they can alter the regulation of various proteins which associate with several neuronal processes and actions. They have been shown to regulate essential genes involved in seizure susceptibility. Therefore, exosomes contained the agents which have anti-seizure activity can be potentially applied to manage and treat epilepsy. However, more studies are needed to address their use in clinical trials [84, 98].

Neuromodulation in pharmacoresistant epilepsy

Correctly selected antiepileptic drugs (AEDs) show good clinical improvement in most individuals with epilepsy, although almost a third of these patients at some point will present an inadequate or insufficient response to current AEDs [99]. In individuals with drug-resistant epilepsy the percentage of general morbidity and mortality is significantly higher when compared to drug responsive cases. Therefore, there is a consistent need for improving therapeutically management in this population. From a pathophysiological point of view, epilepsy is a result of abnormal neuronal network activity in the brain due to a pathological increase in excitatory synapses (Glutaminergic) with a decrease in inhibitory activity (GABA-ergic). Thus, approaching this paradigm it is assumed that inhibitory neuromodulation could induce the phenomenon of LTD (long-term excitatory depression) with normalization of the excitation threshold in hyperactive areas and as a result obtain the expected therapeutic effect. Modulation of brain activity can be achieved by impacting specific intra/ extracranial targets, either the peripheral elements of sensitization or direct cortical-subcortical activity as shown in Figure 1. By influencing the activity of these components, a modulation of either widespread brain networks or direct modulation of network nodes can be achieved.

Vagal nerve stimulation (VNS) involves intermittent electrical stimulation of the afferent fibers of the left cervical vagus nerve in a transcutaneous (tVNS) or non-invasive (nVNS) way or by means of an implanted helical electrode connected to a pulse generator. The therapeutic efficacy of VNS appears to be mediated by the activation of fast myelinated fibers in the vagus nerve. Its anticonvulsant effect can be explained by several mechanisms, such as the modulation of neurotransmitter expression with increased inhibition and reduced excitability, changes in cerebral blood flow, desynchronization of electroencephalographic (EEG) rhythms, and anti-inflammatory effects mediated by norepinephrine [100].

Transcranial direct current stimulation (tDCS) is a non-invasive method that modulates cortical excitability using a weak constant electric current passing through two electrodes (anode and cathode) applied over the skull. Cortical excitability may increase following anodal stimulation, while it generally decreases after cathodal stimulation. Considering this principle, hyperpolarization using cathodal tDCS has been proposed as therapy to suppress epileptiform discharges [101].

Trigeminal nerve stimulation (TNS) similar to VNS and tDCS uses electrical stimuli but targets trigeminal sensory roots within the facial tissue (ophthalmic nerve, supratrochlear nerve, infraorbital nerve). Considering the projections of the trigeminal pathway, it is thought to generate an arousal-like effect from stimulation of the reticular activating system which leads to a shift of cortical activity from a "synchronized" state to a "desynchronized" state. As seizures present highly synchronous activity, it was hypothesized that desynchronization of the neocortex would have an anticonvulsant effect [102].

Transcranial magnetic stimulation (TMS) is a non-invasive brain stimulation technique which using electromagnetic induction phenomenon is able to module electrical activity of targeted cortical areas. The application of low-frequency (<1Hz) repetitive TMS or continuous theta burst stimulation (cTBS) modulates the cortical excitability and produces its relatively long-term depression-like mechanisms in the cortex [103].

Deep brain stimulation (DBS) is an invasive neuromodulation technique that uses electrical stimulation through electrode implantation within the anterior nucleus of thalamus, centromedian nucleus of thalamus, cerebellum, hippocampus, or subthalamic nucleus to interfere with neuronal synchronized oscillations, thus inducing modulation of pathological neural networks [104].

Responsive neurostimulation (RNS) is a closed loop, invasive brain stimulation method that aims to suppress ictal activity by delivering stimulation directly in response to electrographic activity; it is highly time- and area-specific, therefore providing stimulation only when needed [100] (fig. 1).

By reducing cortical excitability, targeted neuromodulation has enormous therapeutic potential in the treatment of epilepsy, especially in drug-resistant epilepsy.

Perspectives

The issue of drug resistance to epilepsy remains relevant to date. Thus, the use of an integrated approach, personalizing the therapy of patients with refractory epilepsy, can achieve significant positive results.

Therefore, a therapeutic strategy for drug-resistant epilepsy should be based on the one hand, on the suppression of epileptogenesis and, on the other hand, on overcoming drug resistance. An integrated approach to the problem of drug resistance will allow not only obtaining new data on the mechanisms of the pathogenesis of epilepsy, but also improving the algorithm for treating patients and increasing its effectiveness.

Although the previous decades have noted remarkable developments in the neurosciences, technological improvements in diagnostics (genetics, imaging, electrophysiology)

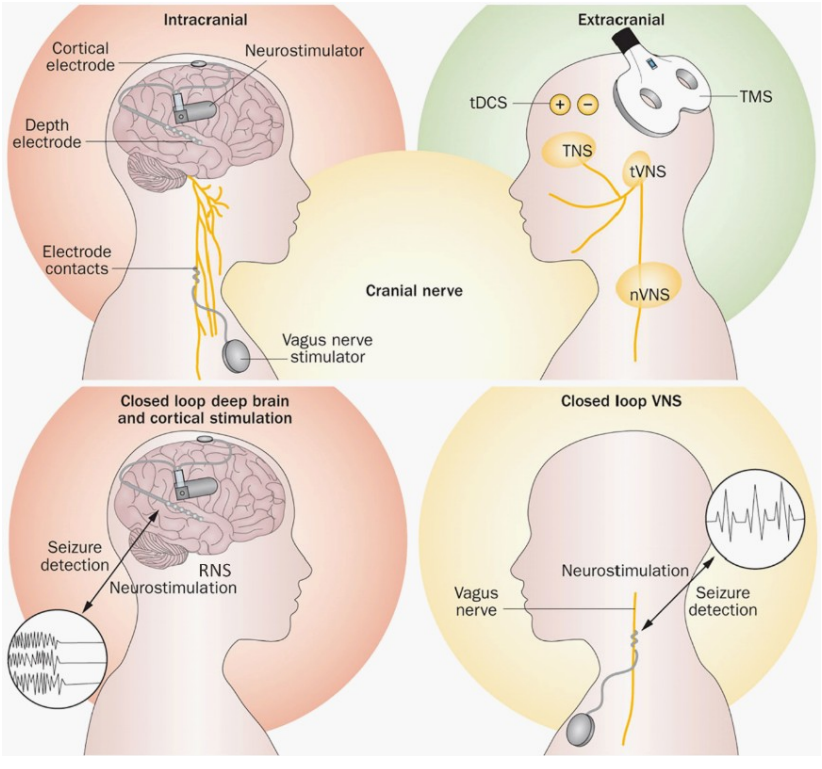

Fig. 1. Types of neurostimulation and targets used for achieving neuromodulatory effect in patients with epilepsy. Adapted from [100]. Transcranial direct current stimulation (tDCS); Trigeminal nerve stimulation (TNS); Transcutaneous vagus nerve stimulation (tVNS); Non-invasive vagus nerve stimulation (nVNS); Transcranial magnetic stimulation (TMS); Deep brain stimulation (DBS); Responsive neurostimulation (RNS)

and increased options of drug therapies, epidemiological studies suggest that this has not translated in wide benefits for drug-resistant patients [105].

Conclusions

Neuroscience of drug-resistant epilepsy faces many challenges. Inflammatory mediators, biomarkers, and genes might allow the identification of new treatment targets, contribute to an earlier diagnosis, and assess the clinical outcomes. New therapeutic approaches, with personalized therapy for each patient, offer better perspectives for patients and their families. Thus, drug resistance in epilepsy is an urgent scientific and practical issue that requires fundamental and interdependent clinical research. The further development of new surgical methods and non-surgical treatment of drug-resistant epilepsy remains relevant, including the modernization of neurorehabilitation methods.

References

1. Fisher RS, Acevedo C, Arzimanoglou A, et al. ILAE official report: a practical clinical definition of epilepsy. Epilepsia. 2014;55(4):475-82. doi: 10.1111/epi.12550.
2. Beghi E, Giussani G, Nichols E, et al. Global, regional, and national burden of epilepsy, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. Lancet Neurol. 2019;18(4):357-375. doi: 10.1016/S1474-4422(18)30454-X.
3. Beghi E. The epidemiology of epilepsy. Neuroepidemiology. 2020;54(Suppl 2):185-191. doi: 10.1159/000503831.
4. Shankar R, Rowe C, Van Hoorn A, et al. Under representation of people with epilepsy and intellectual disability in research. PLoS One. 2018;13(6):e0198261. doi: 10.1371/journal.pone.0198261.
5. Stangl AL, Earnshaw VA, Logie CH, et al. The Health Stigma and Discrimination Framework: a global, crosscutting framework to inform research, intervention development, and policy on health-related stigmas. BMC Med. 2019;17(1):31. doi: 10.1186/s12916-019-1271-3.
6. Tedrus GMAS, Pereira RB, Zoppi M. Epilepsy, stigma, and family. Epilepsy Behav. 2018;78:265-268. doi: 10.1016/j.yebeh.2017.08.007.
7. Chahal CAA, Salloum MN, Alahdab F, et al. Systematic review of the genetics of Sudden Unexpected Death in Epilepsy: potential overlap with sudden cardiac death and arrhythmia-related genes. J Am Heart Assoc. 2020;9(1):e012264. doi: 10.1161/JAHA.119.012264.
8. Lhatoo S, Langan Y, Sander J. Sudden unexpected death in epilepsy. Postgrad Med J. 1999;75(890):706-709. doi: 10.1136/pgmj.75.890.706.
9. Téllez-Zenteno JF, Ronquillo LH, Wiebe S. Sudden unexpected death in epilepsy: evidence-based analysis of incidence and risk factors. Epilepsy Res. 2005;65(1):101-115. doi: 10.1016/j.eplepsyres.2005.05.004.
10. Walczak T, Leppik IE, D'Amelio M, et al. Incidence and risk factors in sudden unexpected death in epilepsy: a prospective cohort study. Neurology. 2001;56(4):519-525. doi: 10.1212/wnl.56.4.519.
11. Kwan P, Brodie MJ. Effectiveness of first antiepileptic drug. Epilepsia. 2001;42(10):1255-1260. doi: 10.1046/j.1528-1157.2001.04501.x.
12. Brodie M, Barry SJ, Bamagous GA, et al. Patterns of treatment response in newly diagnosed epilepsy. Neurology. 2012;78(20):1548-1554. doi: 10.1212/WNL.0b013e3182563b19.
13. Kwan P, Arzimanoglou A, Berg AT, et al. Definition of drug-resistant epilepsy: consensus proposal by the ad hoc Task Force of the ILAE Commission on Therapeutic Strategies. Epilepsia. 2010;51(6):1069-1077. doi: 10.1111/j.1528-1167.2009.02397.x.
14. Leppik I. Intractable epilepsy in adults. Epilepsy Res Suppl. 1992;5:7-11.

15. Jallon P. The problem of intractability: the continuing need for new medical therapies in epilepsy. Epilepsia. 1997;38 Suppl 9:S37-S42. doi: 10.1111/j.1528-1157.1997.tb05203.x.
16. Arzimanoglou A, Ryvlin P. Towards a clinically meaningful definition of drug-resistance. In: Kahane P, Berg A, Loscher W, et al., editors. Drug-Resistant Epilepsy. Montrouge: John Libbey Eurotext; 2008. p. 1-6.
17. Malek N, Heath CA, Greene J. A review of medication adherence in people with epilepsy. Acta Neurol Scand. 2017;135(5):507-515. doi: 10.1111/ane.12703.
18. Nakken KO, Kostov H, Ramm-Pettersen A, Heminghyt E, Bakke SJ, Nedregaard B, Egge A. [Epilepsy surgery: assessment and patient selection]. Tidsskr Nor Legeforen. 2012;132(14):1614-8. Norwegian. doi: 10.4045/tidsskr.11.1149.
19. Lerche H. Drug-resistant epilepsy – time to target mechanisms. Nat Rev Neurol. 2020;16(11):595-596. doi: 10.1038/s41582-020-00419-y.
20. Rogawski MA. The intrinsic severity hypothesis of pharmacoresistance to antiepileptic drugs. Epilepsia. 2013;54:33-40. doi: 10.1111/epi.12182.
21. Schmidt D, Löscher W. New developments in antiepileptic drug resistance: an integrative view. Epilepsy Curr. 2009;9(2):47-52. doi: 10.1111/j.1535-7511.2008.01289.x.
22. Brodie MJ. Road to refractory epilepsy: the Glasgow story. Epilepsia. 2013;54 Suppl 2:5-8. doi: 10.1111/epi.12175.
23. Marson A, Jacoby A, Johnson A, et al. Immediate versus deferred antiepileptic drug treatment for early epilepsy and single seizures: a randomised controlled trial. Lancet. 2005;365(9476):2007-2013. doi: 10.1016/S0140-6736(05)66694-9.
24. Remy S, Beck H. Molecular and cellular mechanisms of pharmacoresistance in epilepsy. Brain. 2006;129(1):18-35. doi: 10.1093/brain/awh682.
25. Rogawski MA, Löscher W, Rho JM. Mechanisms of action of antiseizure drugs and the ketogenic diet. Cold Spring Harb Perspect Med. 2016;6(5):a022780. doi: 10.1101/cshperspect.a022780.
26. Remy S, Gabriel S, Urban BW, et al. A novel mechanism underlying drug resistance in chronic epilepsy. Ann Neurol. 2003;53(4):469-479. doi: 10.1002/ana.10473.
27. Löscher W, Potschka H, Sisodiya SM, et al. Drug resistance in epilepsy: clinical impact, potential mechanisms, and new innovative treatment options. Pharmacol Rev. 2020;72(3):606-638. doi: 10.1124/pr.120.019539.
28. Remy S, Urban BW, Elger CE, et al. Anticonvulsant pharmacology of voltage-gated Na+ channels in hippocampal neurons of control and chronically epileptic rats. Eur J Neurosci. 2003;17(12):2648-2658. doi: 10.1046/j.1460-9568.2003.02710.x.
29. Doeser A, Dickhof G, Reitze M, et al. Targeting pharmacoresistant epilepsy and epileptogenesis with a dual-purpose antiepileptic drug. Brain. 2015;138(2):371-387. doi: 10.1093/brain/awu339.
30. Brooks-Kayal AR, Shumate MD, Jin H, et al. Selective changes in single cell GABA A receptor subunit expression and function in temporal lobe epilepsy. Nature Med. 1998;4(10):1166-1172. doi: 10.1038/2661.
31. Tang F, Hartz A, Bauer B. Drug-resistant epilepsy: multiple hypotheses, few answers. Front Neurol. 2017;8:301. doi: 10.3389/fneur.2017.00301.
32. Loscher W, Luna-Tortós C, Römermann K, et al. Do ATP-binding cassette transporters cause pharmacoresistance in epilepsy? Problems and approaches in determining which antiepileptic drugs are affected. Curr Pharm Des. 2011;17(26):2808-2828. doi: 10.2174/138161211797440212.
33. Sun Y, Luo X, Yang K, et al. Neural overexpression of multidrug resistance-associated protein 1 and refractory epilepsy: a meta-analysis of nine studies. Int J Neurosci. 2016;126(4):308-317. doi: 10.3109/00207454.2015.1015724.
34. Aronica E, Gorter JA, Ramkema M, et al. Expression and cellular distribution of multidrug resistance–related proteins in the hippocampus of patients with mesial temporal lobe epilepsy. Epilepsia. 2004;45(5):441-451. doi: 10.1111/j.0013-9580.2004.57703.x.
35. Zhang C, Kwan P, Zuo Z, et al. The transport of antiepileptic drugs by P-glycoprotein. Adv Drug Deliv Rev. 2012;64(10):930-942. doi: 10.1016/j.addr.2011.12.003.
36. Zhang C, Kwan P, Zuo Z, et al. *In vitro* concentration dependent transport of phenytoin and phenobarbital, but not ethosuximide, by human P-glycoprotein. Life Sci. 2010;86(23-24):899-905. doi: 10.1016/j.lfs.2010.04.008.
37. Löscher W, Potschka H. Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. Prog Neurobiol. 2005;76(1):22-76. doi: 10.1016/j.pneurobio.2005.04.006.
38. Tishler DM, Weinberg KI, Hinton DR, et al. MDR1 gene expression in brain of patients with medically intractable epilepsy. Epilepsia. 1995;36(1):1-6. doi: 10.1111/j.1528-1157.1995.tb01657.x.
39. Clinckers R, Smolders I, Meurs A, et al. Quantitative *in vivo* microdialysis study on the influence of multidrug transporters on the blood-brain barrier passage of oxcarbazepine: concomitant use of hippocampal monoamines as pharmacodynamic markers for the anticonvulsant activity. J Pharmacol Exp Ther. 2005;314(2):725-731. doi: 10.1124/jpet.105.085514.
40. Owen A, Pirmohamed M, Tettey JN, et al. Carbamazepine is not a substrate for P-glycoprotein. Br J Clin Pharmacol. 2001;51(4):345-349. doi: 10.1046/j.1365-2125.2001.01359.x.
41. Baltes S, Fedrowitz M, Tortós CL, et al. Valproic acid is not a substrate for P-glycoprotein or multidrug resistance proteins 1 and 2 in a number of *in vitro* and *in vivo* transport assays. J Pharmacol Exp Ther. 2007;320(1):331-343. doi: 10.1124/jpet.106.102491.
42. Luna-Tortós C, Fedrowitz M, Löscher W. Several major antiepileptic drugs are substrates for human P-glycoprotein. Neuropharmacology. 2008;55(8):1364-1375. doi: 10.1016/j.neuropharm.2008.08.032.
43. Marchi N, Guiso G, Rizzi M, et al. A pilot study on brain-to-plasma partition of 10, 11-dyhydro-10-hydroxy-5H-dibenzo (b, f) azepine-5-carboxamide and MDR1 brain expression in epilepsy patients not responding to oxcarbazepine. Epilepsia. 2005;46(10):1613-1619. doi: 10.1111/j.1528-1167.2005.00265.x.
44. Potschka H, Baltes S, Löscher W. Inhibition of multidrug transporters by verapamil or probenecid does not alter blood-brain barrier penetration of levetiracetam in rats. Epilepsy Res. 2004;58(2-3):85-91. doi: 10.1016/j.eplepsyres.2003.12.007.
45. Baltes S, Gastens AM, Fedrowitz M, et al. Differences in the transport of the antiepileptic drugs phenytoin, levetiracetam and carbamazepine by human and mouse P-glycoprotein. Neuropharmacology. 2007;52(2):333-346. doi: 10.1016/j.neuropharm.2006.07.038.
46. Luna-Tortós C, Rambeck B, Jürgens UH, et al. The antiepileptic drug topiramate is a substrate for human P-glycoprotein but not multidrug resistance proteins. Pharm Res. 2009;26(11):2464-2470. doi: 10.1007/s11095-009-9961-8.
47. Cerveny L, Pavek P, Malakova J, et al. Lack of interactions between breast cancer resistance protein (bcrp/abcg2) and selected antiepileptic agents. Epilepsia. 2006;47(3):461-8. doi: 10.1111/j.1528-1167.2006.00453.x.
48. Nakanishi H, Yonezawa A, Matsubara K, et al. Impact of P-glycoprotein and breast cancer resistance protein on the brain distribution of antiepileptic drugs in knockout mouse models. Eur J Pharmacol. 2013;710(1-3):20-28. doi: 10.1016/j.ejphar.2013.03.049.
49. Römermann K, Helmer R, Löscher W. The antiepileptic drug lamotrigine is a substrate of mouse and human breast cancer resistance protein (ABCG2). Neuropharmacology. 2015;93:7-14. doi: 10.1016/j.neuropharm.2015.01.015.
50. Van Vliet EA, Redeker S, Aronica E, et al. Expression of multidrug transporters MRP1, MRP2, and BCRP shortly after status epilepticus, during the latent period, and in chronic epileptic rats. Epilepsia. 2005;46(10):1569-1580. doi: 10.1111/j.1528-1167.2005.00250.x.
51. Wang-Tilz Y, Tilz C, Wang B, et al. Influence of lamotrigine and topiramate on MDR1 expression in difficult-to-treat temporal lobe epilepsy. Epilepsia. 2006;47(2):233-239. doi: 10.1111/j.1528-1167.2006.00414.x.
52. Wen T, Liu YC, Yang HW, et al. Effect of 21-day exposure of phenobarbital, carbamazepine and phenytoin on P-glycoprotein expression and activity in the rat brain. J Neurol Sci. 2008;270(1-2):99-106. doi: 10.1016/j.jns.2008.02.016.

53. Lazarowski A, Ramos AJ, García-Rivello H, et al. Neuronal and glial expression of the multidrug resistance gene product in an experimental epilepsy model. Cellular and molecular neurobiology. 2004;24(1):77-85. doi: 10.1023/b:cemn.0000012726.43842.d2.
54. Lazarowski A, Czornyj L, Lubienieki F, et al. ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. Epilepsia. 2007;48 Suppl 5:140-9. doi: 10.1111/j.1528-1167.2007.01302.x.
55. Ghosh C, Puvenna V, Gonzalez-Martinez J, et al. Blood-brain barrier P450 enzymes and multidrug transporters in drug resistance: a synergistic role in neurological diseases. Current drug metabolism. 2011;12(8):742-749. doi: 10.2174/138920011798357051.
56. Vazquez SE, D'Giano C, Carpintiero S, et al. Increase 99mTc-SESTAMIBI (MIBI) liver clearance could have identified epileptic pharmacoresistant patients. A preliminary study. Epilepsia. 2004;45:120.
57. Chiosa V, Groppa SA, Ciolac D, et al. Breakdown of thalamo-cortical connectivity precedes spike generation in focal epilepsies. Brain Connect. 2017;7(5):309-320. doi: 10.1089/brain.2017.0487.
58. Chiosa V, Ciolac D, Groppa S, et al. Large-scale network architecture and associated structural cortico-subcortical abnormalities in patients with sleep/awake-related seizures. Sleep. 2019;42(4):zsz006.. doi: 10.1093/sleep/zsz006.
59. Ciolac D. Reorganization and resilience of brain networks in focal epilepsy. Mold Med J. 2020;63(5):5-8. doi: 10.5281/zenodo.4018890.
60. Juvale IIA, Che Has AT. Possible interplay between the theories of pharmacoresistant epilepsy. Eur J Neurosci. 2021;53(6):1998-2026. doi: 10.1111/ejn.15079.
61. Cardenas-Rodriguez N, Carmona-Aparicio L, Pérez-Lozano DL, et al. Genetic variations associated with pharmacoresistant epilepsy (Review). Mol Med Rep. 2020;21(4):1685-1701. doi: 10.3892/mmr.2020.10999.
62. Chouchi M, Kaabachi W, Klaa H, et al. Relationship between ABCB1 3435TT genotype and antiepileptic drugs resistance in Epilepsy: updated systematic review and meta-analysis. BMC Neurol. 2017;17(1):1-14. doi: 10.1186/s12883-017-0801-x.
63. Fernandez-Marmiesse A, Sánchez-Iglesias S, Darling A, et al. A *de novo* heterozygous missense BSCL2 variant in 2 siblings with intractable developmental and epileptic encephalopathy. Seizure. 2019;71:161-165. doi: 10.1016/j.seizure.2019.07.019.64. Goto A, Ishii A, Shibata M, et al. Characteristics of KCNQ 2 variants causing either benign neonatal epilepsy or developmental and epileptic encephalopathy. Epilepsia. 2019;60(9):1870-1880. doi: 10.1111/epi.16314.
65. Yang X, Yan Y, Fang Set al. Comparison of oxcarbazepine efficacy and MHD concentrations relative to age and BMI: Associations among ABCB1, ABCC2, UGT2B7, and SCN2A polymorphisms. Medicine (Baltimore). 2019;98(12):e14908. doi: 10.1097/MD.0000000000014908.
66. Esmaeilzadeh H, Farjadian S, Alyasin S, et al. Epidemiology of severe cutaneous adverse drug reaction and its HLA Association among pediatrics. Iran J Pharm Res. 2019;18(1):506-522.
67. Myers CT, Mefford HC. Advancing epilepsy genetics in the genomic era. Genome Med. 2015;7(1):91. doi: 10.1186/s13073-015-0214-7.
68. Waldbaum S, Patel M. Mitochondria, oxidative stress, and temporal lobe epilepsy. Epilepsy Res. 2010;88(1):23-45. doi: 10.1016/j.eplepsyres.2009.09.020.
69. Janmohamed M, Brodie MJ, Kwan P. Pharmacoresistance – Epidemiology, mechanisms, and impact on epilepsy treatment. Neuropharmacology. 2020;168:107790. doi: 10.1016/j.neuropharm.2019.107790.
70. Kobow K, Reid CA, van Vliet EA, et al. Epigenetics explained: a topic "primer" for the epilepsy community by the ILAE Genetics/Epigenetics Task Force. Epileptic Disord. 2020;22(2):127-141. doi: 10.1684/epd.2020.1143.
71. Hauser RM, Henshall DC, Lubin FD. The epigenetics of epilepsy and its progression. Neuroscientist. 2018;24(2):186-200. doi: 10.1177/1073858417705840.
72. Miller-Delaney SF, Bryan K, Das S, et al. Differential DNA methylation profiles of coding and non-coding genes define hippocampal sclerosis in human temporal lobe epilepsy. Brain. 2015;138(Pt 3):616-31. doi: 10.1093/brain/awu373.
73. Morris G, Reschke CR, Henshall DC. Targeting microRNA-134 for seizure control and disease modification in epilepsy. EBioMedicine. 2019;45:646-654. doi: 10.1016/j.ebiom.2019.07.008.
74. Vezzani A, Rench J, Bartfai T, et al. The role of inflammation in epilepsy. Nat Rev Neurol. 2011;7(1):31-40. doi: 10.1038/nrneurol.2010.178.
75. Vezzani A, Viviani B. Neuromodulatory properties of inflammatory cytokines and their impact on neuronal excitability. Neuropharmacology. 2015;96(Pt A):70-82. doi: 10.1016/j.neuropharm.2014.10.027.
76. Holmes M, Flaminio Z, Vardhan M, et al. Cross talk between drug-resistant epilepsy and the gut microbiome. Epilepsia. 2020;61(12):2619-2628. doi: 10.1111/epi.16744.
77. Medel-Matus JS, Shin D, Dorfman E, et al. Facilitation of kindling epileptogenesis by chronic stress may be mediated by intestinal microbiome. Epilepsia Open. 2018;3(2):290-294. doi: 10.1002/epi4.12114.
78. Xie G, Zhou Q, Qiu CZ, et al. Ketogenic diet poses a significant effect on imbalanced gut microbiota in infants with refractory epilepsy. World J Gastroenterol. 2017;23(33):6164. doi: 10.3748/wjg.v23.i33.6164.
79. Zhang Y, Zhou S, Zhou Y, et al. Altered gut microbiome composition in children with refractory epilepsy after ketogenic diet. Epilepsy Res. 2018;145:163-168. doi: 10.1016/j.eplepsyres.2018.06.015.
80. Lindefeldt M, Eng A, Darban H, et al. The ketogenic diet influences taxonomic and functional composition of the gut microbiota in children with severe epilepsy. NPJ Biofilms Microbiomes. 2019;5(1):1-13. doi: 10.1038/s41522-018-0073-2.
81. Gómez-Eguílaz M, Ramón-Trapero JL, Pérez-Martínez L, et al. The beneficial effect of probiotics as a supplementary treatment in drug-resistant epilepsy: a pilot study. Benef Microbes. 2018;9(6):875-881. doi: 10.3920/BM2018.0018.
82. Yeom JS, Park JS, Kim YS, et al. Neonatal seizures and white matter injury: Role of rotavirus infection and probiotics. Brain Dev. 2019;41(1):19-28. doi: 10.1016/j.braindev.2018.07.001.
83. Larivière S, Bernasconi A, Bernasconi N, et al. Connectome biomarkers of drug-resistant epilepsy. Epilepsia. 2021;62(1):6-24. doi: 10.1111/epi.16753.
84. Henshall DC, Hamer HM, Pasterkamp RJ, et al. MicroRNAs in epilepsy: pathophysiology and clinical utility. Lancet Neurol. 2016;15(13):1368-1376. doi: 10.1016/S1474-4422(16)30246-0.
85. Parras A, de Diego-Garcia L, Alves M, et al. Polyadenylation of mRNA as a novel regulatory mechanism of gene expression in temporal lobe epilepsy. Brain. 2020;143(7):2139-2153. doi: 10.1093/brain/awaa168.
86. Lagarde S, Villeneuve N, Trébuchon A, et al. Anti-tumor necrosis factor alpha therapy (adalimumab) in Rasmussen's encephalitis: an open pilot study. Epilepsia. 2016;57(6):956-966. doi: 10.1111/epi.13387.
87. Woo CW, Chang LJ, Lindquist MA, et al. Building better biomarkers: brain models in translational neuroimaging. Nat Neurosci. 2017;20(3):365-377. doi: 10.1038/nn.4478.
88. French JA, Lawson JA, Yapici Z, et al. Adjunctive everolimus therapy for treatment-resistant focal-onset seizures associated with tuberous sclerosis (EXIST-3): a phase 3, randomised, double-blind, placebo-controlled study. Lancet. 2016;388(10056):2153-2163. doi: 10.1016/S0140-6736(16)31419-2.
89. Zaccara G, Schmidt D. Antiepileptic drugs in clinical development: differentiate or die? Current pharmaceutical design. 2017;23(37):5593-5605. doi: 10.2174/1381612823666170809100524.
90. Krauss GL, Klein P, Brandt C, et al. Safety and efficacy of adjunctive cenobamate (YKP3089) in patients with uncontrolled focal seizures: a multicentre, double-blind, randomised, placebo-controlled, dose-response trial. Lancet Neurol. 2020;19(1):38-48. doi: 10.1016/S1474-4422(19)30399-0.
91. Polster T. Individualized treatment approaches: Fenfluramine, a novel antiepileptic medication for the treatment of seizures in Dravet syndrome. Epilepsy Behav. 2019;91:99-102. doi: 10.1016/j.yebeh.2018.08.021.
92. Szaflarski JP, Bebin EM, Comi AM, et al. Long-term safety and treatment effects of cannabidiol in children and adults with treatment-resistant epilepsies: expanded access program results. Epilepsia. 2018;59(8):1540-1548. doi: 10.1111/epi.14477.

93. Chen JW, Borgelt LM, Blackmer AB. Cannabidiol: a new hope for patients with Dravet or Lennox-Gastaut syndromes. Ann Pharmacother. 2019;53(6):603-611. doi: 10.1177/1060028018822124.
94. Simonato M. Gene therapy for epilepsy. Epilepsy Behav. 2014;38:125-30. doi: 10.1016/j.yebeh.2013.09.013.
95. Riban V, Fitzsimons HL, During MJ. Gene therapy in epilepsy. Epilepsia. 2009;50(1):24-32. doi: 10.1111/j.1528-1167.2008.01743.x.
96. Mollanoori H, Teimourian S. Therapeutic applications of CRISPR/Cas9 system in gene therapy. Biotechnol Lett. 2018;40(6):907-914. doi: 10.1007/s10529-018-2555-y.
97. Rao G, Mashkouri S, Aum D, et al. Contemplating stem cell therapy for epilepsy-induced neuropsychiatric symptoms. Neuropsychiatr Dis Treat. 2017;13:585-596. doi: 10.2147/NDT.S114786.
98. Kalani A, Tyagi A, Tyagi N. Exosomes: mediators of neurodegeneration, neuroprotection and therapeutics. Mol Neurobiol. 2014;49(1):590-600. doi: 10.1007/s12035-013-8544-1.
99. Janmohamed M, Brodie MJ, Kwan P. Pharmacoresistance – Epidemiology, mechanisms, and impact on epilepsy treatment. Neuropharmacology. 2020;168:107790. doi: 10.1016/j.neuropharm.2019.107790.
100. Trinka E, Brigo F. Neurostimulation in the treatment of refractory and super-refractory status epilepticus. Epilepsy Behav. 2019;101(Pt B):106551. doi: 10.1016/j.yebeh.2019.106551.
101. San-juan D, Morales-Quezada L, Orozco Garduño AJ, et al. Transcranial direct current stimulation in epilepsy. Brain Stimul. 2015;8(3):455-464. doi: 10.1016/j.brs.2015.01.001.
102. Fanselow EE. Central mechanisms of cranial nerve stimulation for epilepsy. Surg Neurol Int. 2012;3(Suppl 4):S247-54. doi: 10.4103/2152-7806.103014.
103. Rocchi L, Ibáñez J, Benussi A, et al. Variability and predictors of response to continuous theta burst stimulation: a TMS-EEG study. Front Neurosci. 2018;12:400. doi: 10.3389/fnins.2018.00400.
104. Ellis TL, Stevens A. Deep brain stimulation for medically refractory epilepsy. Neurosurg Focus. 2008;25(3):E11. doi: 10.3171/FOC/2008/25/9/E11.
105. Téllez-Zenteno JF, Hernández-Ronquillo L, Buckley S, et al. A validation of the new definition of drug-resistant epilepsy by the International League Against Epilepsy. Epilepsia. 2014;55(6):829-834. doi: 10.1111/epi.12633.

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VC, DC and SG conceptualized the project and designed the research; VC, DC, VC, DG, AV and CM conducted literature review and drafted the first manuscript. SG revised the manuscript critically. All the authors approved the final version of the manuscript.

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