ISSN 2537-6373 (Print)
ISSN 2537-6381 (Online)

The Moldovan Medical Journal

The Publication of the Scientific Medical Association of Moldova

Frequency – 4 per year
Category – B+

Vol. 66, No 2
December 2023

Welcome to the Moldovan Medical Journal!

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.

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Index for subscription – 32130

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Publisher

"Revista Curier Medical"
192, Stefan cel Mare Avenue
Chisinau, the Republic of Moldova

Printing House "Tipografia No 1"
46, 31 August 1989 str., Chisinau, MD-2001
the Republic of Moldova
www. tipografia.nr1@gmail.com

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ORIGINAL RESEARCHES

https://doi.org/10.52418/moldovan-med-j.66-2.23.01
UDC: 615.322.074:[547.567.2+547.913]:582.949.27

Obtaining thymoquinone and thymohydroquinone from Wild bergamot (*Monarda fistulosa* L.)

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Authors' ORCID iDs, academic degrees and contributions are available at the end of the article

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Manuscript received April 5, 2023; revised manuscript June 7, 2023; published online December 27, 2023

Abstract

Background: Thymoquinone (TQ) and thymohydroquinone (THQ) are natural substances with antibacterial, antimycotic, antiviral, anticancer, antioxidant activity. Previously, was developed the method for obtaining Wild bergamot essential oil with high TQ content. The objective of this study was to create methods for obtaining individual substances TQ and THQ from Wild bergamot herb.

Material and methods: Aerial parts of Wild bergamot have been collected from the plantation of the Scientific Practical Centre of *Nicolae Testemitanu* State University of Medicine and Pharmacy. The hydrodistillation method has been used for essential oil isolation and fractionation, and purification of individual substances. The analytical part of study has been performed by the HPLC method.

Results: THQ was obtained in crystalline form by reducing TQ directly in the essential oil with ascorbic acid, sulphurous acid, or natural components of the Wild bergamot essential oil. Subsequent oxidation of THQ with hydrogen peroxide or nitrous acid gave TQ. Additionally, the yield of TQ was increased by optimising the parameters of plant material fermentation and using two-stage hydrodistillation with intermediate oxidation of the THQ formed at the first stage.

Conclusions: Several technological procedures were created to obtain THQ from the Wild bergamot essential oil with a yield from 68-74% to 93-97% and TQ from THQ with a yield of 87-92%. The procedure of plant material processing has been optimised also to obtain essential oil with 48-56% TQ and not more than 12% residual phenols.

Key words: *Monarda fistulosa* L., essential oil, thymoquinone, thymohydroquinone.

Cite this article
Casian I, Casian A. Obtaining thymoquinone and thymohydroquinone from Wild bergamot (*Monarda fistulosa* L.). *Mold Med J*. 2023;66(2):5-11. https://doi.org/10.52418/moldovan-med-j.66-2.23.01.

Introduction

Thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone, TQ) and thymohydroquinone (2-isopropyl-5-methylhydroquinone, THQ) are phytochemicals found in several genera of plants of the *Lamiaceae* family, such as *Monarda*, *Thymus*, *Satureja*, as well as in the species *Nigella sativa* L. [1, 2].

TQ, studied as a pure substance or as the basic active principle of Black cumin oil (*Nigella sativa* L.), has demonstrated a wide spectrum of actions, including hepatoprotective [3, 4], antioxidant [5-7], antibacterial [8, 9], antimycotic [10], anti-inflammatory [11], anticancer [12, 13], antituberculosis [14], antiviral [15, 16], spasmolytic [17]. The literature describes the obtaining of TQ by chemical synthesis [18, 19] and by isolation from plant material [20].

THQ, being a reduced form of TQ, has a higher antioxidant and prooxidant activity [5]. Antibacterial [8], antimycotic [10], anticancer [13], and antiviral [15] activities have also been found. At the same time, THQ remains less studied in terms of pharmacology.

The TQ content in the Wild bergamot essential oil, obtained by traditional methods, is relatively low, being dependent on drying and storage conditions, as well as on the technological parameters of the plant material processing [21]. The intensification of TQ accumulation in Wild bergamot oil (up to 10-40%) can be achieved by applying some agrotechnical procedures [22, 23]. Previously, was developed the method for obtaining Wild bergamot essential oil with high TQ content (20-32%) from pre-fermented plant material [21], which made it possible to carry out a study for isolation of individual substances TQ and THQ from this oil.

The objective of this study was to create laboratory methods for preparative obtaining individual substances

TQ and THQ from the Wild bergamot herb, and to optimise conditions of plant material processing for obtaining the essential oil most suitable for this purpose.

Material and methods

Plant material. Aerial parts of the Wild bergamot, free of lignified stems, have been collected from the plantation of the Scientific Practical Centre of Medicinal Plants of *Nicolae Testemitanu* State University of Medicine and Pharmacy. A part of the plant material was immediately processed to obtain the essential oil, the rest was air-dried for further processing.

Apparatus. Fermentation of previously humidified plant material has been performed in a cylindrical extractor with an internal volume of 4.5 L, installed in an air thermostat. The plant material temperature and oxygen concentration in the air, blowing through the extractor, were measured with DS18B20 digital thermometer (*Dallas Semiconductor*) and 4OXV oxygen sensor (*City Technology*) respectively, and registered during the entire process with a laboratory-made data logger based on RP2040 microcontroller (*Raspberry Pi Ltd*). Essential oil isolation has been performed by hydrodistillation, using a steam generator with electric power of 1.1 kW, the same extractor, where plant material was fermented, and a glass flow cooler. Analysis of plant material, essential oil, reaction mixes, and the obtained samples of individual substances has been performed using *Agilent 1260* liquid chromatograph with diode-array UV detector in conditions described previously [21].

Chemicals. In the study have been used reference substances: Thymol, Carvacrol, and TQ (*Sigma-Aldrich*). Ascorbic acid was of pharmaceutical grade (*Ph. Eur.*) THQ reference solution was obtained *in situ* as a product of TQ reduction by ascorbic acid. Other reagents of analytical grade have been purchased from *Sigma-Aldrich Chemie GmbH* and *Merck* (Germany).

Results and discussion

The direct isolation of TQ from Wild bergamot essential oil, based on the difference in its physical properties from other components, is notably difficult. Thus, according to the observations, crystallisation of TQ is possible when its content in the essential oil (or any fraction) is higher than 65-70%. Furthermore, the yield of TQ is very low, and its subsequent purification is difficult. The use of preparative chromatography is more efficient but highly expensive. The idea of solving this problem was prompted by the spontaneous crystallisation of THQ, observed during storage of essential oil obtained from dry Wild bergamot herb, as a result of the reduction of TQ, contained in it, with some natural components [21]. The relatively high polarity of THQ causes its low solubility in essential oil, and its non-volatility facilitates its purification from volatile components. Actually, the idea was to reduce quantitatively TQ into THQ with one of the suitable reducing agents, to separate crystalline THQ from the oil phase, and to remove foreign volatile components by distillation with water vapour. Then the obtained THQ can be reconverted to TQ with a suitable oxidising agent.

The solution to this problem required additional optimisation of the previously described technology [21] for obtaining Wild bergamot essential oil rich in TQ. The reason was that residual amounts of thymol and carvacrol increase the solubility of THQ in the reaction mixture (fig. 4), thereby reducing its yield. In this regard, the conditions for the plant material fermentation have been optimised in the direction of the most complete oxidation of volatile phenols into TQ, while avoiding significant wastage of their vapours with air. This was achieved by programming the temperature during the fermentation process, reducing the speed of blowing air, and increasing the overall duration of the process. Additionally, the parameters of hydrodistillation of the fermented plant material were optimised. Instead of blowing with a mixture of steam and air, was used two-stage distillation with clean steam at high speed with intermediate air oxidation. This made it possible to reduce the loss of essential oil during distillation and achieve a high yield of TQ, even when using an extractor with increased volume.

An optimised variant of plant material processing was implemented in laboratory conditions, as the following procedure:

0.45 kg of dried Wild bergamot herb was humectated, mixing and slightly crushing, with 0.6 L of water or hydrolate waste from the previous batch, then loaded into a cylindrical extractor of 4.5 L volume. The extractor was kept at room temperature for a day and at 30-35°C for the next 2-3 days, blowing with air at 0.05-0.07 L/min and changing the air direction to the opposite every day, to prevent drying the segment of the plant material closed to the inlet. The fermentation rate was appreciated by the oxygen consumption. A typical diagram of the outlet oxygen concentration is present in fig. 1. The lower temperature on the first day of fermentation reduces the loss of volatile phenols, allocated outside the peltate trichomes, especially if the plant material was humectated with hydrolate waste. At long-term (more than 2-3 months) storage of plant material, the activity of oxidative enzymes is significantly reduced. In this case, the total duration of the fermentation can be increased to 5-6 days.

The extractor with fermented plant material was wrapped with heat-insulating cloth, connected between the steam generator and flow cooler, and about 250 mL distillate was collected into a narrow-necked flask of 500 mL volume. Immediately after turning off the steam generator, the extractor was blown with air at 0.5 L/min for 3 hours to oxidise the THQ, formed during the first hydrodistillation, into TQ. A small amount of condensate, formed during this operation, was collected into the same flask. Then hydrodistillation was repeated in the same regime until the receiving flask was filled. From the narrow part of the flask with a pipette were taken 11-14 g of essential oil with

TQ content of 48-56% and the amount of residual phenols from 5 to 12%, depending on the activity of enzymes in the plant material. Hydrodistillation was continued until another 100 mL of condensate was obtained, which was combined with hydrolate waste from the main receiving flask and used to humidify the plant material in the next batch. Using waste hydrolate instead of water improves the wettability of the plant material and increases the yield of the essential oil and TQ by about 8-9%.

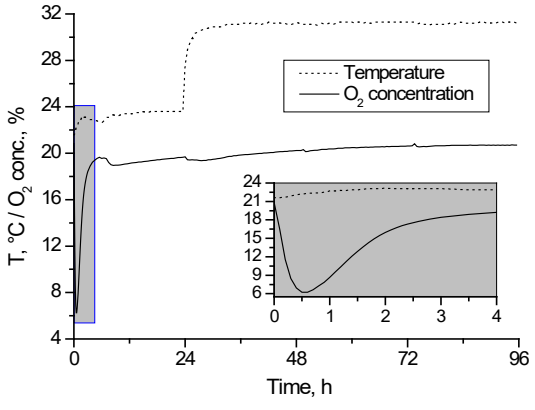

Fig. 1. Temperature and oxygen concentration change during the fermentation process

Obtaining thymohydroquinone. For this purpose, Wild bergamot essential oil with high TQ content has been treated with various reducing agents.

Method 1 – reduction of TQ with ascorbic acid: 20 mL of Wild bergamot oil rich in TQ and 10-20 mL of diluent were added to a 100 mL flat-bottom flask. Separately, a buffered solution of ascorbic acid was prepared as follows: Ascorbic acid in an amount of 1.29 g per gram of TQ, added with essential oil, (20% excess *vs.* stoichiometric amount) and sodium phosphate dodecahydrate in an amount of 0.2 g per gram of TQ was dissolved in 2.5 Mol/L sodium hydroxide solution, taken in an amount of 2.5 mL for each gram of TQ. The solution was cooled to room temperature, adjusted, if necessary, to pH 6.0-6.3 with sodium hydroxide or phosphoric acid, then completed with water to a volume approximately equal to the volume of the oil mixture (essential oil + diluent). The resulting solution was transferred into the flask with the oil mixture and intensively mixed with a magnetic stirrer for 1.5-2.5 hours. The end of the reaction was detected by the cessation of colour change of the reaction mixture and was confirmed by determination of the residual amount of TQ in the oil layer, which usually did not exceed 0.2%. The chemical reaction proceeds according to fig. 2.

THQ forms as a crystalline precipitate in the oil layer. Too low pH values of the aqueous layer slow the reaction, while too high values result in the formation of a fine precipitate of THQ and significant amounts of dark-coloured by-products.

Method 2 – reduction of TQ with sulphurous acid: In a 100 mL flat-bottom flask, 20 mL of Wild bergamot oil rich in TQ and 10-20 mL of diluent were added. Separately, potassium metabisulphite, taken in an amount of 0.75-0.81 g for each gram of TQ added with the Wild bergamot oil (10-20% excess), was dissolved in 30-40 mL of water. This solution was transferred into the flask with the oil mixture, and a small amount (0.5-0.7 mL) of 2.5 Mol/L sulphuric acid was introduced into the aqueous phase to initiate the reaction. Then a sufficient amount of sulphuric acid, necessary for the formation of sulphurous acid, is formed as one of the products (fig. 3). The contents of the flask were intensively mixed with a magnetic stirrer for 2-3 hours until the colour change ceased. As in the previous method, THQ is formed as a crystalline precipitate in the oil layer.

In both described methods, the primary role of the diluent is to reduce the viscosity of the oil layer during the formation of the THQ suspension and to allow efficient mixing of the layers. Its other function is to decrease the concentration of phenolic components of the essential oil, which promotes the crystallisation of THQ. Studies with different fractions of the oil layer, separated after finishing the reaction, and with their model mixtures showed that the solubility of THQ in the oil phase almost linearly depends on the total amount of thymol and carvacrol (fig. 4). This has a direct impact on the loss of the target product

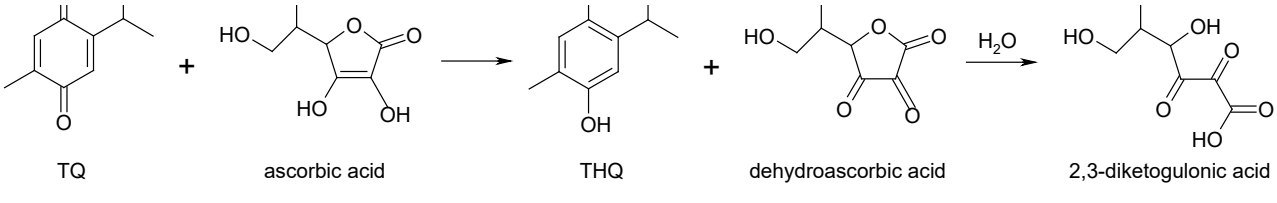

Fig. 2. Reduction of TQ with ascorbic acid

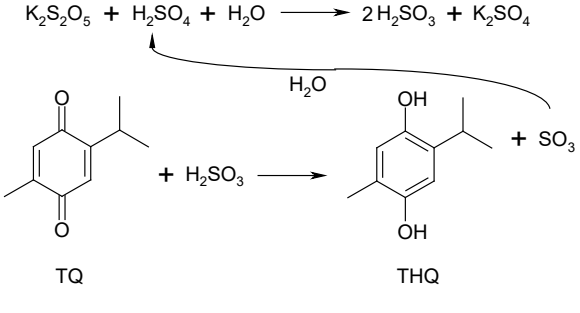

Fig. 3. Reduction of TQ with sulphurous acid

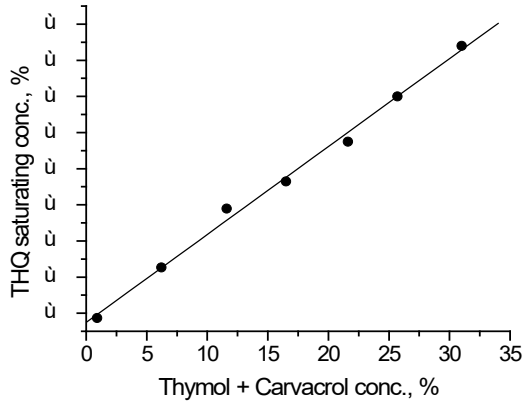

Fig. 4. Solubility of THQ in the oil phase in dependence on the phenols content

with the oil layer, and has become the main reason for optimising the conditions of plant material fermentation to minimise the residual amount of phenols in the essential oil.

Saturated hydrocarbons, such as n-hexane, can be used as a diluent. But, for environmental reasons, individual low-polar components of essential oils, such as pinene, limonene, p-cymene, as well as a light fraction of the waste oil layer of the reaction mixture from previous batches, are more suitable. The last option is the most attractive since it does not require additional solvents and reduces the amount of unused waste.

Waste fractionation was carried out by hydrodistillation according to the following procedure: 160 g of the waste oil layer from several experiments for the THQ obtaining and 0.8 L of water were distilled from a 2 L flat-bottom flask with a dephlegmator, collecting two distillate fractions. The first fraction with a volume of 250 mL was obtained at a heating power of 0.3 kW, and 95.4 g of light components mixture with a total phenol content of 0.9% was separated from it. The second fraction with a volume of 600 mL was distilled at a heating power of 0.5 kW, and 51.4 g of oil with 27.3% phenols was separated from it.

The presence of natural components with reducing properties in the composition of Wild bergamot essential oil is of particular interest. Unfortunately, these components are almost completely oxidised during the fermentation of plant material, but they are preserved in the essential oil obtained from fresh Wild bergamot herb. Their content is especially high in the light fraction of the essential oil. This fact led to the development of another way to obtain THQ. Since the chemical nature of the reducing components is currently unknown, the limited task was set to determine their total reducing power in reaction with TQ.

Method 3 – reduction of TQ with components of Wild bergamot essential oil: Wild bergamot essential oil rich in TQ and essential oil from the fresh herb or (preferably) its light fraction were mixed in a hermetic vial of suitable volume in a ratio, calculated from a predetermined value of reducing power. The vial was kept at 60°C for 3 days, then allowed to cool and left for 1-2 days at 4°C to complete the crystallisation of THQ, usually forming a monolithic crystalline mass, passing into suspension with mixing or shaking.

Fractionation of Wild bergamot essential oil to perform TQ reducing: 103 g of essential oil from fresh Wild bergamot herb and 0.3 L of water were distilled from a 2 L flat-bottom flask with a dephlegmator at a heating power of 0.28 kW, collecting two distillate fractions of 100 mL each. From the first fraction, 27.7 g of the oil layer was separated and used to reduce TQ. The second oil fraction (16.4 g) was similar in its composition to the initial oil and could be re-fractionated. The third oil fraction was separated from the vat residue in the amount of 54.0 g. It contained about 80% of phenols and can be studied as a potential active substance with antimicrobial properties.

Determination of reducing power: In a hermetic 2 mL vial for HPLC samples, 0.5 mL of Wild bergamot essential oil rich in TQ and 0.5 mL of the essential oil from fresh Wild bergamot herb or 0.3 mL of its light fraction were mixed. The vial was thermostated at 60°C for 24 hours, then the concentration of TQ in its content was determined in parallel in the initial Wild bergamot oil. The required volumetric ratio of the two types of essential oil was calculated using the formula:

$V_X = V_1/V_0 * C_0/(C_0 - K * C_1)$, where $K = (V_0 + V_1)/V_0$.

Definitions:

V_X – the volume of the oil from fresh Wild bergamot herb or its light fraction (in mL), required to reduce TQ in 1 mL of the oil from fermented plant material;

C_0 – the concentration of TQ in the initial oil from the fermented plant material;

C_1 – the concentration of TQ in the mixture after the test experiment;

V_1 and V_0 are, respectively, the volumes of oil from fresh Wild bergamot herb, or its light fraction, and the oil from fermented plant material, taken in the test experiment.

Separation and purification of THQ. In all three methods, the THQ precipitate was separated from the liquid phase by filter centrifugation, followed by triple washing with small amounts of water. The last operation allows the removal of water-soluble products of the chemical reaction (for methods 1 and 2) and a part of the oil phase. To remove the residues of the essential oil, the wet precipitate of THQ was transferred to a flat-bottom flask of sufficient volume and placed on a heating magnetic stirrer. Water was added in an amount of 15 mL per gram of TQ in the composition of used essential oil, and ascorbic acid in an amount of about 10 mg per gram of TQ, to protect THQ from oxidation. Then, with continuous stirring, at least half of the added amount of water was evaporated. The flask was allowed to cool slowly and kept until the next day at 4°C to complete the crystallisation. The precipitate was separated on a glass filter under a weak vacuum, washed with cooled 0.1% acetic acid solution, and dried to a constant weight at 40°C.

The resulting product is an odourless white crystalline powder, with a melting point of 142-142.5°C, easily soluble in ethanol and acetonitrile, slightly soluble in chloroform, very slightly soluble in water, and practically insoluble in hexane. The substance contains about 98% THQ, and the main impurities are related hydroquinone derivatives formed by the reduction of corresponding benzoquinones from Wild bergamot essential oil.

Reduction of TQ with ascorbic acid (method 1) can be recommended as the most efficient (93-97% yield of the target product) and safe enough way to obtain THQ.

Method 2 (reduction with sulphurous acid) is the cheapest. However, was observed a significant slowdown of the reaction toward the end of the process, especially under a slight excess of the reducing agent. As a result, it was not possible to achieve complete consumption of TQ. At the same time, a significant excess of sulphurous acid at the end of the reaction greatly increases the solubility of THQ in the aqueous phase and its loss with the aqueous layer waste. As a result, the final yield of THQ was only 68-74%. Another disadvantage of this method is the emission of toxic sulphur dioxide into the atmosphere.

Reduction of TQ with natural components of the same plant (method 3) is very attractive in sense of personal and environmental safety since it does not involve the use of any chemicals. The yield of THQ is quite good (about 82%). However, the need to obtain additionally the essential oil from the fresh plant material and the long duration of the procedure, carried out at an increased temperature, make this method the most expensive.

Obtaining thymoquinone can be performed by oxidation of THQ with various oxidising agents. The main problem was to achieve a sufficiently high rate and completeness of the reaction with a minimum amount of by-products. In this study, were used two oxidising agents: hydrogen peroxide and nitrous acid, which have their advantages and disadvantages.

Method 1 – oxidation of THQ with hydrogen peroxide in aqueous suspension: Into a 250 mL flat-bottom flask were added 15 g of ground and sieved THQ, 0.15 g of sodium lauryl sulphate (for better wetting of THQ), 75 mL of water, and 15 mL of concentrated hydrochloric acid. The mixture was sonicated and cooled to 4°C. Then 1.5 mL of 1 Mol/L copper (II) chloride solution and 59 mL (15% excess) of chilled 6% hydrogen peroxide were added at stirring. The mixture was stirred for 2 hours, cooling the flask with flowing water, and then kept at 4°C for the next 2 hours. The precipitate was separated on a glass filter and washed with chilled water.

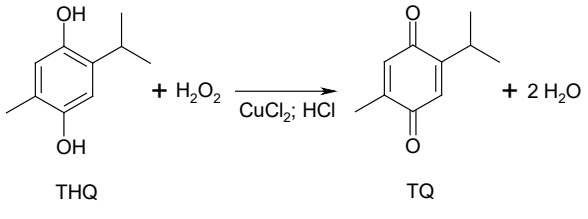

Fig. 5. THQ oxidation with hydrogen peroxide

The main chemical reaction proceeds according to the scheme in fig. 5.

Copper (II) chloride is used as a catalyst. Actually, the catalytic activity belongs to the anionic complex $[CuCl_4]^{2-}$ formed in the presence of hydrochloric acid. Cooling the reaction mixture and slow addition of hydrogen peroxide contribute to the formation of a smaller amount of by-products.

Purification of TQ: The wet precipitate of crude TQ was transferred to a 2 L flat-bottom flask, 700 mL of water was added, and about 500 mL of liquid was distilled without a dephlegmator at a heating power of 0.5 kW, maintaining the condensate temperature within 47-55°C to prevent crystallisation of TQ in the cooler. The distillate was kept until the next day at 4°C, crystalline TQ was separated on a glass filter, washed with chilled water, and dried at 35°C. 13.2 g of purified product were obtained (about 88% yield).

It is also possible to obtain thymoquinhydrone (TQH) by introducing in the reaction exactly half of the stoichiometric amount of hydrogen peroxide, then separating and drying the precipitate without further purification. TQH is obtained as a black-coloured crystalline powder and presents a hydrogen bond complex of equimolar amounts of TQ and THQ. It melts in the range of 88-95°C with decomposition into its constituent components. It is easily soluble in ethanol and acetonitrile, sparingly soluble in chloroform, and slightly soluble in water. It is partially soluble in hexane with decomposition.

Method 2 – oxidation of THQ with hydrogen peroxide in aqueous-acetonitrile solution: 15 g of THQ was added to a 250 mL flat-bottom flask, dissolved in a mixture of 60 mL acetonitrile and 15 mL of concentrated hydrochloric acid. The following operations were performed in an ice bath. 1.5 mL of 1 Mol/L copper (II) chloride solution was added, then, with continuous stirring, 56 mL (10% excess) of 6% hydrogen peroxide solution was injected with a peristaltic pump at 1.5 mL/min. Stirring was continued for another 1 hour (until the liquid turned yellow). The reaction mixture was saturated with 15 g of sodium chloride and allowed to stand to separate the layers. TQ is contained in the acetonitrile (upper) layer.

Isolation and purification of TQ: The upper liquid layer (57 mL) was transferred to a 2 L flat-bottom flask, 120 mL of water was added, and acetonitrile was distilled off with a dephlegmator at a heating power of 0.25 kW up to the vapour temperature of 94°C. The distillate (46 mL), containing about 83% acetonitrile and about 0.5% TQ, can be used in the next batch instead of a part of acetonitrile.

600 mL of water was added to the residue in the flask and about 500 mL of liquid was distilled without a dephlegmator at a heating power of 0.5 kW, maintaining the temperature of the condensate within 47-55°C to prevent crystallisation of TQ in the cooler. The distillate was kept until the next day at 4°C, crystalline TQ was separated on a glass

filter, washed with chilled water, and dried at 35°C. 13.1 g of the purified product was obtained (about 87% yield).

Method 3 – oxidation of THQ with nitrous acid: In a 100 mL flat-bottom flask, 15 g of THQ was added and dissolved in a mixture of 60 mL acetonitrile and 20 mL of concentrated hydrochloric acid (10% excess). With continuous stirring, 42 mL of 30% sodium nitrite solution (about 1% excess) was injected with a peristaltic pump at 0.7 mL/min (until finishing the intensive elimination of nitric oxide, which has been decomposed in the flame of a gas burner). Alternatively, the nitric oxide can be absorbed with a potassium permanganate solution. Stirring was continued for another 10 min. The mixture was allowed to stand for layers' separation, and the upper layer, containing TQ, was taken. In this case, saturation with sodium chloride is not required, since its sufficient amount is formed in the chemical reaction (fig. 6).

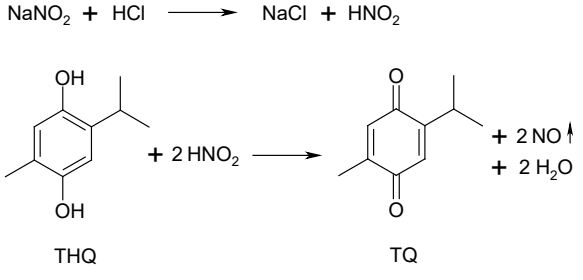

Fig. 6. THQ oxidation with nitrous acid

Isolation and purification of TQ were carried out the same way as described in method 2. In this experiment 13.8 g of the purified product was obtained (about 92% yield).

The obtained TQ is an orange-yellow crystalline powder or conglomerates of crystals with a slight odour. Its vapours are irritant to the mucous membranes of the eyes and respiratory tract. The melting point of TQ is 45-46°C. It is very easily soluble in acetonitrile and chloroform, freely soluble in ethanol and hexane, and very slightly soluble in water. The substance contains about 98% of TQ, and the main impurities are related substances of the benzoquinone structure.

Method 1 is the most environmentally friendly since it is not associated with organic solvents or highly toxic substances. At the same time, passing the reaction in a suspension leads to reduced robustness of the process and an increased amount of by-products. The situation is complicated by the poor wettability of THQ in aqueous solutions and its tendency to float. The last problem is solved by adding a surfactant (sodium lauryl sulphate), but its excess increases the loss of TQ with the filtrate of the reaction mixture and can lead to the formation of a fine precipitate that is difficult to filter.

Method 2 gives well-reproducible results, but has a slightly higher labour intensity and requires an organic solvent (acetonitrile).

Method 3 is characterised by the fastest and most selective passing of the chemical reaction and, as a result, by the formation of the smallest amount of by-products. However, its serious disadvantage is the release of highly toxic nitric oxide, which must be captured and rendered harmless.

Conclusions

Several technological procedures were created to obtain thymohydroquinone from the Wild bergamot essential oil with a yield from 68-74% to 93-97% and thymoquinone from thymohydroquinone with a yield of 87-92%. The procedure of plant material processing has been optimised also to obtain essential oil with 48-56% of thymoquinone and not more than 12% of residual phenols.

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Authors' contributions
IC designed the study, conducted the laboratory work and performed its technological part, interpreted the data, and drafted the first version of the manuscript. AC collected and processed the plant material, performed the analytical part of the laboratory work, and revised the manuscript. The final version of the manuscript was approved by both authors.

Funding
This study was a part of the project No 20.80009.8007.14 "Complex researchers for the elaboration of the new autochthonous anti-infectious pharmaceutical products for the optimisation of the pharmacotherapy of the dental, oropharyngeal, and auricular disorders" within the State Program of the Republic of Moldova (2020-2023).

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

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.02
UDC: 616.314.18-08

Modern nickel-titanium rotary systems in endodontic treatment

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Manuscript received April 13, 2023; revised manuscript June 07, 2023; published online December 27, 2023

Abstract

Background: One of the most important steps in endodontic treatment is the preparation of a uniform space, the use of endodontic irrigant and creating space for the endodontic filling. Therefore, improving endodontic treatment techniques is one of the most important tasks of modern dentistry. The success and effectiveness of endodontic treatment is largely determined by the quality of chemo-mechanical root canal treatment. In recent years there has been a major emphasis on the use of rotary mechanical instrumentation systems, namely the use of nickel-titanium (NiTi) alloys, which due to their properties represent a favorable flexibility in the instrumentation of difficult anatomies.
Material and methods: 20 patients were examined and treated. They were divided into 2 groups: for the treatment of 6 patients was applied the Protaper Universal rotary system. In the second group of 14 patients to whom mechanical instrumentation was applied, were 7 patients following the Protaper Next and the other 7 Dc Taper with different pulpal and periodontal diseases.
Results: Mechanical instrumentation was performed on different nosological entities, the prevalence being acute pulpitis. Among instrumented teeth, the prevalence was on the side of pluriradicular teeth.
Conclusions: The practical application of different NiTi rotary systems has to be determined individually, as each clinical case has its own practical properties. But the success of an endodontic treatment depends not only on the rotary instruments used and, on the method, chosen, but also on the practitioner's experience, detailed knowledge of the properties and step-by-step instrumentation protocols.
Key words: nickel-titanium alloy, rotary systems, endodontic instrumentation.

Cite this article
Buga E, Buga E, Uncuta D. Modern nickel-titanium rotary systems in endodontic treatment. Mold Med J. 2023;66(2):12-19. https://doi.org/10.52418/moldovan-med-j.66-2.23.02.

Introduction

The success of endodontic treatment is based on a clinician's skill in diagnosis, treatment planning, and advanced knowledge of root canal anatomy and morphology. This allows the most effective protocol to be chosen in daily practice and is supported by the pillars of endodontic treatment represented by instrumentation, irrigation and obturation [1, 2]. With the development of microtomography (MicroCT), the internal morphology of the tooth has acquired more complex and extensive nuances. Analyzing in detail the MicroCT performed by Marco Versiane, we truly understand the difficulties faced by a rotating needle on its way to the apical foramen [1, 3].

Undoubtedly, steel endodontic pins are important tools in endodontic practice, being useful in both primary treatment and endodontic retreatment. At the same time, steel endodontic needles have a high tendency to carry the original canal axis, creating thresholds, false pathways, perforations and other iatrogenics, due to the tendency to straighten once we advance in endodontic needle size. With the introduction of nickel-titanium (Ni-Ti) alloys, they have become indispensable in endodontic treatment. Because of this, more and more clinicians have switched to full or partial mechanical preparation of the endodontic space with more flexible and cyclic fatigue resistant alloy needles.

In the early 1990s dental instrument manufacturers launched the revolutionary NiTi rotary endodontic needle system with increased flexibility and cutting properties, which significantly increased the clinicians' efficiency in endodontic treatment, making it faster and easier [4, 5].

An endodontic file, in the process of instrumentation, is continuously subjected to two stress forces: cyclic fatigue and torsional fatigue. Rotating endodontic needles made of NiTi, having a much higher flexibility than those made of steel, still have one property that needs considerable improvement, namely torsional strength. Moreover, as the size of the NiTi rotary needle advanced, the resistance to cyclic fatigue, equivalent to flexibility, also decreased considerably. For this reason, in subsequent studies, it has been shown that 10% of NiTi endodontic needles break on first use. Following this in the desire to improve the physical properties of endodontic file, a new type of wire was introduced for making the rotating file stem: M-wire, which heat-treated during production also gave it memory control, which led to an increase in the physical properties of the endodontic needle, and with them the speed, efficiency and control of endodontic preparation [6].

The combination of modern technologies in endodontic file manufacturing, using heat-treated alloys and appropriate cross-sectional design, provides a highly effective opportunity to negotiate the complex endodontic space for any practitioner.

As a result, the next 3 decades have seen an explosion in the development of NiTi endodontic rotary file systems. Improved properties in metallurgical technology have enabled the development of various new rotary instrument systems with innovative designs with a wide geometric range of blades and differently thermomechanically treated alloys [7, 8].

So individual design features differ from one endodontic needle system to another, as taper, helical angle, cross-sectional shape, tip (aggressive, non-aggressive), pitch of coils. Those endodontics that were among the first implemented on the dental market, many are already no longer used in the clinician's daily practice, giving way to innovative ones with a design much more enriched in properties [9].

According to Priyanka J. (2016) classification there are 5 generations of NiTi rotary systems [5, 9]:

1**. Generation I** – appeared in the mid to early 1990s, which is characterized by:

– They have passive non-marking guide facets, which stabilize the instrument and ensure its central position in the channel;
– They represent a constant taper along the length of the file;
– The angle of attachment is negative or neutral;
– During machining, a sequence of tools is determined which requires several rotating tools.

Examples: ProFile, GT Files, LightSpeed, Quantec, BioRace.

2. **Generation II (2001)** – is characterised by:

– Active cutting edges, no guide facets;
– Fewer rotary instruments are used during treatment;
– Some rotary instruments have been treated by electropolishing.

Examples: Flexmaster, BioRaCe, ProTaper U, Hero, Mtwo, BioRaCe.

3. **Generation III (2007) –** is characterised by:

– They are active rotary instruments;
– They are thermomechanically processed, such as M-wire or CM-wire;
– Decreased incidence of tool fracture due to reduced cyclic fatigue through improved elastic properties and durability.

Examples: HyFlex K3, Twisted file, GT Vortex, Twisted-Files.

4. **Generation IV (2010) –** is characterized by:

– In this generation the mode of action of the rotating system was changed from 360 rotary to a reciprocating motion;
– Single rotary tool technique.

Examples: Reciprocal rotation systems: Endo-Eze M4, Endo-Express.

Single-file systems: Self-adjusting, Wave One, Reciprocal.

5. **Generation V (2013) –** is characterised by:

– Centre of rotation is offset, balanced;
– Includes instruments with an irregular cross-sectional shape.

Examples: ProTaper Next (Dentsply) (2013), One shape, Revo-S (Micro-Mega), DC-Taper.

NiTi rotary files differ greatly in shape, size and mode of action. In order to be used effectively it is necessary to use them in certain sequencing [10]. Thus, many rotating systems are systematized and used in a certain sequencing developed by the production company according to a mathematical principle [11]. The exception is the mutual motion instruments, which according to the manufacturing companies use a single rotary file.

From the point of view of existing designs, it was decided to expose the design properties of various rotary systems and to analyze them in order to expose the diversity (tab. 1).

The aim of this paper is to study the properties of different varieties of rotary systems used in the mechanical preparation of the endodontic space in patients with different pulp diseases.

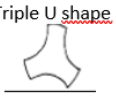
Material and methods

The study was conducted in *Pavel Godoroja* Department of Dental Propaedeutics, *Nicolae Testemitanu* State University of Medicine and Pharmacy, where 20 patients were examined and treated, 9 men and 11 women, aged between 20 and 40 years. From the group of 20 patients treated, teeth with one root canal (incisors, canines and premolars) had 6 patients, 13 patients had 3 root canals (molars) and one had 4 root canals (upper molar).

Clinical history, inspection, palpation, percussion and paraclinical data were used to diagnose the nosological forms. In exobuccal clinical examination, great attention was paid to facial appearance and symmetry, in endo-oral examination the condition of the problem tooth was analyzed, on probing the response was followed, also percussion and thermal samples (cold sample) were performed. From the paraclinical examinations the radiological method was used by performing retroalveolar radiography and orthopantomography (OPG).

Endodontic treatment with mechanical root canal treatment was carried out with manual K-files to create glide paths and the ProTaper Universal, Protaper Next and DC Taper rotary systems. Medicated processing was performed with Sodium hypochloride solution 5.25%, 17% EDTA and distilled water solutions. Root canals were sealed with AH+ or Dexodent sealer and gutta-percha cones using the "central cone" method.

Table 1. Design features of rotaring systems [5, 10, 12]

Instrument system	Manufacturer/year	Introduced by	Cross-section/ Transvers section	Helical angle	Rake angle	Size	Taper	Speed (rmp)
Profile	Dentsply Tusla Dental/1993	Ben Johnson	Triple U shape 	Open 20	Negative (20)	15-90	2,4,5,6,7,8	150-350
ProTaper	Dentsply TuslaDental/ 2001	P. Machtou,C. Ruddle, J West	Convex triangular 	Variable (grows from tip to shaft)	Negative	17-30	Variable SX-3.3-19, S1-2-11 S2-4-11.5 F1-7-5.5 F2-8-5.5 F3-9-5.5	250-350
M two	VDW, Munich Germany/2003	-	Italic S 	Variable (grows from tip to shaft)	Negative	10-40	4,5,6,7	300-350
Hyflex	Coltene Endo/2011	Ricardo Caiecedo Stephen Clark	Double fluted Hedstroem design 	Variable (acelerated flute deesign	Positive	15-40	4,6,8	500
Wave-One	Dentsply Tusla Dental/2011	-	Parabolic (anneabled heat treated) 	Variable (grows from tip to shaft)	Negative	21-40	6,8	300
Reciproc	VDW GmbH, Munich, Germany/2011	-	Double-S-shaped 	Variable (grows from tip to shaft)	Negative	25-50	5,6,8	300
ProTaper Next	Dentsply Tusla Dental/2013	Ricardo Machado	Rectangular (offset design)	-	-	17-40	4,6	300

Results and discussion

After treatment according to the proposed protocols, depending on the rotating instrumentation system used, in each of the 20 cases examined, the clinical signs present at the time of referral, characteristic of each nozological form diagnosed, disappeared. For the forms with periapical disease, it was recommended to perform radiological examination at 6 and 12 months to highlight and note the disappearance of pathological signs.

Of the 20 patients treated, 5 patients had a single root canal (incisors, canines and premolars), 14 patients had 3 root canals (molars) and one had 4 root canals (upper molar).

From the diagnosed noxious forms from the total group of patients, namely acute diffuse pulpitis, chronic

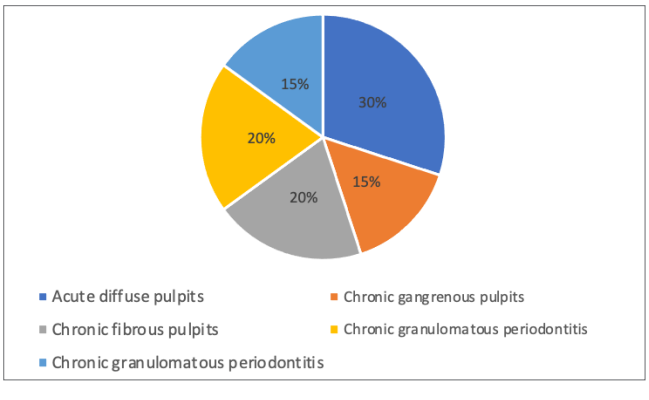


Fig. 1. Ratio of nosological forms of pulpal inflammation and periapical lesions

Table 2. Distribution of nosological forms of pulpal inflammation and periapical lesions

Pulpitis				Apical periodontitis		
Total	Acute diffuse pulpits	Chronic gangrenous pulpitis	Chronic fibrous pulpitis	Total	Chronic granulating periodontitis	Cronic granulomatous periodontitis
13	6	3	4	7	4	3

gangrenous pulpitis, chronic fibrous pulpitis, chronic fibrous periodontitis, chronic granulomatous periodontitis the prevalence was on the side of acute diffuse pulpitis being 6 (30%) patients from the total group as shown in tab. 2 and fig. 1.

Following the group distribution, was analyzed the frequency of instrumentation of one root canal and multiple root canals with rotary systems as well as the frequency of instrumentation with second and latest generation rotary systems (fig.2). The results are shown below by graphical representation in fig. 3, 4, 5, where it was deducted that the frequency of instrumentation was on the side of multiple root canals, with equal use of rotary systems by each manufacturer included in the data analysis.

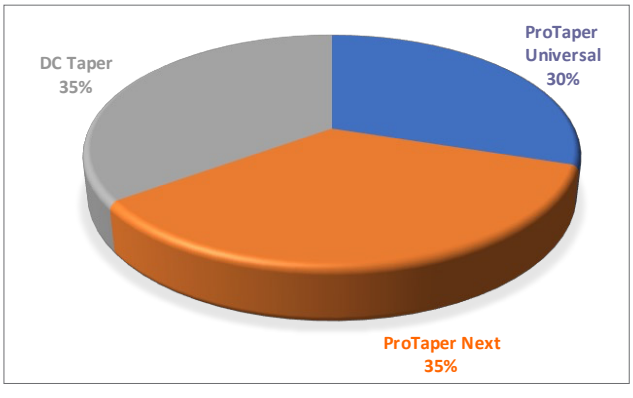


Fig. 2. Frequency of rotary instrumentation used in mechanical root canal preparation

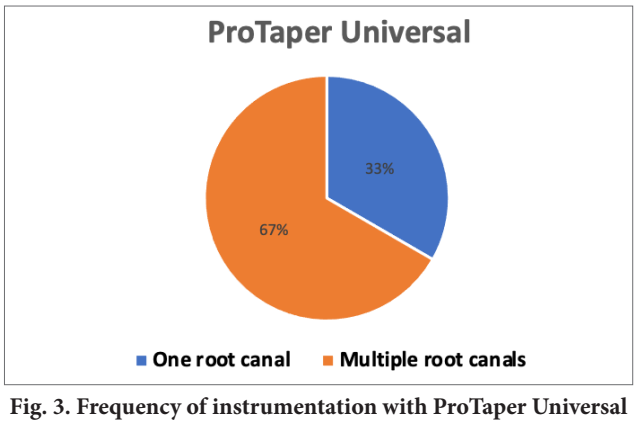


Fig. 3. Frequency of instrumentation with ProTaper Universal depending on the root canal system

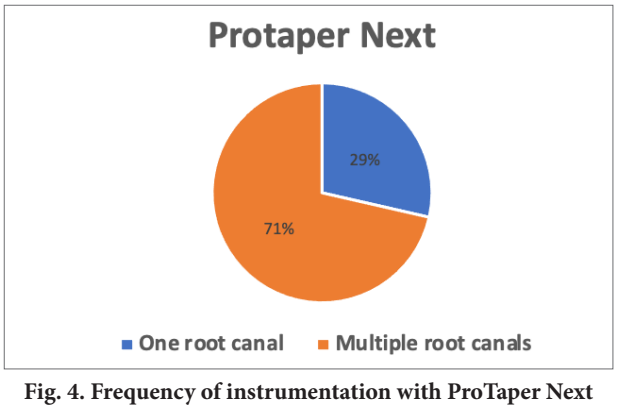


Fig. 4. Frequency of instrumentation with ProTaper Next depending on the root canal system

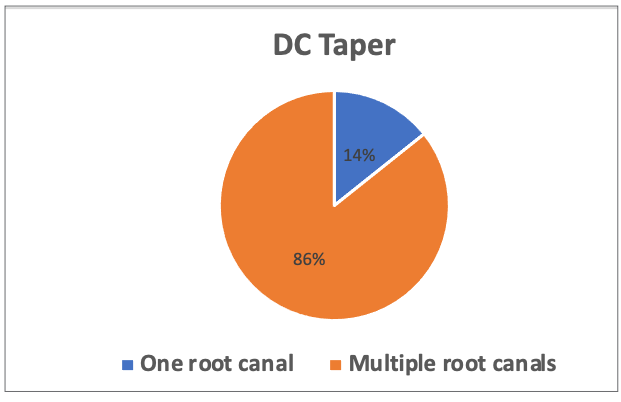


Fig. 5. Frequency of DC Taper instrumentation depending on root canal system

Clinical case

Family Name, First name: Patient X
Date of birth: 12.08.1983
Gender: Female
Residence: Chisinau

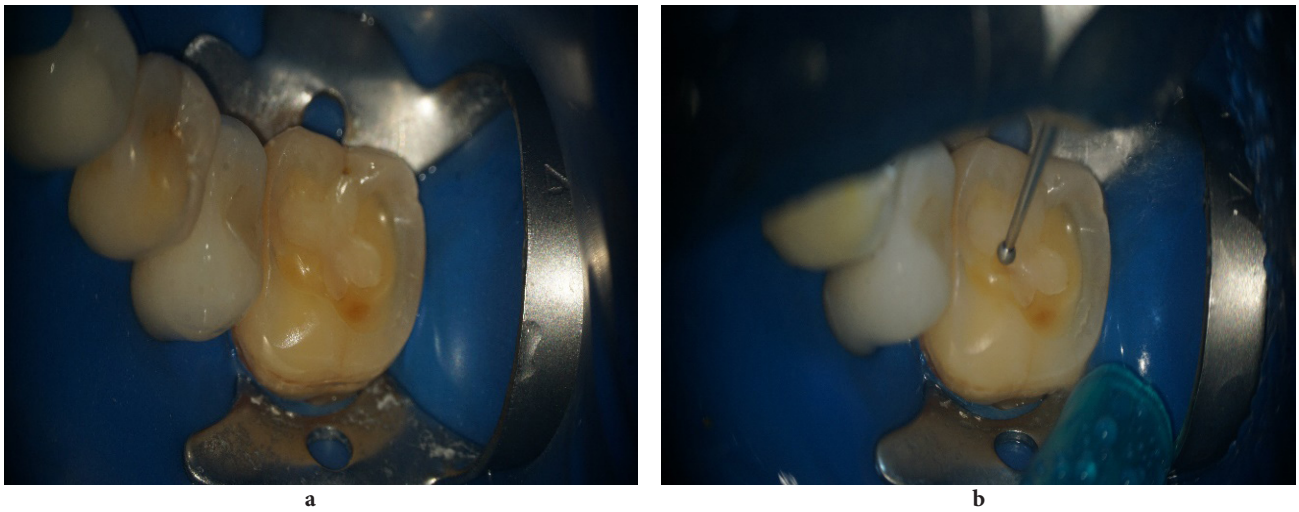
The complaints: the patient presents with an intense pain in the lower jaw on the right side, throbbing, long-lasting with periods of remission, but which are short-lived. The pain may occur spontaneously having a progressive character, but in turn is exacerbated by the action of thermal excitants. The pain is more pronounced at night, when the patient cannot sleep. The pain has a radiating character and radiates towards the temporal region. Painkillers and analgesics practically do not subside.

Endo-oral examination

The mucosa of buccal cavity vestibule, hard palate, pinkish-pale buccal plane without pathological changes. There was an older filling in tooth 37. On probing tooth 37 there was pain on the entire surface of the filling. On percussion, insignificant pain was present. On thermal sampling (cold with ColdSpray) the tooth in question was identified, where the pain persisted even after removal of the excitant. EOD was performed with the Pulpotest device or values of 50 μA were obtained.

Diagnosis: Following subjective clinical examination (violent pain, persisting for a long time, with short periods of remission, which worsens on the action of thermal excitants and is maintained when they are removed) and objective data (pain probing, positive thermal sample, EOD 50 μA.), the diagnosis was: acute diffuse pulpitis in tooth 37.

Treatment: the treatment method involved vital extirpation of the pulp of the tooth 37. The treatment was carried out in a single session. Infiltrative anaesthesia with Septanest 4% 1/100000-1.7 ml was performed. The tooth was isolated with cofferdam (fig. 6), the access was created by removing the filling and opening the pulp chamber (fig. 7). Three root canals were detected in tooth 37: distal and 2 mesial (fig. 8). The instrumentation of the root canals for the creation of the initial glide path was carried out with the help of K-files of 08/02, 10/02 15/02 (fig. 7).



a b

Fig. 6. a) Ruber Dam image application; b) access creation

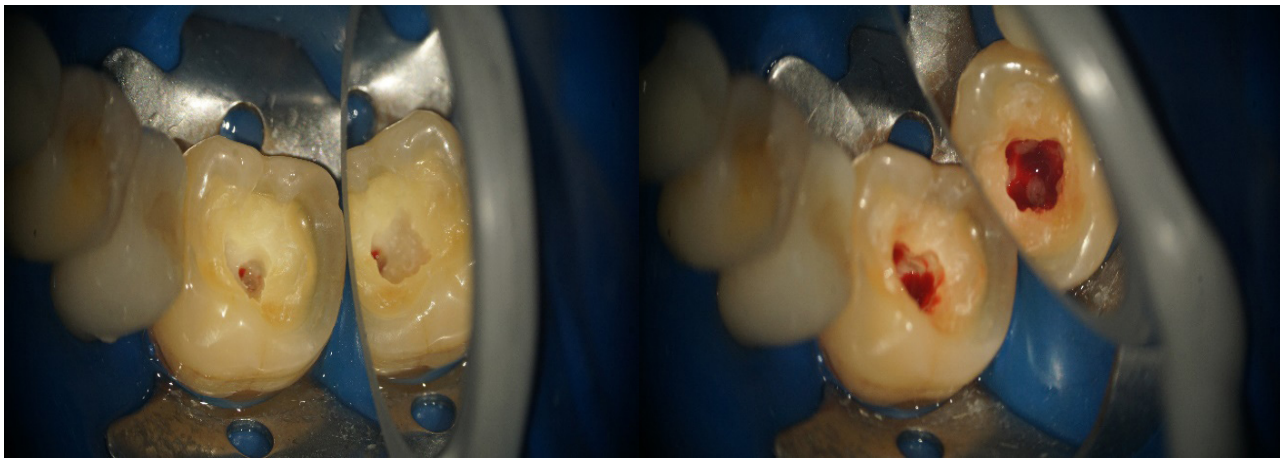


Fig. 7. Opening the pulp chamber

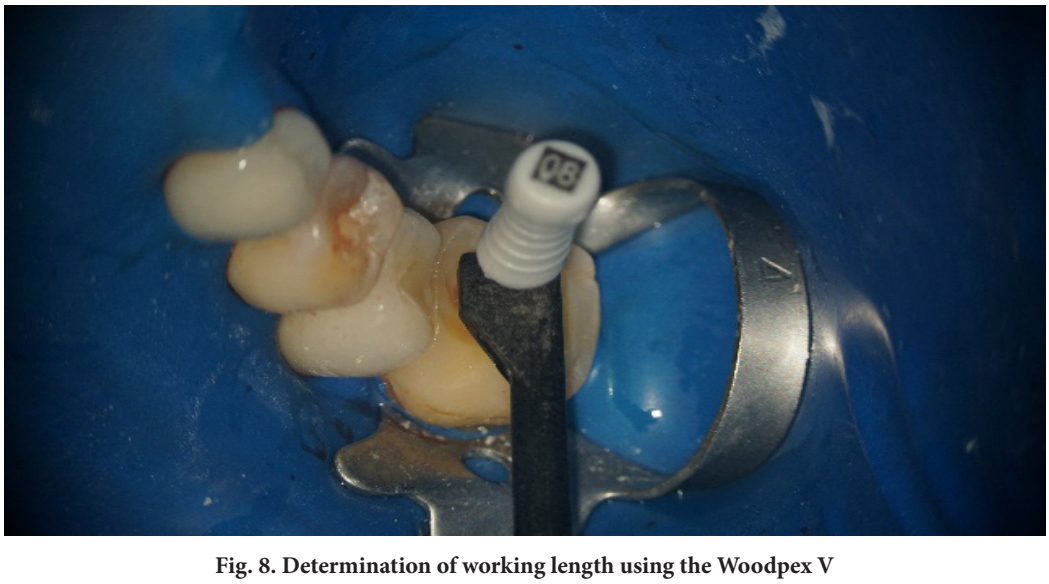


Fig. 8. Determination of working length using the Woodpex V

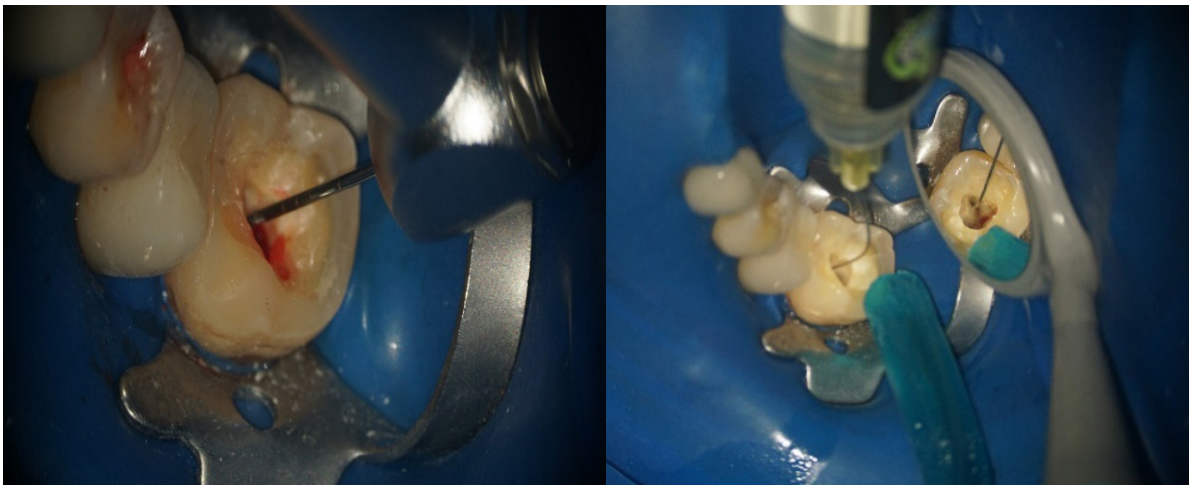


Fig. 9. Mechanical instrumentation with rotary needle 17/04 and irrigation with CHLORAXID 5.25%

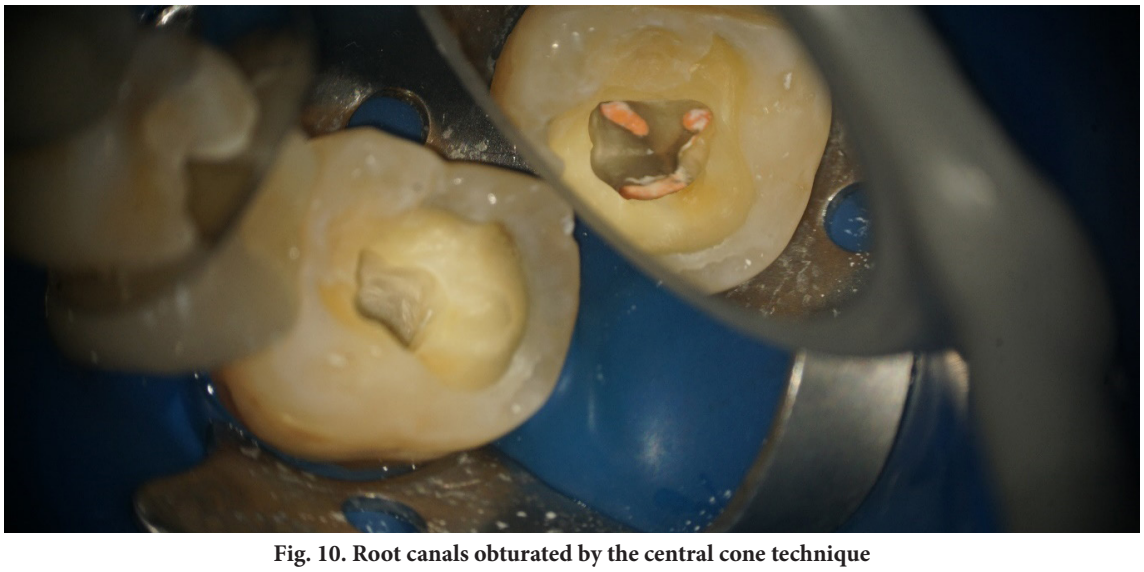


Fig. 10. Root canals obturated by the central cone technique

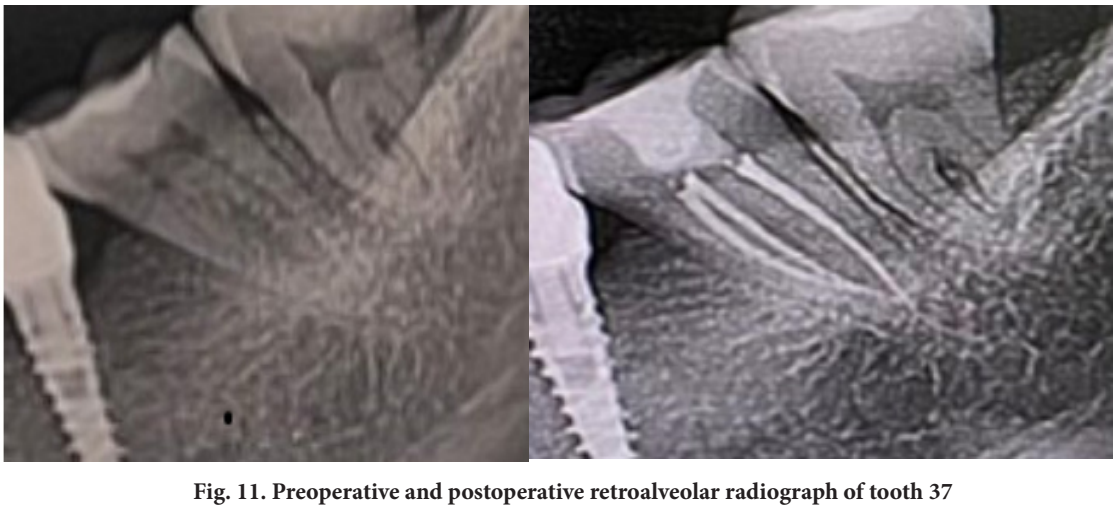


Fig. 11. Preoperative and postoperative retroalveolar radiograph of tooth 37

Then the DC Taper rotary system was used. The technique used was standardized until the apical size of 25/04 was reached according to the manufacturer's protocol (fig. 9). The working length of the root canals was determined by the electronic apex-locator method. Sodium hypochloride solution 5.25% in combination with 17% EDTA gel and distilled water was used for canalicular irrigation (fig. 9). The canals were dried using sterile paper cones, after which they were sealed by the "central cone" method, using size 20 gutta-percha cones with conicity 06 as filler and Dexodent as sealer (fig. 10). The coronal restoration was carried out by the direct method with light-curing composite material "Clearfil-AP-X Esthetics".

According to publications and studies, the advent of nickel-titanium (NiTi) alloys in the late 1980s led to a revolution in endodontics, as these files have been shown to have considerable advantages over stainless steel (SS), particularly in terms of instrument safety [13]. NiTi files were able to overcome the problem of stiffness and low resistance to cyclic fatigue associated with stainless steel instruments. Apart from the advantage of increased flexibility and shorter treatment time, NiTi threads also resulted in fewer procedural errors, such as zippering, corrugations or transport, due to their superelasticity, compared to SS threads [13, 14].

Reference to the study carried out and other comparative studies made in the literature by professionals in mechanical instrumentation with different rotary systems suggested that endodontic instruments made with M-Wire have faster flexibility and mechanical instrumentation staging and higher wear resistance than similar instruments made with conventional NiTi wire, due to its unique nanocrystalline martensitic microstructure [6].

Conclusions

1. According to contemporary literature the emergence and evolution of modern rotary endodontic instruments is intensively studied and analyzed in the literature. Rotating endodontic instruments have a variety of design, taper characteristics that contribute to their excellent clinical performance in modern endodontics. It is concluded that many studies conducted in the analysis of various NiTi rotary systems have shown that the innovation of rotary systems with properties, considerably increase the cyclic and torsional fatigue strength of modern rotary systems.

2. According to the modern endodontic treatment concept, rotary systems are classified according to generation. Both early and late generation rotary endodontic instruments are effective in removing debris from the walls of root canal systems and achieving a conical, continuous canal shape. First generation rotary instruments have certain disadvantages compared to the newer generation instruments as they use more rotary instruments in the instrumentation protocol which increases working time, fatigue. Plus, the enrichment of the new generations with properties compared to the first generations increase effectiveness due to increased flexibility and resistance to cyclic and torsional fatigue.

3. The practical application of different NiTi rotating systems has to be determined individually, as each clinical case has its own practical properties. But the success of an endodontic treatment depends not only on the rotary instruments used and the method chosen, but also on the practitioner's experience, detailed knowledge of the properties and step-by-step instrumentation protocols.

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EB and EB collected the data, wrote the first version of the manuscript; DU conceptualized the idea, completed the final text, and revised critically the manuscript. All the authors approved the final version of the manuscript.

Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy of the Republic of Moldova. The study was the authors' initiative. The authors are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

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 2 of 12.09.2022.

Conflict of interests
The authors have no conflict of interests to declare.

https://doi.org/10.52418/moldovan-med-j.66-2.23.03
UDC: 618.396-07:577.17.083.3+612.017.1

Are Lactoferrin and Interleukine-6 preterm birth participants?

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Manuscript received June 07, 2023; revised manuscript August 25, 2023; December 27, 2023

Abstract

Background: Preterm birth remains a leading obstetrical complication because of the incomplete understanding of its multifaceted etiology. It is known that immune alterations toward a proinflammatory profile are observed in women with preterm birth, but therapeutic interventions are still lacking because of scarcity of evidence in the integration of maternal and placental interrelated compartments. Objective: To investigate value of Lactoferrin (LF) and Interleukine-6 (IL-6) in the preterm labor.
Material and methods: The study comprised 65 women with spontaneous preterm labor and 65 women with term labor. Maternal plasma concentrations of Lactoferrin and Interleukine-6 were detected by standard test system Aeskulisa Lactoferrin and Best-Vector A-8768 for Interleukine-6 Ref 3307 which (GmbH & Co, Germany) gave an analytical sensitive of 1.0 U/ml for Lactoferrin and 0.131 pg/ml for Interleukine-6.
Results: Plasma levels of Lactoferrin in women with preterm labor were lower (μ_{median} = 0.90 U/ml) ($p<0.001$) than in the control subjects (μ_{median} = 40.68 U/ml). Plasma levels of Interleukine-6 in the plasma in women with preterm were predictably higher (μ_{median} = 51.90 pg/ml) than that in the control subjects with term delivery (μ_{median} = 21.51 pg/ml) ($p<0.001$).
Conclusions: The study findings suggest that plasma levels of Lactoferrin and Interleukine-6 in women with preterm labor may be considered as a promising early biomarker for preterm labor.
Key words: preterm labor, term labor, polyfunctional immunomodulatory proteins, lactoferrin.

Cite this article
Cotelea V, Corolcova N, Mihalcean L, Burac M, Profire L, Dondiuc I. Are Lactoferrin and Interleukine-6 preterm birth participants? Mold Med J. 2023;66(2):20-23. https://doi.org/10.52418/moldovan-med-j.66-2.23.03.

Introduction

Preterm birth (PTB) remains a leading cause of neonatal morbidity and mortality. PTB pertains to birth that occurs before 37 weeks of gestation, a definition that the World Health Organization has endorsed. In the European countries the rate of PTB is significantly lower (approaching 6% of all live births) than in the USA (approaching 12 % of all live births) [1-5]. Children born prematurely have an augmented risk of mortality before their fifth year of life. The financial burden of neonatal intensive care is substantial, and its emotional toll on families can last for years [1, 5].

Despite the clinical and public health significance, the etiology of PTB remains largely enigmatic. Notably, multiple causes, including environmental exposure, fetal and/or maternal genetics, stress, and immune/inflammatory conditions, have all been associated with induction of premature delivery.

Inflammation is essential throughout pregnancy – from early fetal development to labor onset [4, 5]. Inflammatory mechanisms are tightly regulated by local/systemic immune cells and mediators in uncomplicated pregnancies. However, evidence shows impaired inflammatory responses in gestational tissues and maternal circulation in pregnancy complications – notably PTB [6-10].

Although the underlying causes of pregnancy-associated complication are numerous, it is well established that infection and inflammation represent a highly significant risk factor in preterm birth. It is estimated that inflammation at the placental-maternal interface is directly responsible for or contributes to the development of 50% of all premature deliveries.

Inflammation can be induced by infections, which are detected in 20% to 30% of PTB cases [11, 12]. PTB can also occur without detectable microorganisms (sterile inflammation), whereby endogenous danger signals derived from cellular stress or necrosis, known as damage-associated molecular patterns or alarmins, are often detected [13-17].

Even more, infection and infection-driven activation of inflammatory responses are thought to be the leading risk factor of "spontaneous" PTB [9, 12, 13]. Consequently, increased production of proinflammatory cytokines has been associated with uterine activation and PTB, whereas production of anti-inflammatory cytokines has been shown to play an essential role in uterine quiescence during gestation

[2, 10]. What sets apart term and preterm labor could be an early imbalance of decidual inflammatory signals or a powerful aberrant stimulation (internal or external) that initiates inflammatory pathways. Anti-inflammatory mediators (including IL-10 and IL-4), in contrast to proinflammatory mediators (IL-1, IL-6, IL-8, TNF- and INF-), are downregulated in PTB [12, 13]. Bacterial flora in the placenta is similar to that found in the mouth rather than the vagina. Inflammation and infection have been tied to as much as one-fourth of all preterm births. The unique triple "I" approach, which represents intrauterine inflammation, infection or both, emphasizes the fact that intrauterine inflammation can manifest itself in the absence of overtly harmful intrauterine infection [18]. The findings suggest that Lactoferrine (LF) may play a role in inflammatory protection in human pregnant cervical tissue. It was clarified that LF suppresses PTB and improves the prognosis of pups in the inflammation-induced PTB animal models. Thus, we have identified the first ever clinical application of LF, a prebiotic contained in breast milk, for the purpose of suppressing PTB in humans.

In summary, a complex interplay between infection/inflammation (both systemic and i.u.) and pathogen/host biologic processes appears to play a central role in defining pregnancy outcomes. Further studies are clearly needed to better define the immune mechanisms underlying infection and/or inflammation-driven PTB [8].

The aim of the study was to investigate the levels of *Lactoferrin* and *Interleukine-6* in the maternal plasma in spontaneous preterm labor and term birth (TB).

Material and methods

During the prospective observational cohort research was performed the evaluation of the serum levels of *Lactoferrin* and *Interleukine-6* in the women who were consecutively admitted and got delivery preterm and term in the Perinatal Center of the 1st Municipal Hospital, Chișinău and gave written informed consent at the time of their admission for delivery. Birth before 37 weeks of completed gestation was considered preterm.

Maternal plasma concentrations of *Lactoferrin* and *Interleukine-6* were detected in the Biochemistry Laboratory of *Nicolae Testemitanu* State University of Medicine and Pharmacy by standard test system Aeskulisa Lactoferrin and Best-Vector A-8768 for Interleukine-6 Ref 3307 which (GmbH & Co, Germany) gave an analytical sensitive of 1.0 U/ml for *Lactoferrin* and 0.131 pg/ml for Interleukine-6.

A total of 130 women, 65 women with premature spontaneous labor were included in the 1st group; 65 women with term delivery were included in 2nd group.

The numerical values of the parameters were numbered in Excel table, after which – imported into the statistical analysis software R studio, using descriptive, variation, and correlational analysis. Applied statistic tests: non-parametric Mann-Whitney tests (with effect size determination) were used for comparative evaluation among the groups and ρ Spearman tests were applied for correlation analysis, p-values less than 0.05 being considered as threshold for statistical significance determination.

The research project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (favorable opinion dated of 21.11.2017, No 16).

Results and discussion

A detailed analysis of the immune mediators in the maternal circulation revealed important differences in PTB in comparison with TB participants.

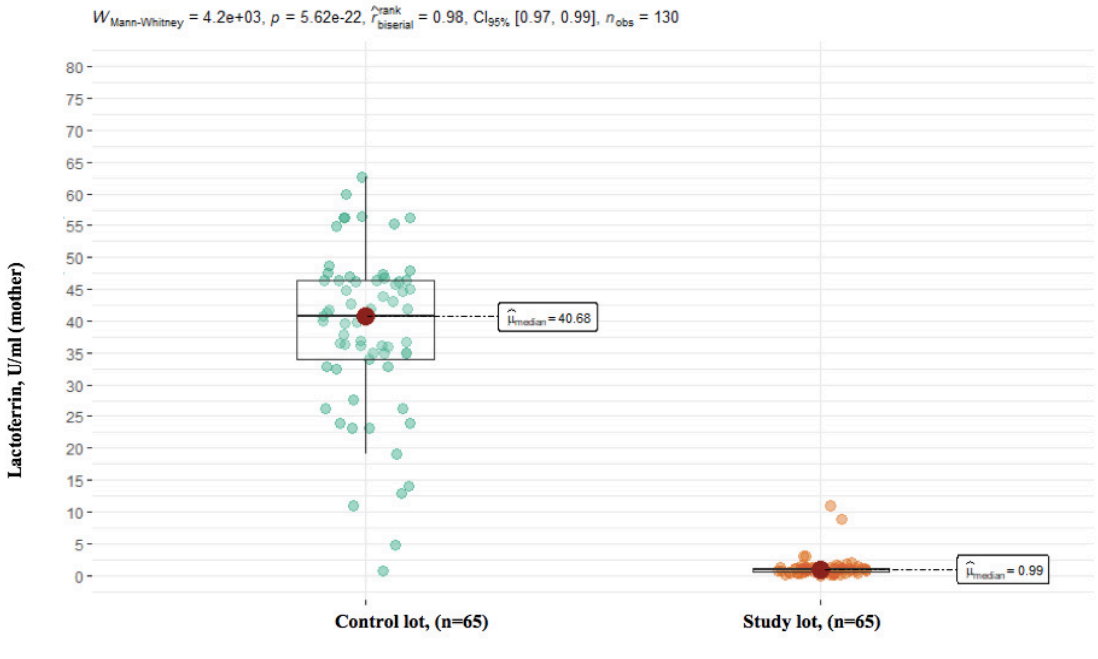

Fig. 1. Serum values of mother Lactoferrin in women with preterm delivery (control lot) and term delivery (study lot)

The study has shown that following the analysis, it was determined that in the study group (women with preterm spontaneous delivery) the level of *Lactoferrin* serum of women constituted in average 0.99 U/ml (median) being less in comparison to 40.68 U/ml (median) in the control group (women with term spontaneous delivery) (p<0.001) (fig. 1), effect size being large one ($r_{rank\ biserial}$=0.98 CI95% 0.97, 0.99). All these data show practical significance of the *Lactoferrin* serum oscillations in PTB group. Possibly, this phenomen is conditioned by the fact that *Lactoferrin* appears to play a critical role in the first line of host defense by modulating innate immune response. In connection with immune co-factors, maternal *Lactoferrin* modulates chemokines real to amplify host defense during pregnancy. *Lactoferrin* could interact with both maternal and fetal microenvironments to establish physical as well as immunological barriers to evade microbial pathogenesis.

Analysis of the immune mediators at the maternal-fetal interface revealed increased levels of proinflammatory *IL-6*. Thus, the data of this study demonstrated a significant increase in *Interleukine-6* values in the base group (women with preterm spontaneous delivery) – 51.90 pg/ml (median), compared to the control group (women with term delivery) – 21.51pg/ml, (median), (p<0.001), effect size being large one ($r_{rank\ biserial}$=0.85 CI95% -0.89, -0.78). The obtained results of the analysis show practical significance of the *IL-6* serum oscillations in PTB group.

At the same time, the correlational analysis was performed between *Lactoferrin* and *IL-6* in both groups in women with PTB and TB. Thus, the following results were obtained and are shown in table 1.

The serum levels of *Lactoferrin* were negatively associated with serum levels of *Interleukine-6* (-0.377 95%CI -0.580, -0.149, p=0.002) which denotes an alteration of the innate immune system by decreasing its defense capacity, represented by *Lactoferrin* possibly on the background of an asymptomatic infectious process, with the subsequent triggering of the inflammatory cascade and the secretion of pro-inflammatory cytokines such as *Il-6*. These active pro-inflammatory compounds induce the secretion of local prostaglandins and thrombin, decrease the concentration of progesterone, therefore there is an increase in uterine contractility, thus inevitably premature labor begins.

Table 1. The indicators of the molecular plasmatic profiles. Correlation's analysis (p Spearman) for the study group

	IL-6, pg/ml
Lactoferrin **U/ml**	-.377**
Sig. (2-tailed)	0.002
95% CI	-0.580
	-0.149

Legend: **Correlation is significant at the 0.01 level (2-tailed).

Conclusions

Preterm delivery remains a serious public health issue. Inflammation represents a major pattern related to several factors essential to triggering labor. However, the molecular triggers and mechanisms underlying the activation of immune pathways associated with induction of preterm delivery remain poorly understood.

Several types of pathogens that disseminate systemically or through the placenta play an important role in induction

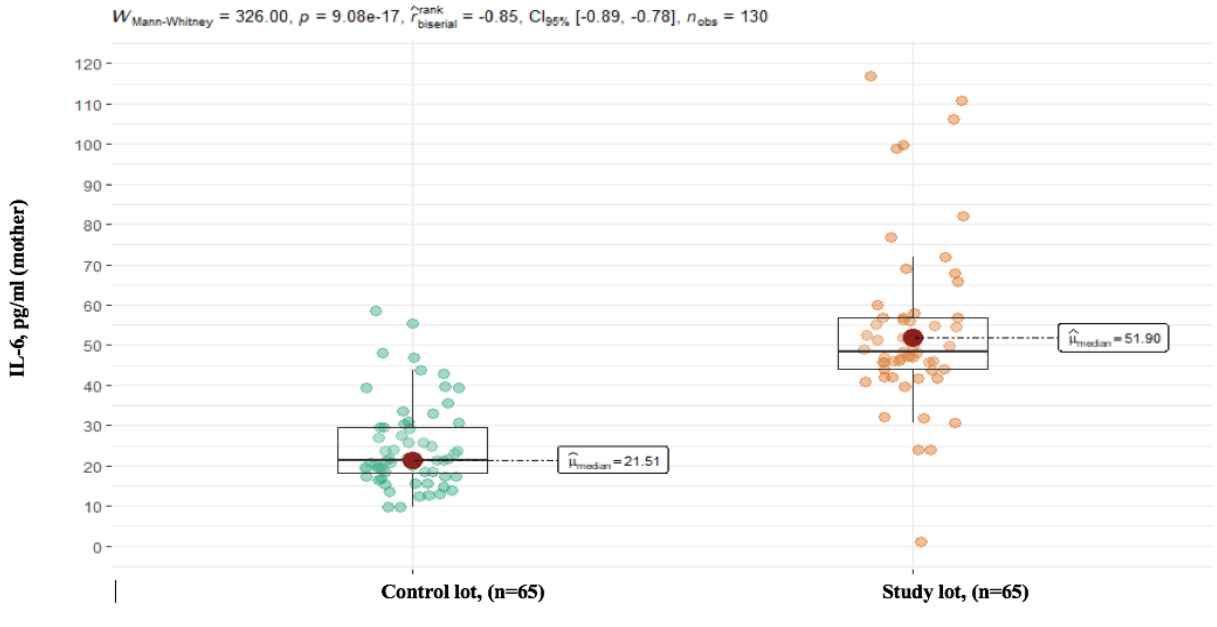

Fig. 2. Serum values of mother Interleukine-6 in women with preterm delivery (control lot) and term delivery (study lot)

of preterm delivery. The sensing of pathogen or endogenous ligand (uncovered during tissue injury and/or inflammation) by innate immune receptors and subsequent induction of immune mediators play an important role for shaping the phenotype and activity of various innate immune cells are known to participate in the labor process. Furthermore, such studies imply that a disruption of homeostasis, either systemically or at the maternal/fetal interface, by an infection and/or inflammatory triggers, contributes to adverse pregnancy outcomes. Therefore, it is important to mention the fact that medical screening of pregnant women for signs of infections and infection-associated immune mediators, such us, the serum levels of *Lactoferrin* and *Interleukine-6* thus may lead to the discovery of novel biomarkers, identify possible at-risk pregnancies, and help to define specific drugs (e.g., specific inhibitors and antibiotics, respectively) for an effective intervention.

Strengths and limitations

Many combined techniques and several compartments strengthened the obtained findings. By design, term pregnancies were used as controls because there is no ethical way to obtain gestational age matched tissues from healthy pregnancies. However, it was previously reported that the inflammatory changes associated with term physiological labor were significant when compared with those occurring in pathologic pregnancies, such as those with PTB.

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Authors' contributions

VC conceptualized the project, drafted the first manuscript and interpreted the data. CN, LM, MB added some conceptual ideas and corrected the text, LP, ID critically revised the manuscript. All the authors revised and approved the final version of the manuscript.

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 16 of 21.11.2017.

Conflicts of interest. No competing interests were disclosed.

Acknowledgments. We thank all the patients who participated in this study.

https://doi.org/10.52418/moldovan-med-j.66-2.23.04
UDC: 616.127-008.1-085.22

Influence of the cytoprotective drug meldonium on diastolic dysfunction of the myocardium

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Manuscript received June 15, 2022; revised manuscript July 10, 2023; published online December 27, 2023

Abstract

Background: The use of myocardial cytoprotectors (meldonium) in patients with exertional angina is a scientific-practical dilemma.
Material and methods: An open randomized clinical trial was conducted involving 160 patients with chronic heart faliure (117 men and 43 women) aged 37 to 81 years. Of them, 142 patients had angina pectoris of stable effort from different functional classes, and 21 – unstable angina pectoris. Study groups were comparable according to the frequency of indication of background drugs and meldonium.
Results: The number of patients with normal diastolic function in both groups, but with a net superiority to meldonium combination administration, has considerably increased: 41 patients (91.11%) in group II vs 33 (58.93%) in the group treated with basic treatment after 9 months of medication; 43 patients (95.56%) group II vs 36 (64.29%) in group I at 12 months of medication. During this period, no patients with pseudonormal type of diastolic dysfunction were registered, these passing into a "more" favorable category – delayed relaxation.
Conclusions: The data obtained confirmed the benefit of using cardiocitoprotection in inducing the reverse-remodelling of the myocardium of left ventricle regardless of the initial ventricular geometric pattern, but the administration of mildronate combination demonstrated a significantly superior efficiency to the basic treatment in hypertrophy of left ventricle regression, an event notable towards the end of the research period.
Key words: cardiocitoprotector, cardiac metabolism, ischemic heart disease.

Cite this article
Chetrus O, Calin G, Sirbu O, Sasu D, Gavriliuc S, Istrati V. Influence of the cytoprotective drug meldonium on diastolic dysfunction of the myocardium. *Mold Med J*. 2022;65(1):24-28. https://doi.org/10.52418/moldovan-med-j.66-2.23.04.

Introduction

Contemporary guidelines for the management of patients with stable exertion angina include several classes of recommendations and levels of evidence, which allow the clinician to objectively assess the benefit and effectiveness of various diagnostic and treatment measures. The contemporary standard of treatment of patients with angina pectoris of stable effort is presented according to the recommendations of the National Clinical Protocol. When determining the priorities of drug treatment, experts have focused on the principles of evidence-based medicine, and in the absence of high veracity data on one or another problem, the consensus of opinions of several experts is taken into account.

In case of development of heart disease – ischemic heart disease (IHD), hypertension, heart failure, the substrate of cardiac energy intake changes, which is reflected by the process of cardiac adaptation to the conditions of energy deficiency [1-3]. Thus, in case of sufficient oxygen intake, the basic energy substrate in the heart of an adult person are fatty acids. In case of myocardial ischemia, all metabolic adaptive mechanisms are oriented towards the transition of energy metabolism from the use of fatty acids to the use of carbohydrates [4-7]. In conditions of insufficient oxygen, the cell is more convenient to oxidize glucose than fatty acids, since this process requires a smaller amount of oxygen [2, 5, 8].

Meldonium in contrast to trimetazidine stimulates not only the oxidation of glucose, but also other hexoses, does not lead to the accumulation in tissues of lactate and activated fatty acids, enhances the effect of angiotensin converting ensyme inhibitors, acts independently of the concentration of fatty acids, prolongs by 30% the life span of animals with myocardial infarction, improves the survival index in experimental chronic heart failure, stimulates calcium-dependent ATP-aza of the sarcoplasmatic reticulum, which improves myocardial contractility, induces nitric oxide biosynthesis and decreases the peripheral resistance of blood vessels [1, 3, 6, 9]. Meldonium blocks carnitine biosynthesis from gamma-butyrobetaine. The decrease in the concentration of carnitine, the transporter of fatty acids through the mitochondrial membranes, determines the oxygen-saving effects of the preparation. Increasing the concentration of gamma-butyrobetain leads to acetylcholine receptor excitation and stimulation of nitric oxyd biosynthesis [9-12]. The ability of mildronate to correct mitochondrial dysfunction was detected, mainly by limiting the oxidation to free radicals of membrane lipids

[10-12]. As a rule, meldonium is not a remedy used in chronic heart failure (CHF) and CHF monotherapy, but it is used in the composition of the widely accepted complex treatment of these diseases, contributing to the increase of its effectiveness [13-15]. The efficacy and harmlessness of meldonium in patients with moderate heart failure on CHF phonon were confirmed in a randomized, double blind, placebo-controlled clinical trial, which was conducted in four medical centers [2, 5, 6, 9]. The positive effects of the use of meldonium in patients with myocardial infarction and manifestations of heart failure have also been achieved in the research described in the litarature of speciality [3, 5, 11]. In the clinical trials of other authors, the positive coronarodilatatory and inotropic effects of this preparation have also been demonstrated [11, 15], the ability of meldonium to increase patients' tolerance to physical exertion [7, 9, 10, 13], to reduce the electrical instability of the heart [5, 8], to increase the antioxidant protection activity of the myocardium [2, 4, 6], to potentiate the effects of hypotensive preparations [2, 4], to improve the quality of life of the sick [11, 14]. The insulin-like action of meldonium [1, 3, 7], its ability to produce a direct beta-2-adrenosensitizing action on smooth muscle [7, 8] and stimulate endothelial acetylcholine receptors [1, 3, 5] expands the possibilities of application of this drug.

Thus, the use of myocardial cytoprotectors (meldonium) in patients with exertion angina pectoris in the preoperative and postoperative periods of coronary bypass is a scientific-practical dilemma [9-11]. The results of scientific research consist in the study of the influence of meldonium on the clinical course, general and local contractility of the left ventricle myocardium, cardiac arrhythmias, biochemical lesions of the myocardium and lipid peroxidation indices in patients with stable angina pectoris in preoperative and postoperative periods of coronary bypass [5, 15]. It is shown the decrease in the number of angina pectoris on the phonon of cytoprotector administration in the preoperative period and their absence in the postoperative period, the reduction of the number of ventricular rhythm disorders, the increase of the ejection fraction of the left ventricle, the decrease of the local contractility index of the myocardium [1, 13, 14]. Evidence of an increased efficacy of meldonium compared to trimetazidine with respect to all the parameters mentioned above is provided [6, 12].

Despite the pathogenetic argumentation of the use of preparations of the metabolic group in the complex treatment of ischemic heart disease, interest in cardiocitoprotectors is more characteristic for scientists of post-Soviet space countries. In European countries, preparations that have not demonstrated their effect on life expectancy, with a ‚dubious' or ‚philosophical' mechanism of action, do not cause particular confidence, as evidenced by the low frequency (no more than 1 %) of the indication of metabolic remedies for the treatment of angina pectoris in the countries of Europe [1-3]. Lately, scientists have also begun to notice the ambiguous efficacy of this group of preparations, papers have appeared that indicate the limited effectiveness of metabolic preparations. Since a "panacea" was not obtained, the attitude towards these preparations became a skeptical one. But real science begins precisely where there is ambiguity.

The purpose of the study: increasing the effectiveness and harmlessness of the pharmacotherapy of ischemic heart disease by developing personalized approaches for indicating drug of metabolic order – meldonium.

Material and methods

An open randomized clinical trial was conducted that included 160 patients with chronic heart failure (CHF), 117 men and 43 women, aged 37 to 81 years. Of them, 142 patients had angina pectoris of stable effort from different functional classes, and 21 – unstable angina pectoris. In most patients angina pectoris was associated with hypertension (HTA) (143 [89.4%]), rhythm disturbances (39 [24.4%]), postinfarct cardiosclerosis (CSPI) (78 [48.8%]), CHF (151 [94.4 %]), some with diabetes mellitus (DM) type II (37 [23.1 %]). The average age of patients was 59.26±0.74 years. The control group involved 30 practically healthy people. The patients were on inpatient treatment in the cardiology department in the years 2011-2015, they continued the outpatient treatment. The observation period was 6 weeks. Each participant was introduced to the research program and signed an informed agreement.

Meldonium was administered at a dose of 0.5 g/24 hours for a period of 6 weeks: in the first 10 days the preparation was administered intravenously in the stationary, after which the outpatient drug was continued as capsules. Study groups were compared according to the frequency of indication of background drugs.

Results

At the stage of enrollment in the study, the groups were homogeneous according to the E/A parameter, which was reduced compared to normal in both groups of patients: in the first group the E/A ratio was 0.67±0.16, and in group II – 0.69±0.29, $p>0.05$. When analyzing the cohort, this index recorded statistically significant improvement in both groups of patients throughout the treatment period: at 3 months in group I E/A increased by +7.46% compared to the initially compared to group II, where this index increased by +10.14%, $p<0.001$ from the initial; at 6 months – +13.43% in group I and +21.64% in group II, $p<0.001$; at 9 months – +29.85% and +44.93% in groups I and II, respectively, $p<0.001$; at 12 months – +67.16% and +75.36%, respectively, $p<0.001$. At the comparative analysis between groups, the dynamics of the E/A ratio was as follows: at 3 months there was no statistically significant difference in the improvement of the E/A ratio between lots (group I – 0.72±0.13 vs 0.76±0.23 in group II, $p>0.05$); but starting with the 6th month of treatment, it was noted a progressive and continuous improvement of this parameter in both groups, but consistently more

obvious in the group treated with meldonium association: at 6 months in the first group the E/A ratio was 0.76±0.09 vs 0.84±0.15, in lot II, p<0.05; at 9 months – 0.87±0.16 vs 1.00±0.19 in groups I and II, respectively, p<0.05; at 12 months – 1.12±0.22 in group I vs 1.21±0.27 in group II, p<0.001). At the same time, the normalization of the E/A ratio occurred earlier in the group, under medication with meldonium association, a phenomenon already observed at the 3rd month of treatment (0.76±0.23 in group II vs 0.72±0.13 in group I), (tab.1).

Table 1. Evolution of the E/A ratio according to medication

E/A ratio					
	Initial	3 months	6 months	9 months	12 months
Group I	0.67±0.16	0.72±0.13* (+7.46%)	0.76±0.09* (+13.43%)	0.87±0.16* (+29.85%)	1.12±0.22* (+67.16%)
Group II	0.69±0.29	0.76±0.23* (+10.14%)	0.84±0.15* (+21.64%)	1.00±0.19* (+44.93%)	1.21±0.27* (+75.36%)
P-value between groups					
	p>0.05	p>0.05	p<0.05	p<0.05	p<0.001

Note: * – p<0.001 from the initial

At the initial stage of recruitment to the study, the lots were homogeneous after telediastolic (TDE) – in the first group it was 253.03±27.59 ms vs 257.78±33.09 ms in group II (p>0.05). When analyzing the cohort at 3, 6, 9 and 12 months of treatment, the statistically true reduction of this parameter in both groups was assessed. The comparative analysis between the batches did not note statistically authentic differences at 3 months of medication (in the first group with -7.23% at 3 months of uninterrupted medication vs -7.67% in group II, p>0.05), but starting with the 6th month of treatment, the medication with meldonium association proved to be more beneficial in reducing this parameter, obtaining also statistical significance, constituting -12.92% in group I vs -17.67% in group II, p<0.05; at 9 months the reduction of the TDE duration was -19.37% in group I vs -23.31% in group II, p<0.05; at 12 months -23.89% in group I vs -28.88% in group II and constituted 192.59±18.61 ms in group I vs 183.33±14.78 ms in group II, p<0.05. It is worth mentioning that the recovery to the values considered normal for TDE was achieved towards the 6th month of uninterrupted medication in both batches of patients (tab. 2).

According to the E/A and TDE ratio, the distribution of patients by type of diastolic dysfunction was as follows: at the initial stage out of 56 patients in the first group, 53 (94.64%) had diastolic dysfunction LV delayed relaxation type, and 3 patients (5.36%) – pseudonormal type of ventricular filling. In the second group, out of 45 patients enrolled to the study, 43 (95.56%) presented at the initial stage with diastolic dysfunction type delayed relaxation, and 2 patients (4.44%) – pseudonormal type of transmittral pattern. After 3 months of treatment, the share of patients with the pathological pattern of ventricular filling type delayed relaxation was reduced in both groups: group I – 42 patients (75%), group II – 33 patients (73.33%), and the number of patients with pseudonormal impairment of diastolic function remained unchanged (3 patients in group I and 2 patients in group II). Already after 3 months of medication, it was noted the normalization of ventricular filling in 11 patients (19.64%) of the first group and in 10 patients (22.23%) in group II. After 6 months of medication, the process of „migration" of patients from a (more) pathological pattern of ventricular filling into a (more) physiological one continued. Thus, the number of patients with echocardiographic presentation typical of delayed relaxation has decreased both in the first group (32 patients), but especially in group II (10 patients). At the same time, the share of patients with pseudonormal type of diastolic vs dysfunction in both groups was reduced: 2 patients (3.57%) in group I and 1 patient (2.22%) in group II. After 6 months of continuous medication, the number of patients with normal ventricular filling pattern increased dramatically: 22 patients (39.29%) in the group treated with basic treatment compared to 34 (75.56%) in the group treated with meldonium association. The same beneficial trend was maintained at 9 and 12 months of uninterrupted medication, with a major gap in favor of medication with meldonium association. Thus, the number of patients with normal diastolic function in both groups, but with a net superiority to meldonium combination administration, has considerably increased: 41 patients (91.11%) in group II vs 33 (58.93%) in the group treated with basic treatment after 9 months of medication; 43 patients (95.56%) group II vs 36 (64.29%) group I at 12 months of medication. During this period, no patients with pseudonormal type of diastolic dysfunction were registered, these passing into a "more" favorable category – delayed relaxation. This last ventricular filling pattern marked a continuous decrease

Table 2. Evolution of TD index depending on medication

TD, ms					
	Initial	**3 months**	**6 months**	**9 months**	**12 months**
Group I	253.03±27.5	234.73±6.9* (-7.23%)	220.35±25.3* (-12.92%)	204.02±22.4* (-19.37%)	192.59±18.6* (-23.89%)
Group II	257.78±33.0	238.0±8.7** (-7.67%)	212,22±23,7* (-17.67%)	197.67±16.1* (-23.31%)	183.33±14.7* (-28.88%)
	P-value between groups				
	p>0.05	p>0.05	p<0.05	p<0.05	p<0.05

Note: * – p<0.001 compared to the initial, ** – p<0.05 compared to the initial

Table 3. Distribution of patients by type of diastolic left ventricle dysfunction

	Initial		3 months		6 months	
	Gr. I	Gr. II	Gr. I	Gr. II	Gr. I	Gr. II
Delayed relaxation	53 (94.64%)	43 (95.56%)	42 (75%)	33 (73.33%)	32 (57.14%)	10 (22.22%)
Normal pseudo-damage	3 (5.36%)	2 (4.44%)	3 (5.36%)	2 (4.44%)	2 (3.57%)	1 (2.22%)
Normal diastolic function	0	0	11 (19.64%)	10 (22.23%)	22 (39.29%)	34 (75.56%)

	9 months		12 months	
	Gr. I	Gr. II	Gr. I	Gr. II
Delayed relaxation	23 (41.07%)	4 (8.89%)	20 (35.71%)	2 (4.44%)
Normal pseudo-damage	0	0	0	0
Normal diastolic function	33 (58.93%)	41 (91.11%)	36 (64.29%)	43 (95.56%)

in both groups, reaching its peak towards the end of the study and more evident in the group, in which meldonium association was administered: 20 patients (35.71%) in group I vs 2 (4.44%) in group II (tab. 3)

Thus, both types of medication have beneficially influenced the compromised lusitropia of left ventricle, but a clearly superior potency in the restoration of physiological parameters of diastolic function was demonstrated in the group treated with mildronate association.

Discussion

The analysis of the peculiarities of the influence of meldonium on the metabolism of cardiomyocytes in myocardial ischemia in young and old patients leads to the idea of an amazing harmony of changes. The explanation of the results obtained may be the mechanism of action of meldonium. This drug preparation blocks carnitine biosynthesis from the butyrobetain range, causing a double positive effect [3-6]. First of all, it reduces the concentration of carnitine, a transporter of fatty acids through the mitochondrial membrane, which causes energy-saving effects [2, 4, 10]. Secondly, it increases the concentration of gamma-butyrobetaine, which excites acetylcholine receptors and stimulates nitric oxide biosynthesis – the mediator of the stress-limiting NO-ergical system, universal regulator of the adaptation process [2, 5, 9]. In the conducted clinical trials, the ability of meldonium to provide an adaptogenic effect by regulating the biosynthesis of NO has been demonstrated [1-8]. Probably this mechanism has a certain contribution to the realization of such a harmonious influence of the preparation on the metabolism of cardiomyocytes in the conditions of myocardial ischemia in both young and old patients.

In the result of the study, the distribution of patients after the geometry of the myocardium LV remodeling was as follows: at the initiation of the study, all types of left ventricular remodeling had a comparable incidence between the groups. In this context, it was noted the prevalence of patients with ventricular geometry type concentric hypertrophy: 52 patients (92.85%) in group I vs 42 (93.33%) in group II. The maladaptation of the left ventricle myocardium to the pressure overload determined the development of concentric remodeling in 1 patient each in the first group (1.79%) and II (2.22%), eccentric hypertrophy – 3 patients (5.36%) in group I and 2 patients (4.45%) in group II. After 3 months of treatment, it was noted the reduction of the share of patients with the pathological pattern of remodeling LV concentric LVH type in both groups: group I – 50 patients (89.28%) vs 39 (86.66%) in group II. At the same time, it was noted the absence of patients with concentric remodeling LV in both groups, as well as the presence of reverse reshaping of the LV myocardium with the achievement of the values considered normal. After 6 months of continuous medication, the trend of reducing the number of patients with the geometric pattern of myocardial remodeling LV concentric LVH type (group I – 78.58% vs 75.56% in group II) was maintained, with the increase in the number of people expressing normal ultrasound phenotype of LV in both batches of patients (10.71% in group I vs 13.33% in group II), but with a slight prevalence in the group treated with meldonium combination. At the same time, it was noted the presence of patients with concentric remodeling LV in both groups (1 patient each), and the eccentric LVH was designated in equal proportions in both groups: 5 patients (8.93%) in group I and 4 patients (8.89%) in group II. Towards the 9th month of treatment, the share of patients with normal aspect of the LV in both groups increased considerably, but with a major gap between the groups in favor of meldonium association: in 12 patients of group I (21.43%) vs 20 patients (44.44%) in group II. At the same time, the number of patients with pathological remodeling of the concentric type LV was reduced to 37 (66.07%) in the first group and 21 patients (46.67%) in group II, but the share of patients with concentric remodeling left ventricle from the account of migration from a (more) pathological category to a (more)

physiological one (3 patients in group I and 4 in group II) increased numerically. Towards the end of the treatment period, only 33.93% (19 patients) of the group treated with basic treatment presented at the normal-looking left ventricle echocardiographic examination, compared to the group treated with meldonium association, where 60% of the patients (27 patients) demonstrated a normal geometric pattern of the left ventricle.

Thus, the data obtained confirmed the benefit of using cardiocitoprotection in inducing reverse-remodeling of the myocardium LV regardless of the initial ventricular geometric pattern, but the administration of mildronate association demonstrated a much superior efficiency compared to the basic treatment in the regression of LVH, a notable event towards the end of the research period.

Conclusions

1. The molecular structure of the pharmacologically studied drug of the metabolic series – meldonium, has a duality of action; under certain conditions, the metabolic corrector is able to exhibit complex pharmacodynamic effects.
2. The data obtained confirmed the benefit of using cardiocitoprotection in inducing reverse reshaping of myocardium left ventricle regardless of the initial ventricular geometric pattern, but meldonium combination administration demonstrated a significantly superior efficiency compared to the basic treatment in hypertrophy of left ventricle regression, a notable event towards the end of the research period.
3. A general concept of personalization of the metabolic pharmacotherapy of meldonium has been developed, according to which it is able to present a cytoprotective effect depending on the initial state of the functional adaptation system.

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Authors' contribution. OC conducted literature review, wrote the first version of the manuscript; GC, 0S, DS collected the data, interpreted the data; SG and VI revised the manuscript critically.

Funding. This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy. The research was the initiative of the authors. The authors are independent and take responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics approval and consent to participate. Favorable opinion of the Research Ethics Committee of Nicolae Testemitanu State University of Medicine and Pharmacy No 11 of 14.05.2015.

Conflict of Interests. There is no known conflict of interests and financial or non-financial support associated with this publication.

https://doi.org/10.52418/moldovan-med-j.66-2.23.05
UDC: 616.126.42-089

Repair surgical techniques in degenerative cardiac valve disease

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Manuscript received May 31, 2023; revised manuscript July 17, 2023; published online December 27, 2023

Abstract

Background: Re-evaluation of reconstructive possibilities in the correction of degenerative mitral valve disease is of great clinical necessity nowadays.
Material and methods: Analyzing the nature of the pathologies that determined the development mechanism of valve insufficiency, 136 cases of anterior cusp prolapse and 152 cases of posterior cusp prolapse were determined.
Results: Cord rupture was established in 79 (58.9%) patients, cusp defects ("Cleft") were appreciated in positions A1, A2, A3 – 15 cases (5.9%) and in P1, P2, P3 – in 92 (86.6%) cases. The surgical techniques performed were separated into: (1) resection – for the anterior and posterior cusps – 45 cases and accompanied by the slide – in 30 cases; (2) with Gore-Tex neo-chordal implantation – 115 cases, with cord transfer – 30; (3) Cusp enlargement with autologous pericardium – 5 cases, Alfieri procedure – 8. Implantation of a support ring required 130 (97.0%) patients. The correction of the associated valve disease required 125 patients (De Vega – 89.1%, ring – 8). Coronary bypass was required – 16 patients. There were no postoperative fatal cases.
Conclusions: Based on the data obtained, reconstructive repair surgery can be can recommend for valves of degenerative, post-traumatic, ischemic, post-endocardial etiology as effective and sustainable techniques over time, being a superior alternative to replacement with prosthetic valves.
Key words: mitral valve repair, degenerative valve disease, cardiac valve surgery.

Cite this article
Moscalu VV. Repair surgical techniques in degenerative cardiac valve disease. Mold Med J. 2023;66(2):29-35. https://doi.org/10.52418/moldovan-med-j.66-2.23.05.

Introduction

Evaluation of reconstructive possibilities in the correction of degenerative mitral valve disease is of great clinical necessity nowadays.

Pathogenically, depending on the lesion type, several operative reconstructive techniques successfully applied in mitral valve insufficiency are determined (tab. 1).

Being an important component of the etiology of mitral regurgitation, tissue dysplasia conjunctivitis is a version of a morphologically modified valve structure, leading to functional insufficiency. Synonyms of this pathology are myxomatous mitral valve disease, endocarditis, chronic degenerative valve disease, chronic valve fibrosis [1, 2]. In the pathogenesis of myxomatous lesions of the mitral valve, a special place is occupied by the anatomical and functional concept – mitral valve prolapse (MVP). The MVP symptom reflects the behavior and position of the mitral valve (MV) in the left atrial cavity at the time of left ventricular systole. It is worth noting that degenerative mitral injury due to valve prolapse does not always mean the development of hemodynamically important insufficiency. True regurgitation of the MV occurs in 40-60% of cases of valve dysplasia, more often in women. Morphologically, mitral degenerative disease is reflected in two different nosological entities: fibroelastic deficiency and Barlow's syndrome [3].

Barlow's syndrome reflects a myxomatous proliferative state with excessive tissue, often with involvement of the mitral annulus (dilation), with an echocardiographic pattern referred to as a "floating valve", characterized by an annulus diameter >36 mm and possible periannular valve fibrosis, but also more often calcification of the anterior mitral leaflet. Barlow's disease is characterized by affecting young people with an average age of 30-40 years. Histologically, myxomatous degeneration is characterized by the deposition of polysaccharides (primarily in the spongy layer of the valve cusps), excessive fibrosis, but also inflammatory infiltrate. Macroscopically, valve degeneration begins with the appearance of nodules on the free edges of the valve cusps, which later merge and contribute to the thickening of the leaflets as well as the elongation of the tendinous cords. With the evolution of the disease, the free edges of the valves sink into the cavity of the left atrium, and as a result, mitral insufficiency develops. In the later stages, united fibrosis can cause shortening of the valves, thickening and degeneration of the chordae tendineae with their eventual rupture [4].

Fibroelastic deficiency is a condition associated with a deficiency of fibrous connective tissue, as well as with stretching, lengthening, thinning and rupture of tendon cords, usually without annular damage, with the average age of patients varying between 60-80 years. Echocardiography

Table 1. Mitral valve repair surgical techniques

Type of injury	Surgical technique
Normal mobility	
Annular dilatation Malposition of papillary muscles Perforation of the leaflets	Annuloplasty Annuloplasty Suture/patch
Increased mobility	
Elongated chords	Sliding of papillary muscles Repositioning of the head of the papillary muscle Looping, chords transposition Artificial chords Leaflet resection
Rupture of chords	Resection of the leaflet Chord transposition Artificial strings
Redundant tissue (prolapse, billowing)	Leaflet resection Edge-to-edge technique (Alfieri)
Elongation of the papillary muscles, malposition	Repositioning of the papillary muscles
Rupture of papillary muscles	Reimplantation
Decreased mobility	
Fusion of the commissures	Commissurotomy
Thickening, fusion of the commissures	Commissurotomy Resection Shaving the cusps
Retraction of chords	Splitting of pillars Resection
Thickening of the subvalvular apparatus	Splitting of pillars
The retraction of the leaflet	The sectioning of the secondary chords Widening of the leaflet
Thickening of the papillary muscles Calcifications	Splitting of pillars Resection, debridement

shows isolated cord damage and isolated or combined leaflet thinning [5]. Patients with degenerative MV pathology who develop mitral regurgitation (MR) symptoms have a poor prognosis, and the annual mortality rate is up to 34%. Mitral valve repair can be considered in patients with MR caused by papillary muscle rupture, degenerative and ischemic mitral regurgitation, or in patients with failed repair attempts undergoing reoperation [6].

MV prolapse reflects the behavior and position of the left ventricle (LV) valves in the left atrial cavity at the time of left ventricular systole. MVP is a syndrome determined by the prolapse of one or both valves in the left atrial cavity during left ventricular contraction, associated in most cases with mitral regurgitation [7-9].

The prevalence of MVP among the population varies depending on the author and the diagnostic criteria used – data range from 1.3% to 38% [10].

Material and methods

The study group included 136 patients with degenerative mitral regurgitation (DMR) in the involvement of a cusp undergoing complex mitral valve repair. Considering the severity of the regurgitation, 4 degrees of mitral insufficiency are distinguished: I grade – mild mitral regurgitation; grade II – moderate mitral regurgitation; grade III – pronounced mitral regurgitation; grade IV – severe mitral regurgitation.

The "gold standard" of the quantification of mitral lesions as well as the result of the plastic surgery is the transthoracic and intraoperative transesophageal echocardiography (fig. 1).

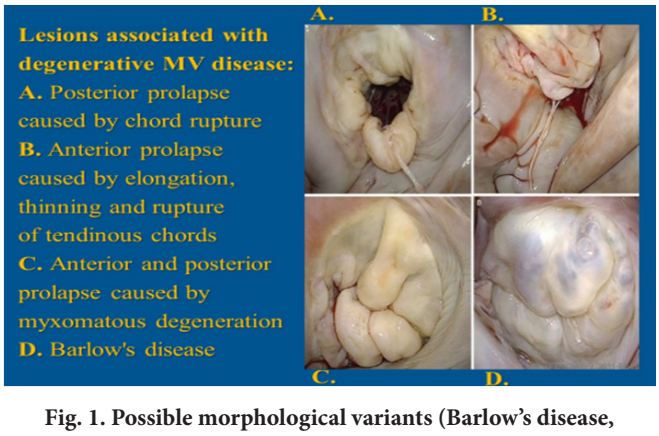

Fig. 1. Possible morphological variants (Barlow's disease, myxomatous degeneration)

Postoperative results

Analyzing the nature of the pathologies that determined the appearance of significant volumetric mitral insufficiency, the following can be mentioned:

– Mitral valve anterior cusp prolapse was dominant in 136 cases with scallop involvement in A1, A2, A3 and both commissures;

– Mitral valve posterior cusp prolapse was dominant in 152 cases; scallops in P1, P2, P3 and anterior and posterior commissure were involved (tab. 2). The scallops A1, P2 were most frequently affected (fig. 2).

Table 2. Operative techniques of mitral valve plastic surgery

Operative techniques	Nr
Support Ring	
26	3
28	22
30	43
32	35
34	19
36	8
Cusp resection	
anterior	1
posterior	44
sliding	30
Neochord	
A_1	13
A_2	30
A_3	19
P_1	8
P_2	28
P_3	17
1	35
2	17
3	13
4	2
Chord transfer	
A_1	6
A_2	9
A_3	10
P_1	1
P_2	1
P_3	3
Secondary-primary	10
Contralateral	5
Cleft suture	
A_1	4
A_2	5
A_3	6
P_1	16
P_2	43
P_3	33
Paracommissural	31
Cusp enlargement with autopericardium	5
Alfieri	8
Commissurotomy, pappilotomy	4

Chord rupture confirmed in the operative field was determined in 34 patients with damage to scallops A1 – 6, A2 – 18, A3 – 10. The most frequent chord suture was

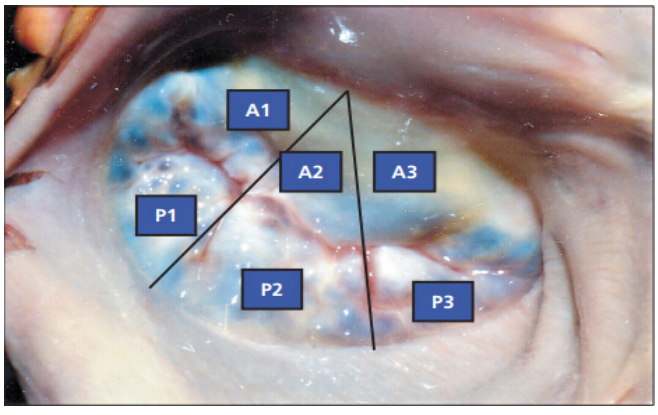

Fig. 2. The conventional division of mitral valve segments into scallops at the anterior (A1, A2, A3), posterior (P1, P2, P3) and commissure (anterior, posterior) cusps, to define the so-called"prolapse score"

established in scallop P2 – 25 cases, followed by scallop P1 – 6 cases and P3 – 14 cases (total 45 patients). Dysplasia were observed, the partial separation of the cusp fragments (cleft) occupying 1-2 scallops, cases that required corrections (application of sutures) to restore the proper coaptation of the valvular complex. The most frequently affected were P1-P2 – 26 cases and P2-P3 – 45 cases, less often the scallops A1-A2 – 2 and A2-A3 – 18 cases (tab. 3).

Table 3. Pathologies diagnosed in cusp prolapse

	Nr
Anterior valve prolaps	102
A_1	24
A_2	64
A_3	39
Comissure	9
Posterior valve prolaps	123
P_1	28
P_2	67
P_3	48
Comissure	9
Chord rupture	79
A_1	6
A_2	18
A_3	10
P_1	6
P_2	25
P_3	14
Cleft	89
A_1-A_2	2
A2-A3	18
P_1-P_2	26
P_2-P_3	43

For the correction of valve diseases, predominantly degenerative, several operative techniques were performed, which aimed to restore valve competence, promote various resection procedures to remove the surplus of redundant tissues according to the planning of the operation and

stabilize the construction with a support ring that was applied according to size depending on the diameter of the hole.

To restore the competence of valve complex, several cleft variants were sutured in positions A1, A2, A3 – 15 and P1, P2, P3 – 92. In all cases, annuloplasty with a support ring was performed. The technique of placing a ring is similar for most types, following a certain algorithm. The procedure begins with the identification of the two fibrous trigones: anterior and posterior and the placement of simple sutures through the valve ring, at this level. The distance between the two trigones is measured, as well as the surface of the anterior area according to this distance, choosing the right size of the ring to be implanted. Sutures are then placed along the entire circumference of the mitral annulus. Due to the proximity of the mitral valve to the circumflex artery, to the anterior aortic valve and to the atrio-ventricular node, the sutures will be made in such a way as to avoid injury to these structures. The wires passed through the mitral ring will then be passed through the annuloplasty ring, after which it is lowered and the wires are tied. The implantation technique is usually standard, the measurement of the diameter of the fibrous ring is respected, and the phenomena of hypercorrection, systolic anterior motion (SAM)> p.8, excessive tensions that can cause dehiscence of the support ring are avoided. Support rings with a diameter of 26 – 3, 28 – 22, 30 – 45, 32 – 35, 34 – 19, 36 – 8 cases were implanted on separate sutures. The device was Medtronic Profile – Future, Carpentier-Edwards – Physio 1, 2, 3, St. Jude medical Saddle Ring, LivaNova Memo (fig. 3).

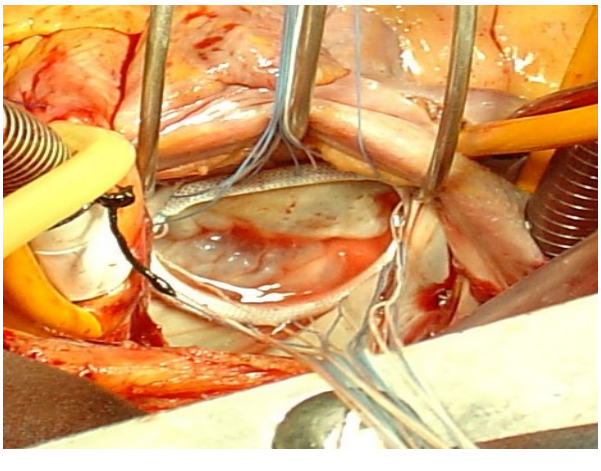

Fig. 3. Mitral annuloplasty with support ring

Annuloplasty with semicircular sutures (3-suture technique) was used as alternative surgical techniques in single cases. Resection techniques (resects) were applied to 1 patient with chord rupture at the anterior cusp, in 44 patients (32.8%) at the posterior cusp. Procedures to lower the coaptation point, sectoral cusp resections, the application of the Sliding technique to avoid the phenomenon of systolic anterior motion were performed in 30 cases.

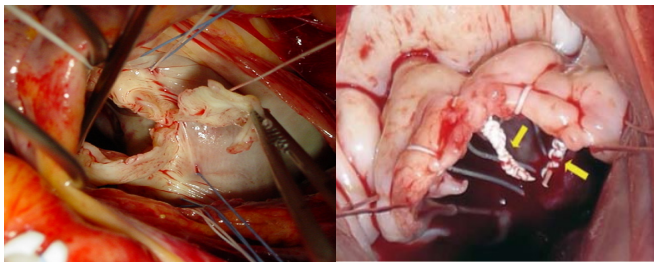

Fig. 4. Resection techniques and the application of neo chord in mitral valve repair

Neochordoplasty techniques (respect) were performed in 62 cases for the anterior scallops and 53 for the posterior ones. Numerically, 35, 17, 13, 4 neochordae were applied using different implantation techniques (fig. 4).

The transfer of native cords was carried out depending on the functional anatomy of the valve disease, the possibilities of replacing affected cords in positions A1, A2, A3 – 25 native cords were transferred, in positions P1, P2, P3 – 5. In 10 cases it was followed the transfer scheme from the secondary-primary position, in 5 – using the contralateral position of the placement of the native cords by performing measurements related to the point of ripening of the valve cusps. In 5 patients, the operative technique was completed with the application of a widening patch from the autopericardium, 8 patients benefited from the so-called Alfieri Sutures (fig. 5). In 4 cases of extensive rheumatic damage, closed mitral commissurotomy with papillotomy was performed to mobilize the valve cusps more effectively.

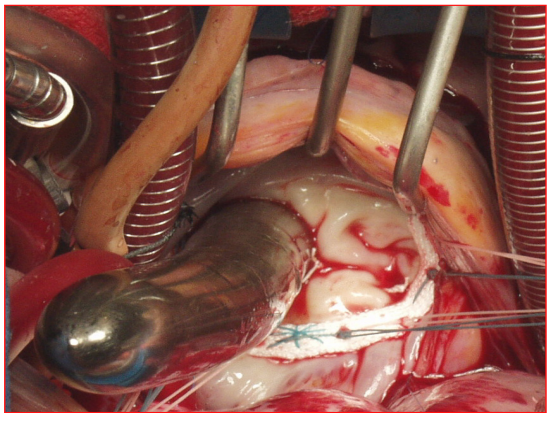

Fig. 5. Application of Alfieri sutures in mitral valve repair

In order to elucidate the most frequent anatomical variants of valve reconstructions, the operative techniques applied in the group of patients with degenerative valve diseases were validated both numerically and cumulatively.

The tricuspid valve presents with moderate fibrous ring dilation in 11 cases (8.0%), with excessive dilatation – in 62 (44.9%), with giant fibrous ring – in 50 cases (42.8%). De Vega (Cabrol) tricuspid valve annuloplasty was performed in 125 cases (89.1%), with support ring – 8 cases (30 mm – 1, 32 mm – 2, 34 mm – 6). Additional tricuspid valve techniques were performed in 39 cases

(cleft suturing – 24 cases, neo chord – 2, Alfieri – 13). In 24 cases with excessive dilatations of the annulus fibrosus, with the formation of a cleft with complex regurgitation mechanisms, separate sutures were applied to restore valve competence. Indications for the application of a widening patch with autopericardium were placed in patients with cusp tethering, in one patient the papillary muscle approximation technique was performed (tab. 4).

Table 4. Additional valve repair techniques

	Nr
Tricuspid annuloplasty	128
De Vega	18
De Vega+Cabrol	102
Support ring	8 (N30-1; N32-2; N34-5)
Cleft suture	24
Neo chord	1
Cusp enlargement with autopericardium	1
Alfieri	13
Coronary bypass	23 (LIMA-11; VENA-12)
ASD, PFO, abnormal drenage	21
Auricula suture	82
Left atrioplasty	12
Thrombectomy	8
Pappilary muscle approximation	6
Ablation	6
Aortic valve repair	3
Secondary chord resection	12

As additional technical procedures, left atrioplasty can also be mentioned in patients with atriomegaly (10 cases), left atrial thrombectomy (4). Carrying out a totalization of the postoperative results that characterize the group of patients who underwent mitral valve reconstruction, some statistical data are taken into account. Thus, at the postoperative examination, it was determined that the diameter of the fibrous ring was – 50 mm (41.1±6.28). The surgical approach through the left atrium was preferred in 127 cases (92.0%), trans-septal – in 11 cases. The aortic valve required correction in 3 cases (annuloplication – 3, cuspoplication – 3, cuspopexy – 1). Coronary bypass was performed in 16 patients (11.6%), including left anterior descending artery (LAD) – 10, diagonal artery (DIA) – 1, autologous vein – 10 cases.

The operations were performed under conditions of extracorporeal circulation with superficial hypothermia – 30-30°, crystalloid cardioplegia was performed in 116 patients (83.3%), blood cardioplegia – in 10, antegrade perfusion distribution was performed in 129 patients. Evaluating the protection of the myocardium during the cardiopulmonary bypass (CPB) period, it can be noted that the spontaneous restoration of the heart rhythm was performed in 91 (63.9%) patients, by single defibrillation in 31 (21.7%), multiple defibrillations – in 8 (5.8%). In the postoperative period, pump assistance was applied in 31 cases, which manifested over a period of time with signs of arterial hypotension, central venous pressure (CVP) increase, tachyarrhythmia or atrioventricular (AV) block with dependence on electro-cardio-stimulator (ECS), cardiotonic and vasopressor treatment. In 87 patients, sources of hemorrhage up to 1000 ml were monitored, average – 351±126.6 ml; 4 patients required resternotomy, 2 with source of hemorrhage and 2 others without. Heart failure was manifested by 36 (34.5%) patients; vasopressors were administered in 75 cases (60.5%), inotropes – in diuretic doses in 45 (32.2%) cases, diuretic doses – in 32 (25.8%) patients. Application of a temporary ECS was performed in 104 (78.6%) patients, permanent implantation required 2 patients. Respiratory insufficiency was manifested by 10 patients (7.8%), hepatorenal – 2, purulent complications or wound infections were not recorded. 6 patients had exudative pericarditis, in 2 cases pericardial drainage was needed. Pleurisy with drainage of the pleural cavity required 5 patients. Pneumonia was recorded in 7 cases (5.1%), stroke – in 3 cases, myocardial infarction, prosthetic endocarditis were not recorded. Postoperative mortality accounted for 1 case (0.7%), the cause of death included a series of postoperative complications, low cardiac output syndrome, hemorrhage, and acute renal failure. At discharge, 67 patients (63.0%) were in sinus rhythm, 44 (31.2%) with atrial fibrillation, 2 (1.4%) with atrial flutter. AV block grade I was recorded in 11 (8%) patients, grade II – in 10 (7.2%). New York Heart Association (NYHA) functional class II was established in 98 (68.8%) cases, functional class III – in 16 (11.6%) cases.

Discussion

Myxomatous degeneration is manifested by elastic fragmentation of collagen, accumulations of spongy connective tissue. Apparently, the dysfunction of the mitral apparatus is a mechanical problem that can be solved surgically, with mitral repair – replacement as the techniques of choice. The rate of reconstructions is increasing in recent years – 51-74% [11-13], and in dedicated centers the individual rate of repair is higher – 92-96%. In the therapeutic attitude, the risk/benefit ratio prevails, the "Respect rather than resect" postulate is well known, the surgical timing is much discussed and recommended for standardization [14-16]. The advantages of repair surgery in prosthetics: fewer bed days; low rate of attributed complications; specific complications (thromboembolism, hemorrhages, prosthetic dysfunctions) reduced; reduced mortality; higher survival rate; pump function preserved. Some retrospective studies determined that compared with other etiologies of mitral regurgitation, degenerative disease is the easiest to repair and has the best survival rate with postoperative longevity equal to the general population [2, 17-21].

Mitral valve repair techniques are perfected over time, which leads to long-lasting results with good functionality. To overcome some of the challenges, it is important that

reconstructive techniques are performed by an experienced team with dedicated, recognized skills. In this study >80% of operations were performed by 1-2 surgeons with more than 25 years of experience in cardiac surgery [9, 22-24].

The mechanisms underlying the survival advantage in patients undergoing correction vs prosthesis are: reduced intraoperative mortality; reservation of the pumping function of the myocardium; low rate of complications attributable to the mitral valve; rate of complications attributed to the procedure. In the description of the study group, the clinical postulations were confirmed with statistical data having confirmed veracity. For a repair that can fail – the following are important: underestimated primary correction, suture dehiscence, systolic anterior motion syndrome, residual mitral regurgitation, hemolysis [20, 25, 26].

The factors that are associated with a higher rate of re-intervention in patients with mitral repair are: mitral regurgitation > moderate postoperatively; annuloplasty ring dehiscence; unjustified intraoperative shortening of tendinous cords; anterior cusp plasty applying resection techniques [27-29].

Anterior repair accompanied by coronary bypass was of longer duration (122±53 vs 109±43 min, P<0.001), the degree of residual regurgitation was greater for the anterior cusp, the cumulative survival index of the patients did not differ among the mentioned groups, the reoperation rate over time (15 years) was 7.5% versus 4.9% after posterior cusp repair (Gray test P=0.26) [30, 31]. Looking at the techniques in which both cusp resections were performed, with cord repair a better preservation of left ventricle (LV) function was obtained for the posterior cusp [16, 22]. In all cases, the prolapse of this cusp was removed; a wide surface of the mitral orifice was obtained, avoiding cases of SAM and residual regurgitation [14, 32]. Similar results were obtained in this study group. Comparing 2 other operative techniques comprising 186 cases (24.9%) of isolated use of neo chords and 560 (75.1%) applying resection techniques, it was found that the probability of a residual regurgitation (20 years of follow-up) was much less in the group with neo chords [10, 31]. In the given study group, there were no cases of dehiscence or rupture of implanted cords. Of great importance to ensure the stability of reconstructive surgical techniques is the observance of an adequate length of the coaptation line, which after implantation of neo chords made up 89-65% vs 11-29% after resection techniques (P<0.001) [22].

Conclusions

Based on the data obtained, reconstructive repair surgeries can be recommended for valves of degenerative, post-traumatic, ischemic, post-endocardial etiology as effective and sustainable techniques over time, being a superior alternative to replacement with prosthetics.

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

Funding
The study was supported by the Institute of Cardiology of the Republic of Moldova. The author is independent and takes responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The study was approved by the Research Ethics Committee of the Institute of Cardiology, protocol No 3 of 16.03.2015. An informed consent was received from every patient.

Conflicts of interest
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.06
UDC: 616-085.273.53

Directions for optimizing the organization of long-term anticoagulant treatment

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Manuscript received July 18, 2023; revised manuscript September 19, 2023; published online December 27, 2023

Abstract

Background: In this study the organization of long-term anticoagulant treatment has been evaluated to estimate whether clinical practice is in accordance with current recommendations for optimal use and effective control of oral anticoagulant (OAC) treatment.
Material and methods: Mixed (quantitative and qualitative), transversal, descriptive, selective study. Samples: quantitative study – 394 adult patients, eligible for anticoagulant treatment; qualitative study – 39 family doctors.
Results: The rate of use of OAC treatment is 68%. The period from the diagnosis of the disease to the initiation of OAC treatment lasted one month or more in 59.1% of patients. 60.6% of patients do not have sufficient knowledge regarding the treatment of OAC. The high price is the most important barrier to direct oral anticoagulant administration (91.1%). Patients' satisfaction with OAC treatment control is low, mainly for vitamin K antagonists (59.8%). 75.5% of respondents claim that OAC treatment control and management is poor. 40.3% do not perform safe therapeutic International Normalized Ratio control, and 54.7% are not in the optimal therapeutic range.
Conclusions: The main barriers to adherence to OAC treatment: the burden of regular monitoring of blood parameters, perceived concern about complications, limited access to laboratory tests and specialist doctors, insufficient information about anticoagulation, and deficiencies in communication with medical staff. There is limited conviction, and uncertainty persists in the initiation and monitoring of OAC treatment by family doctors.
Key words: control of anticoagulant treatment, Warfarin management, oral anticoagulants, atrial fibrillation.

Cite this article
Sumarga N, Belii A. Directions for optimizing the organization of long-term anticoagulant treatment. *Mold Med J*. 2023;66(2):36-40. https://doi.org/10.52418/moldovan-med-j.66-2.23.06.

Introduction

Thrombosis is a global public health problem, and the organization of anticoagulant therapy has evolved considerably, improving the management of OAC treatment [1]. The organization of effective long-term anticoagulant treatment must correspond to the correct and well-justified balance of providing quality services, by avoiding overuse, underuse or misuse [2]. The European and American Societies of Cardiology and Neurology recommend long-term (lifelong) anticoagulation with classical oral anticoagulants, vitamin K antagonists (VKAs) or direct oral anticoagulants (DOACs), with proven efficacy and safety, mainly in atrial fibrillation (AF) to prevent ischemic stroke (IS), as well as in other medical conditions that are associated with thromboembolic complications, such as deep vein thrombosis (DVT), pulmonary thromboembolism (PTE), and mechanical heart valves [3].

According to the Institute for Safe Medication Practices, anticoagulants are a high-priority drug family for optimal medication management, due to their established benefits in reducing the risk of IS due to AF by approximately 70%, the risk of recurrent venous thromboembolism by more than 90% and the risk of death by approximately 25% [4]. OAC is a socioeconomic burden that requires continuous resources, taking into account the increasing demands arising from the aging of the population and the increase in the number of patients with increased thrombotic risk, being a treatment used throughout life, which currently involves approximately 2% of the western population [5].

Successful anticoagulation has always been defined as a scientific balance of the risk of thrombosis and bleeding complications. To maintain such optimal anticoagulation, rational prescription of drugs, institution of therapy monitoring, as well as active participation of patients receiving the therapy is required [6]. Inadequate anticoagulation control is often associated with major complications, such as bleeding, thromboembolic events, and mortality [5]. Most complications related to anticoagulation are preventable, and safety measures are encouraged or mandated [7].

In the Republic of Moldova there are no widely accessible anticoagulation clinics, there are no national disease registries, portable coagulometers are not accessible, and the health insurance fund does not reimburse the use of new OACs at the research stage. The management of anticoagulation (mainly Warfarin) in the long term is quite difficult, being officially assigned to family doctors. Adherence to regular OAC treatment monitoring and Warfarin dose adjustment are time-consuming and may be difficult to achieve in the outpatient setting, especially

for patients with limited mobility. Previous reports from other studies suggested that the use of OAC in the Republic of Moldova was poor [8], but there are no studies that provide a deeper insight into the use and management of OAC treatment in patients who require long-term anticoagulation, especially in the era of new OACs. A better understanding of utilization, treatment patterns, and factors that might influence treatment strategy is essential to know whether clinical practice is in line with current treatment recommendations.

Material and methods

This study presents a mixed (quantitative and qualitative), transversal, descriptive, selective research.

The quantitative study was carried out using the survey as a method, and the questionnaire developed and adapted in the interests of the study as an instrument (it is based on the validated multinational questionnaire of barriers to the use of warfarin in AF, the multinational questionnaire for assessing patients' satisfaction in terms of regarding anticoagulant treatment "Anticoagulant Treatment Perception Questionnaire" (PACT-Q©), a new questionnaire for assessing the quality of life of patients treated with anticoagulants "Anticlotting Treatment Scale", the questionnaire for the Duke Anticoagulation Satisfaction Scale and the evaluation questionnaire of patients' knowledge about anticoagulant treatment).

The representative sample was calculated based on the reduced formula for large populations:

$$n = \frac{z_{\alpha}^2 * p(1-p)}{e^2} n = \frac{z_{\alpha}^2 * p(1-p)}{e^2}$$ where:

n – minimum volume of the representative sample,

p – the probability of occurrence of the phenomenon (in case of 2% **p** will be equal to 0.02),

e – within the margin of error 2 % e will be equal to 0.02, at the 99% confidence level $\mathbf{Z}_{\alpha}$ will be equal to 2.58.

According to the data in literature, approximately 2% of the general population require long-term anticoagulant treatment. Using the respective formula, was obtained **n** approximately equal to 326 respondents. The non-response rate being estimated at 10% of probably invalid questionnaires, the minimum size of the representative sample will be approximately 359 respondents.

The sample of the study included 394 respondents from the entire territory of the Republic of Moldova. Inclusion criteria – adult patients, eligible for anticoagulant treatment from different regions of the Republic of Moldova, who agreed to participate in the survey. Exclusion criterion – people who refused to participate in the survey and incomplete questionnaires.

The tool used to carry out the qualitative study was the focus group interview guide, with the survey developed for this purpose. The research allowed the evaluation of the attitudes and practices of the use and control of anticoagulant treatment by family doctors, the identification of respondents' opinions and the reasons for selecting the answer. Family physicians working in Primary Health Care Institutions (urban and rural) and consenting to participate in the study served as inclusion criteria in the study. The exclusion criterion was the family doctors who refused to participate in the discussions. The study sample consisted of 39 family doctors (24 from the urban regions and 15 from the rural regions).

Data collection period: November 1, 2022 – March 1, 2023. The data obtained were processed using the SPSS 23 (Statistical Package for the Social Sciences) application for statistical data analysis.

The study protocol was discussed and approved at the School of Public Health Management meeting (05.10.2022).

Results and discussion

394 eligible patients were surveyed, selected uniformly throughout the territory of the Republic of Moldova. The average age of the investigated patients was 67 years, ranging between 33 and 84 years. The age of 65-74 years prevails. Of the diseases with thromboembolic risk, with an indication for long-term OAC treatment, the majority of patients in the sample were diagnosed with AF (the largest subset of patients requiring lifelong anticoagulation [9]), constituting 91.8% (CI 95% 81.7%-100%), 11.9% patients with heart valve prostheses, 7.2% with deep vein thrombosis and 3.4% with pulmonary embolism. Of the concomitant pathologies detected in the patients, arterial hypertension predominated, constituting 76.9% (CI 95% 72.6%-81.2%), diabetes 37.8% (CI 95% 33.2%-42, 9%) and chronic heart failure – 35.3% (95% CI 30.7%-40.1%). The obtained data allowed to evaluate the thromboembolic risk of stroke using the CHA_2DS_2-VASc score in patients with AF in this sample, demonstrating that the majority are at high thromboembolic risk – 72.9% or moderate – 17.5% and with indication for long-term OAC treatment. Particular attention was paid to the degree of use of OAC treatment. Patients eligible for OAC treatment were divided into four groups: patients undergoing OAC treatment – 68% (CI 95% 63.5%-71.6%), patients who were not indicated for OAC treatment – 8.6% (CI 95% 5.8%-11.4%), patients who did not follow the indicated treatment – 22.6% (CI 95% 18.8%-26.4%) and patients who did not consult a doctor for treatment until the moment of questioning – 0.8% (CI 95% 0%-1.8%), (fig. 1). Accordingly, in the given study, the degree of use of OAC treatment is 68% of the total eligible patients.

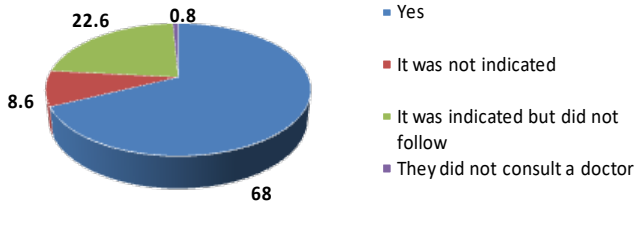

Fig. 1. Evaluation of the degree of use of OAC treatment (%)

The research analysis shows that patients from the rural region have a lower degree of use of OAC treatment

(63.8%), more often they do not follow the indicated OAC treatment (24.3%) or the treatment was not indicated (11%) ($p<0.05$), (fig. 2).

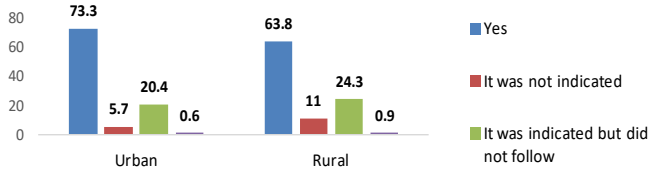

Fig. 2. Degree of use of OAC treatment by region of residence (%)

The age correlation highlights that younger patients have a better utilization rate of OAC treatment. In the category of patients under 75 years of age, the degree of use of OAC treatment is higher (70.1%), compared to those aged 75 and over (59.2%), (fig. 3).

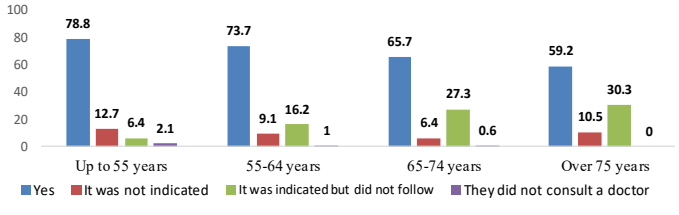

Fig. 3. Degree of use of OAC treatment by age (%)

The evaluation of the degree of use of the OAC treatment revealed a practically uniform use by regions, with a higher degree of use in the North of 71.1% ($p<0.05$), (fig. 4).

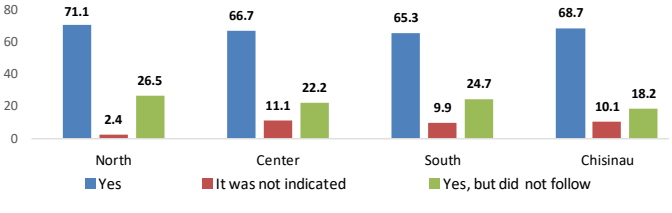

Fig. 4. Evaluation of the degree of use of OAC treatment by region (%).

In the examination group there was a prevalence of use of OAC treatment in women (73.1%) versus men (62.2%) ($p<0.05$). The period from the diagnosis of the disease to the initiation of OAC treatment lasted a month or more in more than half of the patients, constituting 59.1%, increasing the high thromboembolic risk of stroke or other thromboembolic complications (fig. 5).

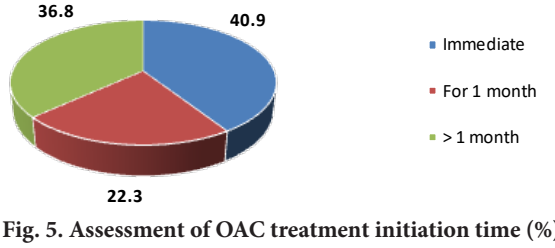

Fig. 5. Assessment of OAC treatment initiation time (%)

During the course of the research, many uncertainties arose regarding who it belongs to and who is responsible for prescribing OAC treatment. In this sample, the majority were cardiologists who indicated anticoagulant treatment, constituting 71.4% (CI 95% 65.2%-74.9%), (fig. 6).

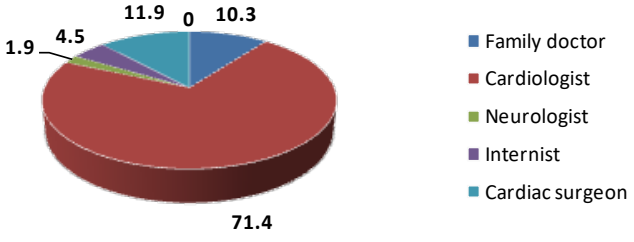

Fig. 6. Healthcare professionals involved in prescribing OAC treatment (%)

Was assessed the patients' level of responsibility in the treatment administering. From the total group of patients, practically half (46.7%) forgot 2-3 times a month or more to receive the medicine on time, and 31.7% of patients skipped (forgot) the treatment 2-3 times per month and more, which influences the degree of use, adherence and quality of AOC treatment. The rate of skipping (forgetting) one dose or more per month in correlation with age demonstrated that the rate of skipping one dose or more increases with age, and young patients, under 65 years of age, administer the medication daily at a higher level and have better treatment adherence ($p<0.005$), (fig. 7).

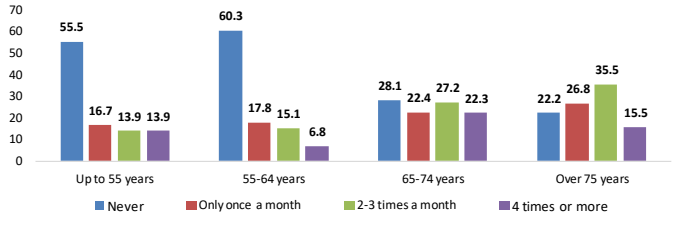

Fig. 7. Omission rate (forgetting) of the monthly dose depending on age (%)

To assess patients' knowledge of OAC treatment was assessed respondents' understanding of the purpose of OAC treatment. Of the respondents who receive OAC treatment, only 81% understand that the main action of OAC is to prevent the formation of blood clots, 53.7% see this treatment as one that can prolong life and 35.9% patients are informed that OAC can prevent stroke. The obtained results highlight that a large group of patients do not have sufficient knowledge related to OAC treatment and do not correctly perceive the purpose of this treatment, these factors being individual determinants in the treatment outcome (fig. 8).

The obtained data highlight that patients in the urban region are better acquainted with the treatment of OAC, constituting 69.2% versus 42.4% in the rural region, which results in the fact that access to information in the urban

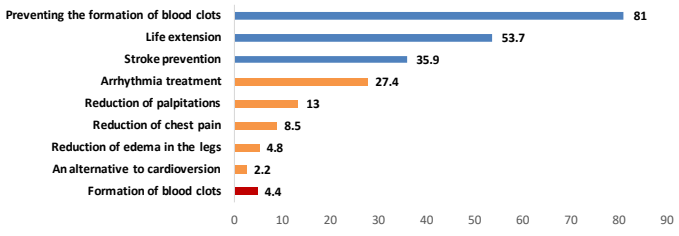

Fig. 8. Evaluation of respondents' understanding of the purpose of OAC treatment (%)

A multitude of factors was assessed with primordial negative impact on anticoagulant adherence, besides frequent blood monitoring, including perceived drug efficacy and safety, anxiety about real drug side effects, patient autonomy, quality of information provided to patients by physicians, the influence of the drug on physical activities and quality of life. Adherence barriers were compared between patients treated with VKAs (vitamin K antagonists) and DOACs (direct oral anticoagulants), demonstrating an increased preference for DOACs (in eligible patients) versus Warfarin among both patients and family doctors. The high price of DOAC, being the most important barrier to administration in 91.1%. Although antiplatelet therapy is not recommended simultaneously with OAC treatment in most cases [10], in the group of patients practically half (44%) received Aspirin or Clopidogrel simultaneously, being subject to an increased risk of bleeding. The complication rate of OAC treatment in the research group was 35.7% (mainly mild hemorrhagic complications, ecchymoses, superficial bleeding, blood in stool or urine), with prevalence in patients using VKA (38.7%), compared to DOAC (20%).

Patient satisfaction with anticoagulant treatment control is low in more than half of patients, mainly for VKA (59.8%). The majority of respondents claim that the task of controlling anticoagulant treatment is defective, and the management of OAC therapy in the medical institution where they are served is difficult (75.5%).

In patients using VKA were assessed preferences for the OAC treatment monitoring and control model. The majority of respondents wanted INR monitoring and control at home through self-monitoring (38.4%) or at specialized anticoagulation centers (36.4%), and only 25.2% claimed for monitoring at the family doctor. Considering that in the Republic of Moldova the management of OAC treatment is assigned to family doctors and there are no monitoring centers or the possibility of self-monitoring, patient preferences cannot be encouraged at the moment.

Time in therapeutic range (TTR) is a tool to assess the quality of treatment, and access to reliable INR monitoring is important for the optimal management of anticoagulation therapy of VKA, with the frequency of testing being assessed individually for each patient. The International Self-Monitoring Association for Oral Anticoagulation recommends a testing interval of no more than 4 weeks for stable patients and no more than 2 weeks in unstable patients [11]. In the research carried out 40.3% of the respondents did not perform safe therapeutic control of International Normalized Ratio (INR), (fig. 9).

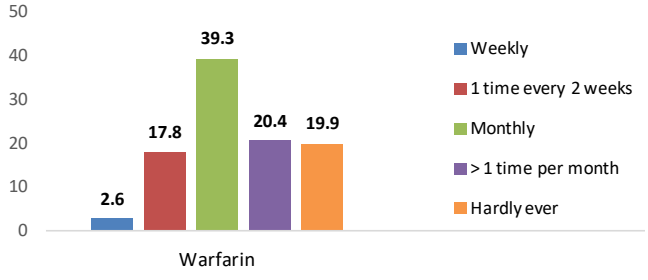

Fig. 9. Frequency of INR testing in the group of patients receiving VKA (%)

54.7% of patients are not in the optimal therapeutic range and do not have adequate treatment control, being subject to a high thromboembolic risk (fig. 10).

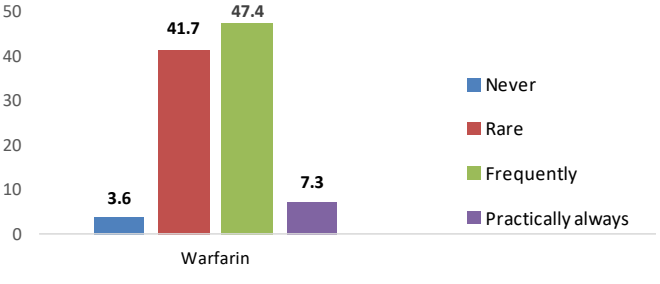

Fig. 10. INR values outside the therapeutic range (%)

Respondents mentioned that in 21.5% of cases the dose is changed practically after each INR test, with a negative impact on treatment adherence (fig. 11).

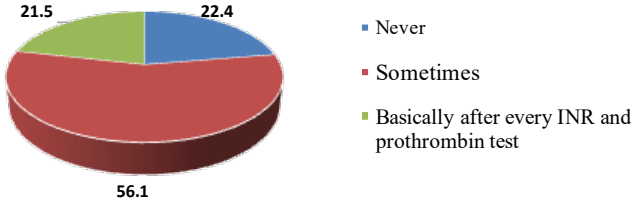

Fig. 11. Dose adjustment rate of respondents using VKA (%)

More than 1/3 of patients have a low level of trust and satisfaction in the healthcare provided (38.2%), which could also have a significant impact on adherence, persistence and therefore the effectiveness of OAC treatment, and 32.6% of patients were not explicitly informed about anticoagulant treatment, which indicates a low level of communication. The majority of respondents consider the cardiologist (224 respondents) and the family doctor (186 respondents) as the main source of information and communication.

Conclusions

1. The study found that anticoagulant therapy is underused in the Republic of Moldova, especially among rural and elderly patients.
2. The main barriers to adherence to OAC treatment are the burden of regular monitoring of blood parameters imposed by Warfarin therapy, the perceived concern of complications and limited access to laboratory tests and specialist doctors.
3. The research conducted identified that there is limited confidence and uncertainty persists in the initiation and monitoring of OAC treatment by family doctors.
4. The use and control of OAC treatment are influenced by the technical-material and laboratory incapacity of the anticoagulation service to ensure the current rigors of diagnosis and treatment.
5. The application of DOAC treatment is associated with a higher level of patient satisfaction and compliance, as well as the safety of family doctors in the use of OAC treatment.
6. The monitoring model and applied practices of OAC treatment in the Republic of Moldova do not correspond to patients' preferences, at the same time there is no access to alternative monitoring and control models.
7. Some of the essential problems faced by patients with thromboembolic diseases are limited access to medical assistance, insufficient information about anticoagulation and deficiencies in communication with medical personnel.

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Authors' contributions
NS conceptualized the project, drafted the first manuscript and interpreted the data. AB critically revised the manuscript. Both authors revised and approved the final version of the manuscript.

Funding
This study was supported by the School of Public Health Management. The study was carried out as part of a master's program in Public Health Management. The authors are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The study protocol was discussed and approved at the School of Public Health Management meeting (05.10.2022).

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.07
UDC: 611.77.018.1+57.085

The effectiveness of the tissue engineering in the obtaining of the biological materials from the extracellular matrix

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Manuscript received November 09, 2023; revised manuscript December 04, 2023; published online December 27, 2023

Abstract

Background: The present work describes the possibility of manufacturing biomaterials from the extracellular matrix for the treatment of the skin wounds. Biomedical collagen-based materials are clinically effective. Collagen is the most abundant and major component of the skin. Porcine collagen is almost similar to the human collagen, it is not immunogenic when used for the therapeutic purposes. Biomaterials can be obtained from the decellularized dermis, being a matrix rich in the collagen and glycoproteins.
Material and methods: 3 parallel groups of biomaterials were established and the average value was calculated. To ensure the effectiveness of the decellularization process, the decellularized porcine dermis was compared with the intact sample using qualitative and quantitative criteria.
Results: Histologically, the decellularized tissues revealed the presence of fewer cells. As a result, were removed approximately 80.5% of the genetic material from porcine dermal structures, demonstrated by the spectrophotometric quantification of deoxyribonucleic acid. *In vitro* graft degradation study in 0.01 M phosphate buffer pH 7.4 combined with collagenase, demonstrated a significant ($p < 0.05$) loss of collagen sponge mass by 100% over one hour in the group II compared to the decellularized dermis in group I which decreased in the weight by 91.3% during 35 hours.
Conclusions: Acellular biomaterials are immunologically inert, have hydrophilic and biodegradable properties, thus they can play a key role in the wound care, exerting the transfer of the bioactive molecules and drugs directly into the wound.
Key words: porcine acellular dermis, collagen sponge, biomaterials, tissue engineering.

Cite this article
Macagonova O, Cociug A, Taralunga T, Ciobanu V, Braniste T, Nacu V. The effectiveness of the tissue engineering in the obtaining of the biological materials from the extracellular matrix. *Mold Med J*. 2023;66(2):41-48. https://doi.org/10.52418/moldovan-med-j.66-2.23.07.

Introduction

The extracellular matrix (ECM) is a structural support network made up of various glycoproteins. It influences a large number of cellular processes, including migration, wound healing, and differentiation, all of which are of particular interest to tissue engineering researchers [1]. Collagen-based biomedical materials are clinically important and effective [2]. Collagen is the most abundant structural protein in animals. It is the major component of the skin [3, 4]. Porcine collagen is almost similar to human collagen, it is not immunogenic when used for therapeutic purposes. The dermis has been used for tendon reconstruction, hernia repair, skin and wound healing in plastic and reconstructive surgery (Belviso I., et al. 2020) [5]. Decellularization or removal of cells from the complex mixture of structural and functional proteins that make up the extracellular matrix can be done by physical (shaking, sonication, freezing and thawing), chemical (alkaline oracids, ionic, nonionic detergents, tri-n-butyl phosphate (TBP), hypotonic or hypertonic treatments, chelating agents) and enzymatic methods (trypsin or protease inhibitors) [3]. The effectiveness of the decellularization procedure is characterized by the following parameters: the absence of cells and nuclear debris, the preservation of matrix integrity, tissue density and the ability of cellular repopulation. The acellular matrix must be compatible with cells and possess phenotypic building material [7]. Scientific articles describe different techniques for extracting collagen from the skin of animals eaten for meat. The journals address the pretreatment and extraction methods that have been investigated for the production of collagen from animal skin. Enzymatic, acid or alkaline processing was used. Chemical hydrolysis extraction, salt solubilization, enzymatic hydrolysis, ultrasound-assisted extraction, and other methods are described. Post-extraction purification methods are also explained. Natural scaffolds allow proper cell population, proliferation and secretion, which is important for their survival and regeneration in the affected tissue [8-10]. The shortcomings of some decellularization methods are: persistence of residual deoxyribonucleic

acid, which has a significant proinflammatory effect [11], inhibitory response on cell proliferation and cytotoxic effect. Researchers have described the factors that can lead to these negative effects on the matrix, being residual detergents, sterilizing chemicals that modify the structure of the scaffold. The behavior of the acellular scaffold applied to the wound will be different depending on the hydrogen indicator in the lesion [12]. Cellular content in the ECM has the potential to cause graft rejection when grafted, so it should be removed prior to transplantation. With the development of decellularization technology, extracellular matrix as a new biomaterial has attracted the attention of many researchers. In the present work will be examined the effects of tissue engineering methods in obtaining biomaterials and the factors that influence the preservation of bioactive properties for the development of biological dressings for skin wounds: (1) Decellularized ECM must have less than 50 ng of double-stranded DNA per mg weight dry, (2) lack of visible nuclear staining when treated with DAPI or H&E. Furthermore, the mechanical properties, including (3) biodegradable and (4) hydrophilic properties, will be considered as well.

Material and methods

Skin preparation. To achieve the aim of the study were examined 30 decellularized porcine dermal grafts and 30 non-cross-linked porcine dermal collagen scaffold fragments (fig. 1, 2). The samples were obtained from piglets weighing up to 10 kg euthanized by blunt trauma, following the recommendations of the university ethics committee (decision No 41 of 03.02.2020). The dimensions of the tissue fragments being 5×5×2 mm and the weight of 87.9 ± 3 mg for the acellulardermis and 15.26 ± 5.0 mg for the collagen sponge.

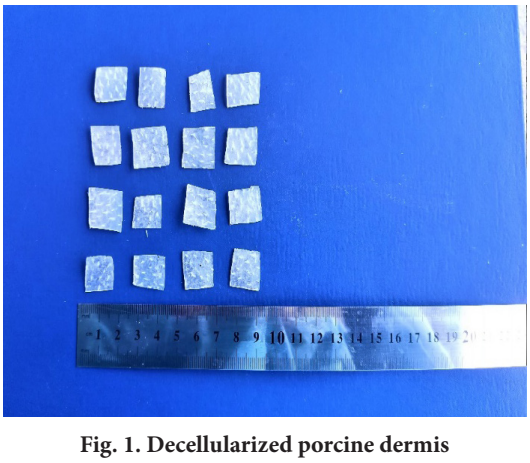

Fig. 1. Decellularized porcine dermis

Separation method. By treating the tissues with 0.3% trypsin solution at 37°C for 30 minutes with minimal mechanical effort, the dermo-epidermal separation of the grafts was obtained, according to the protocol (Wilkinson D., et al. 1974) [13].

Decellularization method. Tissue decellularization was performed by treating porcine dermis with 1% Triton

Fig. 2. Porcine dermal collagen sponge

X-100, 4% sodium deoxycholate and washing thoroughly in sodium phosphate buffer [14].

Staining method with 4′,6-Diamidino-2-phenylindole dihydrochloride (DAPI). DAPI staining was performed to visualize the presence of residual nuclei in normal and decellularized porcine dermis and to qualitatively assess the effects of decellularization, using confocal fluorescence microscopy (Optica, Italy) [15]. Samples were fixed in 4% paraformaldehyde, permeabilized, sectioned with a microtome (7 μm) and stained with DAPI (fig. 3).

Fig. 3. Staining of the dermis with 4′,6-Diamidino-2-phenylindole dihydrochloride

Morphological assessment. Examination of the decellularized samples was performed by the histological examination with hematoxylin-eosin (H&E). Samples were fixed in 4% buffered formaldehyde [15].

Spectrophotometric method. The spectrophotometric method was applied for DNA quantification. Decellularized and native tissues were quantified using a kit (DNA Extraction Kit, Cygnus Technologies, USA). The extracted DNA was quantified spectrophotometrically in a microplate reader at a wavelength of 260/280 nm (NANODROP 2000C). The value obtained was represented as a function of the weight of dry samples ng/mg [16, 17].

Obtaining collagen scaffolds. Collagen was most commonly extracted from the skin through a hydrolysis treatment involving the use of acidic or alkaline solutions. Under acidic conditions, collagen molecules have a net positive shift, and the resulting electrostatic repulsive

force between them facilitates molecular separation [3]. Commonly used organic acids are acetic, chloroacetic, citric and lactic acids. Acetic acid has been widely reported for collagen extraction [2]. Proteolytic enzymes are used in collagen extraction. These enzymes can be of animal origin (trypsin, pepsin), plant origin (e. g. bromelain, papain, ficin) or single or mixed enzymes microbial products (e. g. collagenase, proteinase K, Alcalase® (Novozymes, Bagsværd, Denmark), Nutrase® (Nutrex, Hoogbuul, Belgium), Flavourzyme® (Novozymes, Bagsværd, Denmark) and Protamex® (Novozymes, Bagsværd, Denmark)). Pepsin from animal sources is most commonly used in collagen extraction [3]. Thus, 30 fragments of further decellularized porcine dermis were morcellated and subjected to successive treatment with 0.5 M acetic acid solution and 5% w/w pepsin based on dry tissue weight. Neutral salt solutions are effective in solubilizing collagen and are commonly used in extraction. Examples of salts used are citrates, phosphates, sodium chloride and Tris-HCl. Collagen suspension was filtered and repeatedly solubilized with 2.5 M sodium chloride in 0.5 M acetic acid solution. Afterwards, the obtained collagen was lyophilized for 72 hours until the porous collagen scaffold was obtained (Hakim T., et al. 2021) [18].

***In vitro* degradation of dermal grafts.** Each phase of skin wound epithelialization is influenced by exogenous and endogenous factors. One of the significant endogenous factors that determine the rate and outcome of biochemical reactions during wound recovery is wound pH [12]. A number of the authors claim that the pH changes during the epithelialization process. During the inflammatory phase, the hydrogen value moves to the acidic side and varies from 5.4 to 6.9. During the proliferative phase the pH becomes neutral or alkaline and ranges from 6.9 to 9.0. And in the final phase of healing, the pH takes on the value of healthy skin, which normally ranges from 4 to 6. Thus, the *in vitro* degradation behavior was analyzed by following the weight loss of the biomaterials in 0.01 M phosphate buffer solution (PBS) in acidic, neutral and basic environments. The freeze-dried scaffold was weighed (m0), immersed in a centrifuge tube containing PBS. The pH of the buffer was 7.4, 4.0 and 10.0, the exposure time being 1, 7, 14, 21 and 28 days in the incubator conditions at t 37°C. Comparatively, graft degradation was monitored in 0.01 M PBS pH 7.4 solution combined with collagenase from Clostridium histolyticum (≥250 CDU/mg solid, Sigma-Aldrich, UK) 10 U/ml, the follow-up period being of 1, 5, 8, 24 and 35 hours at t 37°C. The remaining mass fraction (D, %) was calculated using the following formula: $D = mx/m0 \times 100\%$, where mx is the final tissue mass. Four parallel groups were established and the mean value was calculated.

Water absorption test. In the local treatment of skin wounds, it is important to rely on the use of dressing material impregnated with antiseptic drugs. Therefore, this test allowed studying the hydrophilic properties of the scaffolds. The absorption of water revealed the diffusion of the medium into the tissues being necessary for the resorption of exudate from the wound and the cultivation of cells on the ECM obtaining essential nutrients. 0.01 M PBS pH 7.4 was used in the fluid absorption test. The time required to follow the weight dynamics of the samples being 1, 2, 4, 8, 12 and 24 hours at t 25°C. The immersed samples were then removed from the solution and weighed, and the excess water on the surface of the samples was gently blotted with a filter paper to obtain the W_{WET}. The percentage of water absorption for the samples at different times was calculated as follows:

$$\text{Water absorbtion (\%)} = \frac{100\times W_{WET} - W_{DRY}}{W_{DRY}},$$

where W_{DRY} and W_{WET} are the weights of the dry and wet scaffolds respectively at the required times [19]. Four samples were tested for each scaffold and the average values were recorded.

Scanning electron microscopy. The inside of the graft is crucial for appreciating the "microenvironment". Pore size and connectivity affect cell adhesion, nutrient exchange and metabolic waste removal, and skin regeneration. The morphological characteristics of the acellular dermis scaffolds were observed using scanning electron microscopy (SEM) [20]. After washing with phosphate buffer, the decellularized dermis samples were dehydrated and dried under vacuum. Then the dried sample was cut and the cross section was coated with 10 nm of Au. For scanning electron microscopy was used a representative tissue sample from each study group and a non-degraded control sample to monitor ECM disorganization leading to tissue weight loss.

Results

DAPI and hematoxylin eosin analysis

To create the porcine extracellular matrix biomaterials, decellularization was performed to remove only porcine resident cells from the dermal skin devoid of epidermis and hypodermis. To verify the efficiency of decellularization, DAPI and H&E staining was performed. As shown in figure 4, the nuclei of the cells in the normal dermis are stained compared to the image (figs. 5 and 6) where the cells of the decellularized dermis were not highlighted by staining, while the ECM surrounding the cells was maintained.

DNA quantification by spectrophotometry

To quantitatively characterize the effects of decellularization, DNA was quantified from normal and decellularized dermis. Figure 7 shows the result. Residual DNA in the extracellular matrix was 2.43 ± 0.5 ng/µl ng/mg, which was significantly different from 17.43 ± 3.4 ng/µl determined from normal dermis from which the epidermis and adipose tissue have been removed.

Thus, it was managed to remove about 80.5% of the genetic material from the porcine dermal structures according to spectrophotometric DNA quantification. As a

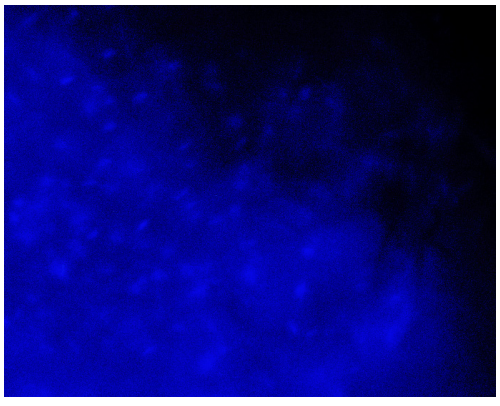

Fig. 4. Normal porcine dermis stained with DAPI

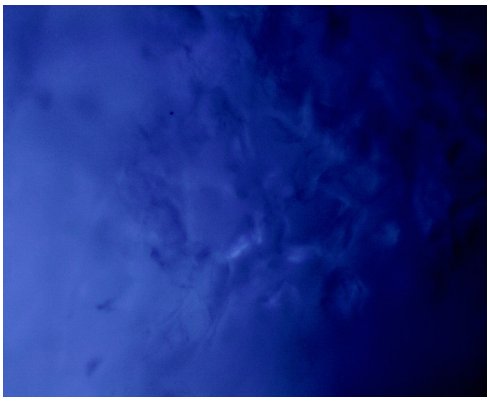

Fig. 5. Decellularized porcine dermis stained with DAPI

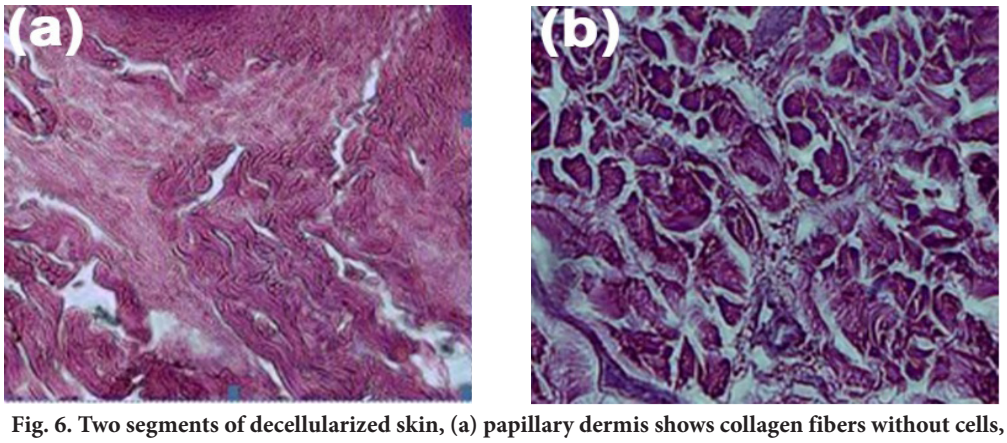

Fig. 6. Two segments of decellularized skin, (a) papillary dermis shows collagen fibers without cells, (b) reticular dermis shows collagen fibers without cells, H-E×140

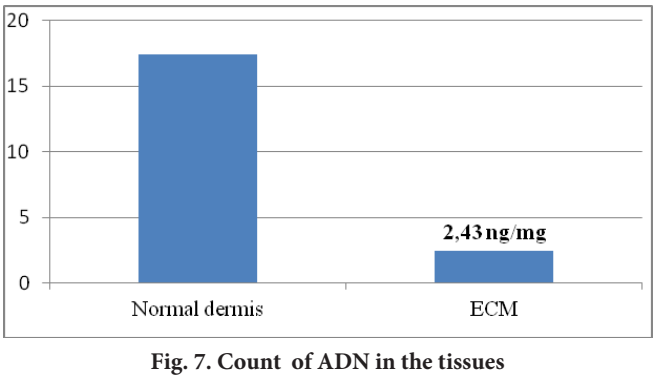

Fig. 7. Count of ADN in the tissues

result, similar to the results of DAPI and H&E staining, the extraction of dermal extracellular matrix was confirmed.

***In vitro* degradation of the biomaterials**

The rate of biomaterial degradation in the skin wound should ideally match the rate of wound regeneration. Thus, if the acellular scaffold degrades rapidly during the early stage of wound regeneration, it will not provide a good barrier for regeneration itself, and this will eventually lead to soft tissue extension into the skin defect, which is not welcome for organized soft tissue regeneration. As shown in Figure 8 and 9, the degradation rate of dermal

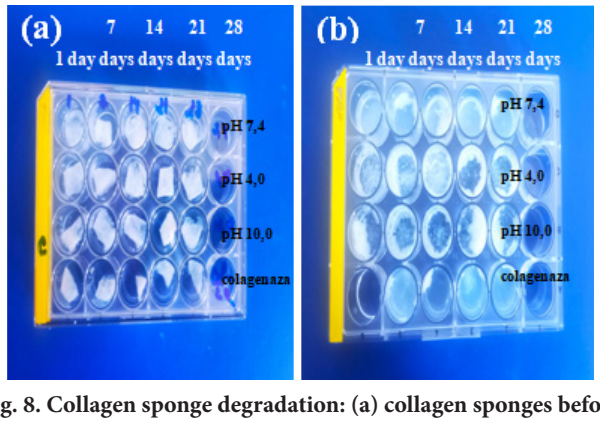

Fig. 8. Collagen sponge degradation: (a) collagen sponges before degradation, (b) collagen sponges after degradation.

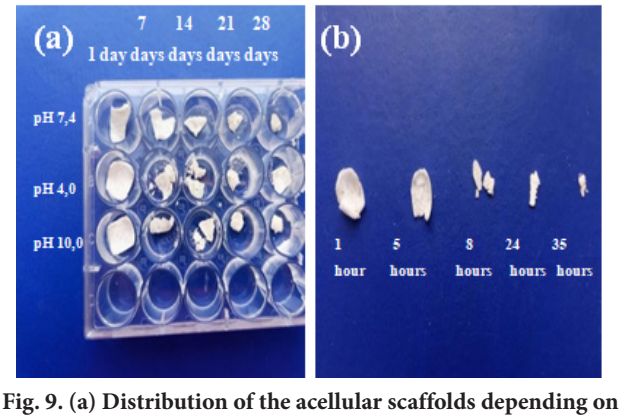

Fig. 9. (a) Distribution of the acellular scaffolds depending on the duration of exposure and the pH of PBS chosen for the study. (b) Degradation behavior of acellular dermis in the presence of collagenase (10 U/ml, PBS pH 7.4, 35 hours).

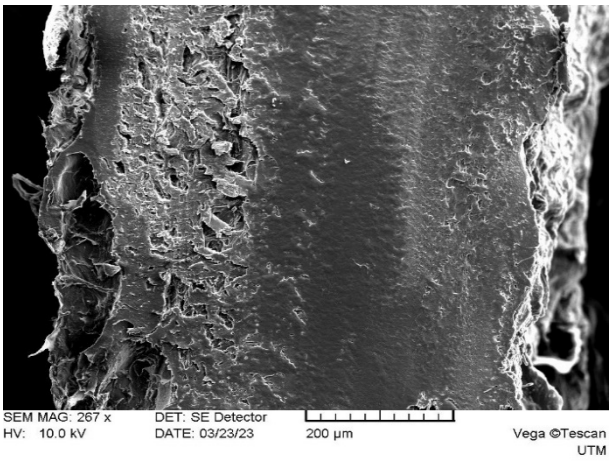

Fig. 10. ECM from the decellularized porcine dermis.

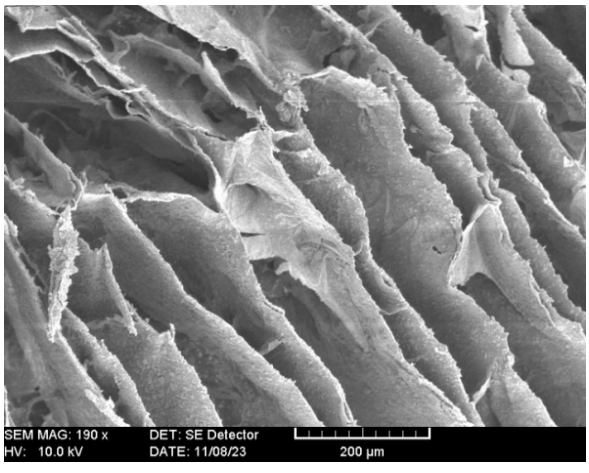

Fig. 11. Collagen sponge from porcine dermis.

collagen sponge in 0.01M PBS pH 7.4 combined with collagenase from Clostridium histolyticum was the fastest, reaching a degradation rate of 100% in the first hour of contact with fluid compared to 91.3% of the extracellular matrix that degraded within 35 hours. At the same time, the degradation volume of collagen sponges in 0.01 M PBS solution with pH 7.4 and 10.0 was 100% and 95% in pH 4.0 at 7 days. Compared to acellular scaffolds that performed differently in 0.01 M PBS solution pH 4.0 and 10 without enzymes, it accounted for 79.8% and 74% of the total sample from the 21st to the 28th day, and then the degradation tended to be slow. In 0.01 M PBS solution with pH 7.4, the degradation of acellular dermis reached 90.3% on the 28th day.

Evaluation of hydrophilic properties

The hydrophilic properties of the biomaterials were evaluated by the absorption test of 0.01 M PBS solution with pH 7.4. A variable depending on the exposure time was obtained, namely the acellular dermis immersed in the solution reached 350 mg at the 4th hour of immersion in the liquid, the initial mass being 87.9±3 mg. The absorption rate of the collagen sponges was nil. The collagen samples hydrolyzed in the PBS solution during the first hour of immersion.

Scanning electron microscopy

Decellularization of the dermis with Triton/SDS removed cells, resulting in a porous appearance of the ECM (fig. 10) and preservation of dermal structures, which is a hallmark of decellularization. The ECM is mostly composed of fibers, has a more dense structure and small pores. Scanning electron microscopy image of the sponges showed collagen-based membrane and fibrous components. It has an irregular and relatively fluffy structure, with large pores (fig.11).

In vivo recruitment of the tissue-forming cells into scaffolds is closely regulated by the physicochemical properties of the scaffolds, e.g., pore size, porosity, bioactivities, stiffness, etc. The dermis, having dense ECM with inherent small pores, would inhibit cellular infiltration and lead to poor tissue regeneration. Therefore, the development of naturally occurring scaffolds with three-dimensional structure of large interconnected pores would be essential for cell infiltration and functional tissue regeneration [21]

Discussion

The skin is the natural barrier between the human interior and the external environment, consisting of the epidermis, dermis and subcutaneous tissue. In daily life, the skin is easily damaged, and the human body has a certain reconstruction function to realize the self-repair of the damaged skin. However, when the affected skin area is large and the degree of damage is severe, such as skin defects caused by severe burns, trauma or some chronic wounds that cannot heal, they may be complicated by infection, and the reconstruction of the structure and skin function can only be ensured by autologous skin transplantation [22-25]. Autograft requires a sufficient supply of skin, however the amount of autologous skin available to the patient is quite limited and the process of removing the skin will cause additional pain and secondary trauma to the patient. Another type of implantable skin is derived from cadaver skin. However, corpse skin is limited and mostly aged and unhealthy. More importantly, the use of cadaveric skin carries the risk of transmitting contagious diseases and is ethically restricted [26]. Therefore, the development of artificial skin has become a hot spot in the field of medical skin tissue engineering. An acellular dermal matrix is used as a skin substitute. For this, it must be de-epithelialized and decellularized removing the cellular components and preserving the three-dimensional collagen and reticular structures in the dermis [27, 28]. Thus, the extracellular matrix reduces the rejection reaction while preserving the native dermal structure, thereby inducing the growth of cellular components, supporting fibroblast infiltration, the formation of new blood vessels, promoting the gradual fusion of fibroblasts and transplanted autologous epidermal components, finally, the complete structure of the skin is formed,

and the original functions of the skin are practically recovered. In the process of preparing the acellular dermal matrix, pig skin is usually selected and treated by a physical, chemical or biological method to obtain the porcine acellular dermal matrix [28]. A key parameter of decellularized ECM materials is balancing strength and biodegradability properties. Decellularized ECM materials have been shown to retain a complex array of proteins present in the original tissue being the cytokines that are preserved at the time of decellularization. Among the several cytokines whose levels are quantified are vascular endothelial growth factor and transforming growth factor beta that are retained in the ECM [29, 30]. After decellularization of the dermis, many ECM proteins remain in the material, including collagen III, collagen IV, collagen VII, laminin, and fibronectin. Glycosaminoglycans are also preserved, including hyaluronic acid. They ensure the bioactive properties of ECM for recellularization, promoting rapid integration and repair in clinical applications [29]. Most decellularization efficacy test reports revealed positive data on X-100 triton treatment compared to 0.1% sodium dodecyl sulfate (SDS) and 0.1% trypsin solutions [32-32]. It was demonstrated that the X-100 triton decellularization method in combination with SDS or tri-n-butyl phosphate solution was the most effective, but also the most destructive in terms of glycosaminoglycan and collagen depletion. This phenomenon was also shown in the study where the skin was decellularized with trypsin, triton, and sodium hydroxide and observed fibrinoid necrosis, fragmentation, and undulation of fibrillar structures in the dermis affirming depletion of the dermal matrix. Although, from the protocols tested in the study, triton X-100 had the least harmful effect on glycosaminoglycan content [35]]. Crapo P., et al. suggested that the densest tissues, such as dermis, tendon, and trachea require decellularization protocols by continuous agitation lasting from days to months [32, 36, 37]. However, in the present study, the desired results were obtained after 48 hours of treatment with biological detergents. Gilbert T. et al. have shown that cells and cell products cannot be completely removed from dense tissues such as dermis, even with the most rigorous processing methods [7]. However, in the present study, complete cell-free membrane was observed after 48 h of treatment, although SDS solubilized cell membranes and dissociated DNA. It is therefore effective in removing cellular material from tissues. Sodium dodecyl sulfate was more effective in removing cell debris and cytoplasmic proteins such as vimentin from the tissue compared to other detergents, but is more aggressive to ECM [35, 38, 39]. Dodecyl sulfate was more effective than Triton-X 100 in removing nuclei from dense tissues. SDS disrupted native tissues and caused a decrease in the concentration of GAGs and depleted collagen. Sodium deoxycholate (SD) is very effective at removing cellular debris. SD has been shown not to alter the structural properties of the ECM but it tends to disrupt the structure of the tissue itself, so it should be used in a lower concentration. Among the freeze-thaw cycle methods with NH (4) OH and triton X-100 with 1.5 M K Cl showed the best effect on the removal of cellular components from the complexes, while the other five methods could only partially remove the component cells. The freeze-thaw method maintained the ECM structure as well as the mechanical strength, but retained a large amount of the cellular components of the ECM scaffold. About 88% of the DNA was left in the ECM after freezing defrosting treatment. *In vitro* inflammatory assays suggested that the amount of DNA fragments in the ECM scaffolds did not elicit a significantly different immune response. All three ECM scaffolds showed a comparable ability to support cell repopulation. There were described the successful results of de-cellularization with SDS and Triton X-100. Total absence of nuclear structures and removal of viable cells was confirmed by hematoxylin-eosin staining and scanning electron microscopy [39]. Macroscopic evaluation of de-epithelialized rat skin with the hypertonic solution for 4 hours found that the epidermis was not separated from the dermis. Thus, after 6 hours, the multilayered epithelium was removed more easily. However, after another 8 hours the epidermis was separated spontaneously with minimal mechanical effort and a completely de-epithelialized dermis was obtained. Treatment of the skin with hypertonic saline for 24 hours resulted in an acellular matrix with collagen fibers of insignificant thickness. In de-epithelialized skin treated with triton X-100, cell debris was detected between the interstitial spaces of the thicker collagen fibers [11]. At 48 hours after treatment with triton X-100, the acellular dermis became more porous. Treatment of skin with sodium dodecyl sulfate (SDS) for 24 hours resulted in a membrane with fewer cells and collagen fibers with significantly preserved thickness. At 48 hours after immersing the dermis in SDS, the collagen fibers became more fragile with large spaces between them. Treatment with 1% sodium deoxycholate (SD) effectively removed cellular debris at 48 hours. Increasing the concentration from 1% to 2% of SD, led to the expansion of the spaces between collagen fibers. No cell nuclei were observed and the tissue was composed of the more porous extracellular matrix. Hypotonic and hypertonic solutions have been reported as ineffective decellularizing agents [39]. 48 hours after immersing the dermis in SDS, the collagen fibers became more fragile with large spaces between them. Treatment with 1% sodium deoxycholate (SD) effectively removed cellular debris at 48 hours. Increasing the concentration from 1% to 2% of SD, led to the expansion of the spaces between collagen fibers. No cell nuclei were observed and the tissue was composed of the more porous extracellular matrix. Hypotonic and hypertonic solutions have been reported as ineffective decellularizing agents. TBP treatment resulted in a displacement of nuclear waste. This led to a decrease in the content of glycosaminoglycans. However, because most tissues are very dense, deoxyribonucleic acid (DNA) is almost impossible to remove 100%. Therefore, DNA remaining after de-

cellularization should be examined quantitatively or qualitatively, that should not yield any staining after treatment with DAPI or H&E [15].

Conclusions

1. The variability of the hydrogen indicator and the involvement of the proteolytic enzyme that is secreted in significant quantities in all epithelial lesions and causes the degradation of collagen polypeptide fibers is important in the resorption of acellular dermal grafts.

2. The samples degraded in contact with the fluids, result in a significant reduction in weight. Non-cross-linked sponges hydrolyze immediately on contact with fluids.

3. Evaluation with DAPI, H&E and SEM assesses the effectiveness of tissue engineering in obtaining biocompatible materials.

4. Obtaining smart biological grafts for skin wound regeneration is one of the research directions of modern tissue engineering and represents an attractive segment of regenerative medicine.

5. Xenograft is easier to obtain in commercial quantities that would cover clinical needs. Scaffolds obtained from decellularized porcine dermis have great potential as a source of bioactive molecules with biocompatible, hydrophilic and biodegradable properties.

6. To show the quality of the scaffolds, it is necessary to perform immunohistomic studies to determine the growth factors on the acellular membrane, cross-linking, recellularization and cytocompatibility of the grafts.

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Authors' contributions
OM proposed the concept and design of the research, selected the literature and contributed to the elaboration and writing of the manuscript. AC, VC and TB performed microscopic images and helped draft the manuscript. VN conceptualized the idea, designed the research and monitored the experiment. All the authors approved the final version of the manuscript.

Funding
The study was supported by the State Program Project 2020-2023: "GaN-based nanoarchitectures and three-dimensional matrices from biological materials for applications in microfluidics and tissue engineering", No 20.80009.5007.20. The trial was the authors' initiative; they are independent and take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The project was approved by the Research Ethics Committee of *Nicolae Testemitanu* State University of Medicine and Pharmacy (Protocol No 41 of 03.02.2020).

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.08
UDC: 618.36-008.64:618.344

Ang1 immunoexpression vs vascular profile in chorio-villous germinative status in early term compromised pregnancies

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Manuscript received September 29, 2023; revised manuscript November 24, 2023; published online December 27, 2023

Abstract

Background: A normal maternal-fetal system is essential for the functioning of the placenta during placentation and the establishment of the maternal-embryo-fetal vascular circulation. The angiopoietin/TIE pathway is involved in vascular morphogenesis through regulation, survival and maturation of endothelial cells concomitant with vascular remodeling. Deregulation of pro-angiogenic factor secretion and expression is associated with disruption of vascular morphogenesis, reduction of vascular bed and installation of primary placental insufficiency. The aim: Evaluation of Ang 1 immunoexpression in early term compromised pregnancies in the context of chorio-villary circulatory dysfunction in primary placental insufficiency.
Material and methods: Abortion product from 61 patients (stagnant pregnancies – 39 cases, early miscarriage – 8 cases, control group – 14 cases of pregnancies solved on social indications/ desire) were immunohistochemically evaluated with the marker for anti-Ang1 and anti-CD31.
Results: The villous syncytiotrophoblast was the most immunoreactive area. Most of cases of the pregnancies terminated on social indications/ desire were anti-Ang1 negative. The levels of anti-Ang1 immunoexpression were statistically significantly different in case of syncytiotrophoblast of early miscarriages and abortions terminated on social indications/ desire. The highest chorio-villous vascular density was noticed in the abortions on social indications/ desire and early misscariages group.
Conclusions: The placental period is characterized by a weak angiogenic Ang1 differentiated cellular environment in the chorio-villous germinal site in the group of short term compromised pregnancies. The selectively immunoexpressed cellular profile statistically significantly correlates with placental vascular index and chorio-villous vascular density in stagnant pregnancies.
Key words: Ang1, angiogenesis, fetal conceptus, compromised pregnancies, primary placental dysfunction.

Cite this article
David V, Fulga V, Petrovici V, Carpenco E, Saptefrati L. Ang1 immunoexpression vs vascular profile in chorio-villous germinative status in early term compromised pregnancies. *Mold Med J*. 2023;66(2):49-55. https://doi.org/10.52418/moldovan-med-j.66-2.23.08.

Introduction

Establishing of a functional maternal-fetal system is essential for the functioning of the placenta during placentation with the establishment of maternal-fetal vascular circulation and reduction of pregnancy complications. The vascularization of chorionic villi is an important morphological indicator of the functional state of the placenta, reflecting the expression of metabolic processes between mother and fetus [1]. The formation of such a competent vascular network in the early period is achieved by two successive processes: vasculogenesis (formation of vessels from endothelial progenitor cells) and angiogenesis (formation of vessels from pre-existing vessels) [2], the destabilization of which contributes to the onset of placental circulatory failure.

The primary placental insufficiency is of particular impact in the development of the human conceptus in prenatal pathology. It is caused by morpho-functional changes and is characterized by lack of or decreased optimal mother-embryo/fetus exchange during the establishment of hemochorionic blood circulation. According to the study by Regnault T. R. et al. (2002), defective angiogenesis is often the cause of intrauterine developmental restriction [3], as a result of vascular disturbances [4].

Angiogenic factors of the angiopoietin family play an important role in stabilizing of the placento-fetal vascular network during placentation. According to the molecular profile, 4 ligands (Ang1, Ang2, Ang3, Ang4) are elucidated, the first two and their tyrosine kinase-like receptors (TIE-1 and TIE-2) having a significant role in vascular morphogenesis during placental period [5]. The regulatory effect of Ang1 on endothelial cell survival and maturation occurs via its specific receptor tyrosine kinase (TIE-2) [6]. Thus, angiopoietins 1 are endogenous ligands of the vascular endothelial-specific tyrosine kinase receptor TIE-

2, which is still expressed in the early period of placentation and has endothelial and non-endothelial effects [7].

At the same time, the expression of angiopoietins is diverse and contradictory, often being elucidated much later in the pathogenesis of pathologies, such as intrauterine developmental restriction, pre-eclampsia, etc. [7-9]. In this context, Schneuer F. J. et al. (2013) found an association with risk of miscarriage at gestational term greater than 10 weeks while evaluating the impact of angiopoietin1 levels in the first trimester of pregnancy [10].

Deregulation of secretion and expression of pro-angiogenic factors during placentation may be associated with disruption of vascular morphogenesis in the chorio-villous compartment because of endothelial dysfunction and reduction of the chorio-villous fetal vascular bed. The above-mentioned ideas make this study of a high topicality.

Thus, the aim of the current study was to assess Ang1 immunoexpression in early term compromised uterine pregnancies in the context of chorio-villous circulatory dysfunction.

Material and methods

Tissue samples were obtained by uterine aspirate from 61 patients with early compromised pregnancies (3-12 weeks). The specimens were collected at the Level III Perinatal Center, Institute of Mother and Child, during 2020. All patients were examined by ultrasonographic investigation and the gestational term was determined based on the first day of the last menstruation. The cases were grouped as follows: stagnant pregnancies (SP) – 39 cases, early miscarriages (EM) – 8 cases and pregnancies solved on social indication/desire (SI/D) – 14 cases, last one being the control group. The age of the patients ranged from 22 to 40 years (mean±std. dev. being 30.5±5.6 years).

Clinical data were obtained from the medical records of each patient. The current research is part of a larger study of early term compromised pregnancies within the state program "Morphological approach by conventional, histo- and immunohistochemical methods of the peculiarities of the pathological profile of early placentogenesis in early term compromised pregnancies", code 20.80009.8007.17 P1P2 0750.

Cases were selected according to the inclusion and exclusion criteria:

Inclusion criteria: terminated pregnancies with gestational term from 3 to 12 weeks (clinically confirmed by ultrasound and terminated in the Institute of Mother and Child); pregnancies with pathological evolution: stagnant, early miscarriage; pregnancies with abortion at social indications/ desire; quality and volume of the aspirate: chorionic villi and decidual plates of a sufficient volume in standard paraffin blocks (1.0x1.0x0.5cm); monofetal pregnancies; no age threshold.

Exclusion criteria: serious somatic pathology; multiple pregnancies; pregnancies terminated on medical indication; lack of clinical-anamnestic data in medical records; lack of gestational term specification and ultrasound confirmation of pregnancy status.

The examination included histoprocessing of tissue samples, application of the usual histological method (haematoxylin-eosin), immunohistochemical method (anti-CD31; anti-Ang1) with evaluation of histopathological features and immunoexpression, and statistical processing.

Primary processing. Tissue material of the conception product was collected in a short time in obstetric department with rapid fixation in 10% formalin, pH 7.2-7.4, to reduce the risk of early lysis of tissue material and bacterial flora growth. The fixation period in 10% buffered formalin solution was 24 hours. The paraffin embedding system was DP500/CIT2002 (Bio-Optica, Italy). Histochemical and histological processing of samples was performed on the histoprocessor "TISSUE-TEK, VIP 6AI" (Sakura, Japan), sectioning on the HM325 microtome (Thermoscientific) (USA). 3.5 μm thick sections were placed on positively charged slides (APTACA, Italy).

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

Immunohistochemical method. Immunohistochemical assays included manually adopted operational procedures for anti-Ang1 (ab8451) antibodies with the application of the Novolink™MaxPolimer detection system, Leika (RE7280-K) [11] and anti-CD31 (JC70A) with the application of the EnVision™FLEX detection system, high pH (K8000) [12]. The conventional immunohistochemical method was applied (Table 1). Deparaffinization was performed in two toluene baths (code UN1294, Sigma-Oldrich), the first bath for 60 min at 59°C in thermostat, followed by the second bath for 5 min at room temperature. Slides were then placed in a mixed bath of toluene and 96% alcohol for 5 min, then – 2 baths of 96% alcohol with 2 rehydrations of 10 min each in distilled water. For the purpose of epitope unmasking, sections intended for application of Ang1 and CD31 antibody were exposed to dissolved Target Flex solution (1ml Target: 49 ml distilled water) at high pH, 20 minutes exposure time at 95°C-96°C with a total pretreatment and post-treatment time of 60 minutes. Neutralization of endogenous peroxidase was performed with peroxidase block for 7 minutes followed by incubation with Novocastra Protein Block for 5 minutes. Next step was incubation with primary antibody (anti-Ang1) for 12 hours at +4°C, 1:1000 dilution, including 5 minutes in thermostat at 59°C. In the case of anti-CD31 antibody, incubation lasted for 20 minutes at room temperature. After incubation with the primary antibody, neutralization of

Table 1. Antibodies used: source, dilution, unmasking system, detection system, incubation time

Antibody / clone	Source/ incubation time/ dilution	Retrieval system / time	Detection system / time
CD31 JC70A	Abcam, Cambridge, UK/ 20 min/ ready- to-use	Solution Target Flex, high pH/ Water bath at 95°C - 96°C / 20 min	EnVision™FLEX, high pH
Ang1 ab8451	Abcam, Cambridge, UK/ 12 hours/ 1:1000	Solution Target Flex, high pH/ Water bath at 95°C - 96°C / 20 min	Novolink™MaxPolimer, Leika / 30 min

endogenous peroxidase with peroxidase block was performed for 5 minutes, followed by application of the secondary antibody (HRP) for 20 minutes and DAB (3,3'-diaminobenzidine) applied as a chromogenic substrate for 5 minutes. Counterstaining of nuclei was performed with Leica haematoxylin (RE7164) when using the Novolink™MaxPolimer detection system and with Mayer's haematoxylin (HEMM-36/21, BIOGNOST, Slovenia) when using the EnVision™FLEX system. The final product of the reaction was stained brown with cytoplasmic pattern for Ang1 and membranous pattern for CD31. Then, the histological slide panel was subjected to the dehydration and clarification procedure using two absolute alcohol baths, one alcohol and toluene mixed bath and three toluene exposures, each exposure being 5 minutes. The final procedure consisted of mounting the slides with BMC-100 solution. In the manual immunohistochemical staining procedure, Sequenza™ Immunostaining Center was applied using Thermo Shandon Coverplat.

Microscopic evaluation. The CD31 protein (endothelial cell adhesion receptor) was detected at the membrane level, manifested by the presence of brownish colour in the tissue studied. In all sections, blood vessels were quantified by the hot-spot method. For the assessment of Ang1 immunoexpression, initially, areas with the highest density of chorionic villi were determined at ×100 magnification. Immunoexpression was assessed at ×200 magnification using the semiquantitative hot-spot method applied to three representative areas of the germinal site corresponding to chorionic villi (vascularized and avascularized). For the evaluation of anti-Ang1 immunoexpression, the scoring system based on the intensity of immunoreactivity was applied. The reaction was considered to be positive in the presence of brown color in the tissue studied according to the specificity of each antibody. The following score was applied: 0 (no staining); +1 (weak but detectable staining); +2 (moderate or distinct staining); +3 (strong or pronounced staining). The reaction for anti-Ang1 was analyzed in the following areas: cytosyncytiotrophoblast, mesenchymal stromal cells, angiogenic/ endothelial vascular cells.

The above-mentioned structures were counted in each of the 3 study groups (SP, EM, SI/D), grouped according to gestational term into the following groups: 3-5 weeks, 6-9 weeks and 10-12 weeks. Quantification of positive cells was performed on the Axio Imager A2 microscope (Carl Zeiss, Germany) equipped with the AXIOCam MRc5 recording camera.

Data analysis. Statistical procedures (Winstat 2012.1, R. Fitch Software, Bad Krozingen, Germany) included determination of the Spearman's rank correlation coefficient (Spearman (rs)) and differences between groups and subgroups (Mann-Whitney U test). Results were considered statistically significant at $p<0.05$.

Results

The study was carried out on a group of 61 cases of early terminated pregnancies, divided into: stagnant pregnancies – 39 cases (82.9%), early miscarriage – 8 cases (17.1%) and the control batch that included 14 cases of pregnancies solved on social indications/ desire (SI/D) (24.2%).

The intensity of Ang1 immunoexpression varied from 0 to +3 and can be analyzed in the tables 2, 3 and 4.

Table 2. Immunoexpression intensity of anti-Ang 1 (SP batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**
3-5	0	6	1	0	0	6	0	0		4	3	0	1	1	2	0	1	4	2	0
6-9	5	15	4	0	1	18	**5**	0	2	18	4	0	5	15	3	0	5	**17**	2	0
10-12	6	2	0	0	0	**8**	0	0	5	2	1	0	4	3	1	0	6	1	1	0
Total	11	**23**	5	0	1	**32**	5	0	7	**24**	8	0	10	**19**	6	0	12	**22**	5	0

Note: GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

Table 3. Immunoexpression intensity of anti-Ang1 (EM batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**
3-5	0	0	1	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0
6-9	2	**3**	0	0	0	0	**5**	0	2	**3**	0	0	2	3	0	0	2	**3**	0	0
10-12	**2**	0	0	0	0	**2**	0	0	1	1	0	0	1	1	0	0	2	0	0	0
Total	**4**	3	1	0	0	2	**6**	0	**3**	4	0	0	**3**	4	0	0	**4**	3	1	0

Note: GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

Table 4. Immunoexpression intensity of anti-Ang 1 (SI/D batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**
3-5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6-9	**4**	2	1	0	0	**4**	3	0	**4**	1	2	0	**6**	1	0	0	4	1	2	0
10-12	**6**	2	0	0	3	**5**	0	0	**5**	3	0	0	**7**	1	0	0	4	4	0	0
Total	**10**	4	1	0	3	**9**	3	0	**9**	4	2	0	**13**	2	0	0	**8**	5	2	0

Note: GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

In most cases of stagnant pregnancies (SP) (tab. 2), the anti-Ang1 immunoexpression was given the +1 score. The villous syncytiotrophoblast was the most immunoreactive area, the +1 score being assigned in 94.8% of cases. In the early miscarriage (EM) batch (tab. 3), the syncytiotrophoblast was given the +1 score in 75% of cases, and the +2 score was much less frequent (fig. 1). Most of cases of the contol batch (SI/D) were anti-Ang1 negative. Again, the most immunoreative region was the syncytiotrophoblast. To be mentioned that the +3 score was never assigned in any study group.

Then were analyzed the differencies between groups by applying the Mann-Whitney U test. The levels of anti-Ang1 immunoexpression were statistically significantly different in case of syncytiotrophoblast of EM and SI/D (p=0.02). Intragroup statistically significant differencies were also noticed, particularly in case of SP 10-12 weeks vs SP 3-5 weeks: cytotrophoblast (p=0.004) and vascular endothelium (p=0.02); SP 10-12 weeks vs SP 6-9 weeks: cytotrophoblast, vascular endothelium and stroma (p=0.01, p=0.02 and p=0.03, respectively).

Subsequently, to assess the involvement of Ang1 expression in the morphogenesis of the chorio-villous vascular network, placental vascularization index (PVI, %) and chorio-villous vascular density (VD, %) were determined with the application of anti-CD31 antibody. According to the results obtained, anti-CD31 immunoexpression in the chorionic villous stroma determined a maximum PVI mean in the control group (SI/D) (91.72±9.5). The means of PVI in EM and SP

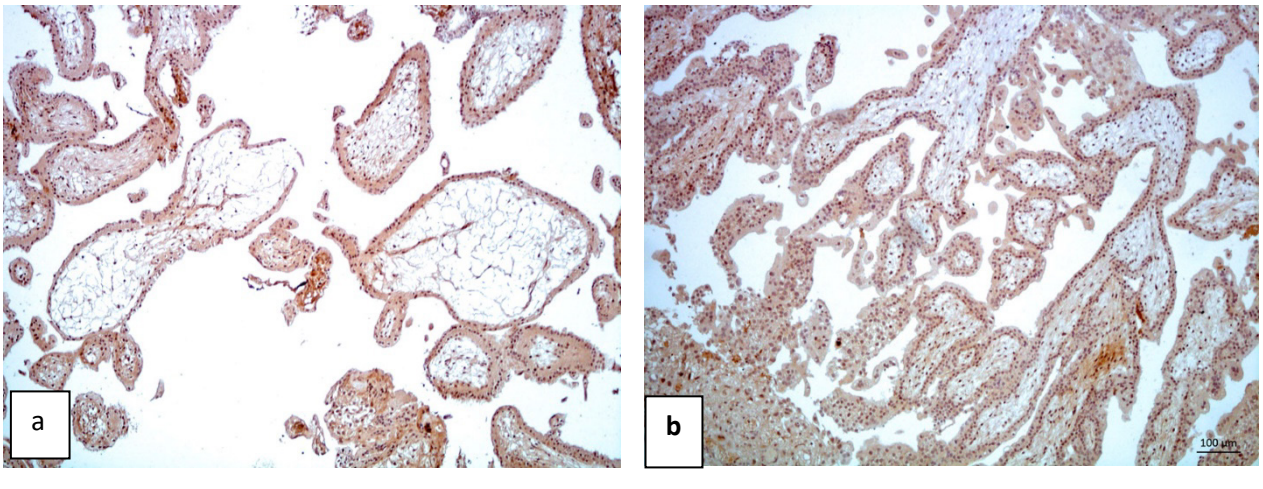

Fig. 1. Differential immunoexpression in the chorio-villous cell profile in early term (6 weeks) compromised pregnancies: a) SP and b) EM. Anti-Ang1 immunoreaction, DAB; x100

Table 4. Descriptive statistical analysis of placental vascular density

Vascular profile	SI/D batch (M±SD), GT			EM batch (M±SD), GT			SP batch (M±SD), GT		
	3-5	6-9	10-12	3-5	6-9	10-12	3-5	6-9	10-12
PVI%	-	92.48 ±7.76	90.97 ±11.25	100 ±0.00	68.1 ±11.39	78.58 ±23.71	70.69 ±29.21	52.64 ±26.83	56.50 ±36.44
PVI% total	-	91.72±9.5		82.22±17.55			59.94±30.82		
VD%	-	6.61 ±1.2	12.1 ±8.78	7.54 ±0.00	6.2 ±3.07	16.91 ±2.24	5.23 ±1.73	5.5 ±3.0	6.79 ±5.97
VD% total	-	9.36±4.99		10.21±2.65			5.84±3.56		

Note: M – mean; SD – std. Dev.; GT – gestational term, weeks; SI/D – abortion on social indications/ desire; EM – early miscarriage; SP – stagnant pregnancies; PVI – placental vascularization index; VD – chorio-villous vascular density.

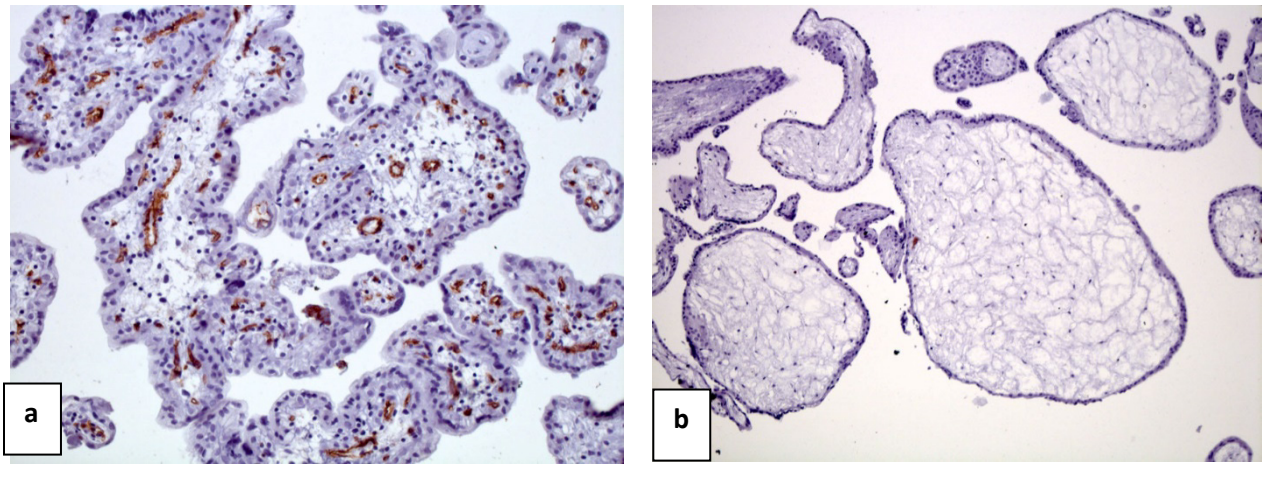

Fig. 2. High positive immunoexpression in stromal vessels of chorionic villi of early compromised pregnancies: a) SI/D (11 weeks) with high PVI and rich vascular profile vs b) SP (10 weeks) with low PVI with virtually avascular chorionic villi. Anti-CD31 immunoreaction, DAB; x100.

batches were 82.22±17.55 and 59.94±30.82, respectively. The highest chorio-villous vascular density was noticed in the SI/D and EM group (9.36±4.99 and 10.21±2.65, respectively). The lowest density was observed in case of SP (5.84±3.56) (tab. 4, fig. 2).

Next step was to analyze the VD and PVI differencies between batches , grouped by gestational term. The correlations can be seen in table 5.

The PVI was inversely dependent on chorionic villi stroma (rs=-0.40, p=0.03) in the 6-9 weeks group. However, same correlation was positive in the 10-12 weeks group (rs=0.76, p=0.01). The EM batch was characterized by a high overall PVI in the syncytiotrophoblast (rs=0.88, p=0.01) (tab. 6). There were no statistically significant correlations in the SI/D batch.

In the case of the statistical analysis of chorio-villous vascular density statistically significant positive correlations were established in the SP group with gestation term of 10-12 weeks, particularly in the vascular endothelium area, Hofbauer cell compartment, and stromal/mesenchymal site (rs=0.87, p=0.002; rs=0.73, p=0.02 and rs=0.65, p=0.04, respectively) (tab. 7). The group of uterine pregnancies solved on social indications/ desire and in early miscarriages showed no statistically significant intragroup correlations.

Table 5. Mann-Whitney U test results, intergroup analysis

GT, weeks	Batch	Correlations obtained for the VD	Correlations obtained for PVI
6-9	SI/D vs EM	p=0.855	**p=0.011**
	SI/D vs SP	**p= 0.021**	**p=0.001**
	EM vs SP	p=0.453	p=0.294
10-12	SI/D vs EM	p=0.086	p=0.283
	SI/D vs SP	p=0.183	**p=0.006**
	EM vs SP	p=0.117	p=0.296
3-5	EM vs SP	p=0.317	p=0.207

Note: GT – gestational term, weeks; SI/D – abortion on social indications/ desire; EM – early miscarriage; SP – stagnant pregnancies; PVI – placental vascularization index; VD – chorio-villous vascular density. Statistically significant correlations were marked as **bold**.

Table 6. Statistically significant correlations of PVI in different areas in the study groups

Batch	GT, weeks	Area studied	r_s	p
SP	6-9	CV-stroma/ mesenchyme	-0.40	0.03
	10-12	CV-stroma/ mesenchyme	0.76	0.01
EM	total	CV-syn-tropho	0.88	0.01

Note: GT – gestational term, CV-stroma – chorionic villi stroma, CV-syn-tropho – chorionic villi syncytiotrophoblast, SP – stagnant pregnancies, EM – early miscarriage, rs – Spearman's correlation coefficient. The results were considered statistically significant in case of $p<0.05$.

Table 7. Statistically significant correlations between choriovascular density and different areas in the study groups

Batch	GT, weeks	Area studied	r_s	p
SP	10-12	CV-vasc-endot	0.87	0.002
		CV-hofbauer	0.73	0.02
		CV-stroma/ mezenchim	0.65	0.04

Note: GT – gestational term, SP – stagnant pregnancies, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma, , rs – Spearman's correlation coeficient. The results were considered statistically significant in case of $p<0.05$.

Discussion

Formation of a functional maternal-fetal system is essential for the progressive functioning of the placenta during placentation with the establishment of maternal-fetal vascular circulation and decrease of pregnancy complications. An important morphological indicator of placental functional status, reflecting the expression of metabolic processes between mother and fetus, is the vascularization of chorionic villi [1]. The development of the functional placental vascular network requires the continuous formation of new blood vessels involving two successive mechanisms: vasculogenesis (formation of vessels from endothelial progenitor cells) and angiogenesis (formation of vessels from pre-existing vessels) [2]. According to the study by Regnault T. R. et al. (2002), defective angiogenesis is often the cause of intrauterine developmental restriction [3] as a result of vascular disturbances [4]. Placental insufficiency related to defective angiogenesis is frequently the cause of severe complications (IUGR, PE, etc.), including placenta accreta [13]. The physiological development of the placental vascular network takes place under the action of various angiogenic factors, the angiopoietin family being one of the most important.

Angiopoietins are a family of extracellular ligands, integral proteins involved in angiogenesis and vascular remodelling. According to the molecular profile, 4 ligands have been elucidated (Ang1, Ang2, Ang3, Ang4), the first 2 and their tyrosine kinase-like receptors having significance in placental vascular morphogenesis (TIE-1 and TIE-2) [5].

Ang1 is a protein made of 498 aminoacids and is having a NH2-terminal coiled domain and a COOH-terminal fibrinogen domain [14], which *in vivo* promotes angiogenesis [15], and *in vitro* is a chemotactic factor for human endothelial cells, not having a cell proliferation effect [14, 16]. Ang1 acts as a paracrine agonist of the Tie2 receptor by phosphorylating and dimerizing the receptor with activation of signaling pathways including the phosphoinositide-3-(PI3)-kinase/Akt pathway and extracellular signal-regulated kinase (ERK) [17]. In this context, angiopoietin-1 represents endogenous ligands of the vascular endothelial-specific tyrosine kinase TIE-2 receptor. It has endothelial and non-endothelial effects and is expressed in the early period of placentation [7].

The Ang1 immunoexpression can be noticed quite early in placentogenesis (week 4) with localization only in syncytiotrophoblast. It is also poorly expressed in the endothelium of blood vessels in immature intermediate chorionic villi, which explains its involvement in vascular promotion and stabilization [7].

The ANG1/ TIE2 signaling pathway promotes endothelial cell survival, endothelial integrity through recruitment and interaction with periendothelial cells, anti-inflammatory/antiapoptotic responses in case of reduced vascular permeability [6, 15] which attributes to it a role in vascular bed formation and stabilization.

In this context, the Ang1 immunoexpression in the germinative site was assessed by immunohistochemical investigation, and the angiogenic profile was described by location and intensity (tab. 2, 3, 4). As a result, the immunoexpression was different both by cellular profile and in relation to the type of early compromised pregnancies, confirmed by statistically significant differences.

Were found some statistically significant correlations between the stromal/mesenchymal cell site and the placental vascularization index (negative in the 6-9 weeks group and positive in the 10-12 weeks group) in the stagnant pregnancies batch. These correlations confirmed the differential angiogenic effect in the formation of the vascular network in the chorionic villous stroma. Thus, differential immunoexpression within groups brings the idea of Ang1 involvement in vascular formation and stabilization during the early period of placentation. This is the case of stagnant pregnancies, in which vascular disruptions are more pronounced and the vascular density is reduced (tab. 4). In order to confirm the given hypothesis, was analyzed the immunoexpression in the research sites vs chorio-villous vascular density. Statistically significant correlations were found in the SP group, in the 10-12 weeks group. The involvement of stromal/mesenchymal cells and Hofbauer cells was noticed in the formation of angiogenic medium. This could be the result of a paracrine effect on the endothelium of blood vessels (tab. 6). The results obtained are in agreement with the authors' data supporting differential cellular immunoexpression of Ang-1 in the placental parenchyma, including the diversity

of the degree of expression, ranging from absent to high degree [7, 18-20].

Conclusions

The placental period is characterized by a weak angiogenic Ang1 differentiated cellular environment in the chorio-villous germinal site in the group of short term compromised pregnancies. The selectively immunoexpressed cellular profile statistically significantly correlates with placental vascular index and chorio-villous vascular density in stagnant pregnancies.

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

Funding. This work was carried out within the framework of the state project "Morphological approach by conventional, histo- and immunohistochemical methods of the peculiarities of the pathological profile of early placentogenesis in early-term compromised pregnancies", Project code 20.80009.8007.17 P1P2 0750.

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

Conflict of interests. No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.09
UDC: 616.131-005.7-08

Optional therapeutic management of intermediate-risk pulmonary embolism patients

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Manuscript received November 17, 2022; revised manuscript December 11, 2023; published online December 27, 2023

Abstract

Background: Few studies have evaluated the thrombolytic treatment in patients with intermediate-high risk pulmonary embolism, making this study more valuable.
Material and methods: It was a prospective, non-randomized, open-label, single-center study. Eligible patients at the age of 18 or older with an acute pulmonary embolism (PE) confirmed by CT pulmonary angiography with onset until 14 day and signs of right ventricular (RV) overload on echocardiography took part in the study. Pulmonary Arterial CT Obstruction Index Rate (PACTOIR) was used to define the localization and the expansion zone of thromboembolism. This study included 18 patients with intermediate risk and acute submassive pulmonary thromboembolism. In thrombolysis (TT) group (n=9) were used 50 mg of tissue-plasminogen activator (t-PA) administered in infusion as 0.4 mg/h for 2 hours. In the standard anticoagulation group, unfractioned heparin (UFH) was administered as a bolus of 70 units/kg or a maximum of 5000 units, followed by continuous infusion at an initial rate of 16 units/kg or a maximum of 1000 units/h.
Results: The mean age for TT group was 69 vs 63 for the UFH group. PACTOIR was 100% in 3 patients in the half-dose rt-PA group and in 2 patients in the UFH group. RV/LV diameter ratio decreased from baseline to 48 h post-procedure (1.55 vs. 1.13; mean difference, -0.42; $p < 0.0001$). Mean pulmonary artery systolic pressure was 55 mm Hg in both groups ($p < 0.05$), with 53 [43–60] in TT group vs. 41.5 [37–45] mmHg in UFH group, $P<0.05$. Also, RV/LV ratio and systolic PAP decreased significantly in both groups. Severe bleeding with a need in red blood cell transfusion was seen in 0.11% (1 patient) in the TT group vs 0 in UFH group. The hospitalization length of stay was significantly shorter in the TT group (3.8±1.8, $p < 0.05$). The rate of secondary endpoints was significantly higher in the UFH group with a high rate of pulmonary hypertension (0 vs. 19%, p=0.003).
Conclusions: Half-dose thrombolytic therapy in patients diagnosed with submassive pulmonary embolism significantly reduced death and hemodynamic decompensation in the first 7 days compared to anticoagulant therapy only. With all that being said, it can be concluded that patients with high-intermediate risk PE could benefit from reduced-dose TT.
Key words: pulmonary embolism, intermediate risk, submassive pulmonary embolism.

Cite this article
Diaconu N, Civirjic I, Sorici G, Grosu A, Cuzor T, Racila-Iatco S. Optional therapeutic management of intermediate-risk pulmonary embolism patients. Mold Med J. 2023;66(2):56-65. https://doi.org/10.52418/moldovan-med-j.66-1.23.09.

Introduction

Acute pulmonary embolism (PE) is a life-threatening disease that usually is a serious complication of venous thromboembolism [1]. Life risk stratification in PE (high risk, intermediate risk, and low risk) is based on clinical presentation and on markers of myocardial dysfunction and/or injury.

In hospitalized patients there is significant mortality rate, with 3-8 % in intermediate risk (a submassive PE) and 25-52% in patients with high-risk pulmonary embolism (massive PE) [2-4]. Patients who survive PE, after hospital discharge, they may develop chronic thromboembolic pulmonary hypertension with functional impairment [5-7].

Thrombolytic therapy is an established treatment option for patients with high-risk pulmonary embolism [8, 9]. However, the use of thrombolytic therapy in patients with intermediate-risk pulmonary embolism is controversial because of the lack of sufficient information about its benefits and risks [2, 9-13]. This group of patients requires special interest because the in-hospital mortality can reach up to 30% [14, 15].

In the PEITHO trial was observed an increased incidence of hemorrhagic stroke and major non-intracranial bleeding, in the thrombolytic group [16]. The aim of this trial was to determine the controversial issue in intermediate-risk pulmonary embolism. Unfortunately, the question of the applicability of thrombolysis in submassive PE was not established.

Most of the evidence supporting the use of thrombolytic therapy for intermediate-risk pulmonary embolism

focuses on minimizing the treatment and helping to return at normal functional status with reduced risk of residual pulmonary hypertension. Three-year follow-up data from the PEITHO study showed no long-term mortality benefit or difference in the incidence of chronic thromboembolic pulmonary hypertension (CTEPH) in either group [2, 5, 10, 11]. The clinical benefits of thrombolytic therapy are less significant than the one of high-risk PE, as 2% and 6% risk of cerebral hemorrhage and major bleeding respectively is higher than in another group [10]. Knowing that the pulmonary vasculature receives the majority of any intravenous thrombolytic dose, it has been suggested that lower-dose thrombolytics may provide sufficient efficacy while reducing the risk of major bleeding [5, 17-20]. This hypothesis is supported by recent evidence suggesting that the use of half the usual dose of alteplase in pulmonary embolism may be effective, with a reduced risk of bleeding [5, 18, 19]. In order to study the usefulness of thrombolysis in PE with high intermediate risk (submassive), was compared treatment approach with half-dose thrombolysis and monotherapy with unfractionated heparin (UFH).

The aim of the study is to evaluate the efficacy and safety of half-dose alteplase versus UFH alone (standard therapy) for the treatment of intermediate-risk acute PE.

Material and methods

Study design

It was a prospective, non-randomized, open-label, single-center study conducted between January 2020 and December 2022. The study included 18 patients diagnosed with submassive acute pulmonary thromboembolism (with intermediate thromboembolic risk). The patients included in the thrombolysis group received half-dose of tissue plasminogen activator (rt-PA) – alteplase. All patients in this group presented a written consent for this procedure. The standard therapy group included patients who refused thrombolysis with acute PE at intermediate risk with similar clinical profile. They received unfractionated heparin according to the scheme. Finally, 9 patients were taken into the half-dose rt-PA group and 9 patients into the UFH group.

Studied population

Inclusion criteria:

– Age 18 or older.

– Acute PE confirmed by pulmonary CT angiography (CTPA) with early onset (maximum 14 days old PE)

– Signs of RV overload: Confirmed pulmonary hypertension (pressure in the pulmonary artery above 40 mm Hg) on echocardiography (ECHOCG) and/or expansion of the right ventricular cavity (end-diastolic diameter of the right ventricle > 30 mm); deviation of the interventricular septum; hypokinesis of the right ventricular free wall; LV/RV ratio ≥ 0.9 mm on CTPA or ECHOCG.

Exclusion criteria:

– Hypotension (Systolic blood pressure <90 mm Hg) or shock.

– Known risk of bleeding.

– Ischemic stroke <3 months.

– The presence of uncontrolled hypertension (Systolic >180 mm Hg and/or diastolic >110 mm Hg).

– Known hypersensitivity to thrombolytic therapy or UFH (including previous HIT).

– Known coagulopathy and vitamin K antagonist treatment, ACOD or platelet count below 100000/mm^3.

– Recent major surgery (<1 week).

Treatment scheme

For the half-dose thrombolysis group: 50 mg of rt-PA was infused for 2 hours, checking the activated partial thromboplastin time (APTT) immediately and after 4 hours. When APTT became less than twice the control value, the UFH therapy was initiated during at least next 24 hours.

In the standard anticoagulation group, UFH was administered as a bolus of 70 units/kg or a maximum of 5000 units, followed by continuous infusion at an initial rate of 16 units/kg or a maximum of 1000 units/h. Every 6 hours the APTT was evaluated, adjusting the dose based on the result received (the target being 60 to 70 s). In rare situations, when the APTT became <50 s, an intermittent mini-bolus of UFH (16 units/kg or maximum 1000 units) of UFH was associated along with a dose adjustment (2 units/kg/h infusion). In case of a longer APTT, more than 80 s, UFH was stopped for 30 to 60 minutes and the dose was adjusted.

All patients continued treatment with warfarin and enoxaparin simultaneously, as standard coagulation scheme requires. When target INR was achieved (>2.0) and was maintained for at least 24 hours, the UFH or enoxaparin was suspended. The treatment with warfarin or DOAC was continued for at least 3 months.

Efficacy

The treatment efficacy was the primary outcome assessed in this study. It is defined as the lack of need in vasopressors, secondary thrombolysis, assisted ventilation, or cardiopulmonary resuscitation, occurring within the first 24 hours after administration of alteplase [3]. Secondary efficacy outcomes included in-hospital mortality, readmission for pulmonary embolism, ICU length of stay, length of hospital stay, and total hospital costs. Safety outcomes were considered major bleeding: a documented cerebral hemorrhage, gastrointestinal bleeding, acute anemia with blood loss, including the need to transfuse red blood cell mass, or aminocaproic acid/ tranexamic acid after the alteplase infusing.

Hemodynamic decompensation (or collapse) was defined as sustained hypotension (i. e., Systolic blood pressure <90 mmHg) that involves the need of initiation of vasopressors/inotropes within 48 hours of ICU admission. Vasopressors/inotropes included norepinephrine, phenylephrine, vasopressin, epinephrine, dopamine, dobutamine and milrinone, including drop in Systolic blood pressure of at least 40 mm Hg in 15 min with signs of end-organ hypoperfusion (cold extremities or low urine output <30 ml /hour) or mental confusion.

In this prospective study were evaluated patients' sociodemographic characteristics, such as age, sex, symptoms, risk factors, vital signs, electrocardiography results and blood gas values, D-dimer and cardiac biomarker (troponin and NT proBNP). The cut-off value for D-dimer was taken as 500 μg/L for those under 50 years age and × 10 μg/L for those over 50 years [21]. The cut-off value for troponin was 0.14 μg/mL [22].

The pretreatment risk assessment test as pulmonary embolism severity index was used to determine the intermediate-low and intermediate-high risk groups. The HASBLED score calculated patients' bleeding risk (hypertension, abnormal kidney or liver function, stroke, bleeding, labile international normalized ratio, age >85 years, using drugs and/or alcohol): 0 = low risk, 1 to 2 = moderate risk, >2 = high risk [23].

As diagnostic tools were used the chest radiography, perfusion scintigraphy, compression ultrasonography of the lower extremities, echocardiography and CTPA. The gold standard – CTPA showed partial or complete vessel occlusion by locating the filling defect in the lobar and segmental branches of the right and left main pulmonary arteries. Pulmonary Arterial Obstruction CT Index (PACTOI) and Pulmonary Arterial Obstruction CT Index Rate (PACTOIR) were calculated using these data [24]. After studying related articles, PACTOI and PACTOIR were calculated:

$$\mathbf{PACTOI=n\times d}$$

$$\mathbf{PACTOIR=PACTOI\times 100/maximum\ total\ score}$$

where **n** is the number of segmental branches in the distal field (1–20) and **d** is the degree of obstruction: 0 (none), 1 (partial), and 2 (complete).

Transthoracic echocardiography (TTE) was performed within the first 2 hours of hospital admission, before t-PA administration, and was repeated at 12–24 hours. The pressure in AD was assessed and classified as 10, 15, and 18 mmHg for mild, moderate, and severe right atrial enlargement, respectively. Systolic pulmonary artery pressure (SPAP) was calculated using the velocity of the tricuspid valve regurgitation jet according to the modified Bernoulli equation [25, 26].

In the simplified Bernoulli equation for measuring pulmonary artery Systolic pressure (PASP), tricuspid regurgitation jet flow and right atrial pressure were used, with systolic PAP calculated using the following formula:

$$\mathbf{PASP = [4\times(tricuspid\ regurgitation\ jet)^2] + AD\ pressure}$$

An M-mode cursor was placed over the lateral tricuspid annulus to measure the longitudinal motion of the annulus at peak systole in the typical apical 4-chamber view. This information was used to determine the systolic excursion of the tricuspid annular plane. The Simpson method was used to determine the left ventricular ejection fraction [27]. A cardiologist not involved in the treatment scheme interpreted the echocardiographic results. Pulmonary hypertension was defined as systolic PAP with a value superior of 40 mmHg. A RV-to-left ventricular (RV/LV) ratio > 0.9 was considered to indicate RV hypertrophy [25-26]. A retrospective analysis was done on the recorded images taken at the time of diagnosis and after TT or heparin. Methods for measuring the RV/LV ratio were developed according to previous definitions [28-30].

Control CTPA and echocardiography were performed to examine residual and/or chronic organized thrombus in the pulmonary arteries and pulmonary hypertension at 6 months.

Follow-up

After discharge, patients were contacted and underwent reexaminations at 3 months. At recall patients underwent ETT and CT investigations to assess systolic PAP, RV size and functional capacity. The follow-up was made retrospectively by review of medical records or by phone call (confirmation of medical record events) and an assessment of any clinical event.

Statistics

The data obtained from the study were analyzed in the SPSS V.15.0 program. While data were evaluated, continuous variables were expressed as mean ± SD, median, and lowest–highest values, and census data were expressed as numbers and percentages. In statistical analysis, the conformity of continuous variables to normal distribution was assessed with the "Kolmogorov-Smirnov Test". When the continuous variables obtained in the study did not follow the normal distribution, they were given the highest and lowest values with the median values, instead of the mean and SD. The Mann-Whitney U test was used in groups that did not follow normal distribution in comparing of arithmetic means of continuous variables. The Pearson $\chi 2$ test was used to compare categorical data. A p-value of 0.05 was accepted as the limit of statistical significance.

Results

The study included eighteen patients. The mean age of half-dose thrombolysis group was 69 years, which was comparable to the age of those in the UFH group, which was 63 years. Five of them (55%) were females and 4 (45%) were males. Patients over 75 years of age constituted 33% (n=6) of all patients. Chronic lung disease was present in 2 (11%) patients and chronic heart disease in 4 (22%). Patients with a body mass index over 30 constituted 33% of all. Four patients (22%) were smokers.

Dyspnea was observed in almost all patients (94.5%), while the second most common symptom was chest pain, which was present in 55% of patients in the half-dose thrombolytic group and 67% in the UFH. A smaller proportion of patients experienced tachypnea (37.3%), syncope (12%) and chest pain (9.6%). Although 14.4% of patients were on antiplatelet therapy, the HASBLED score was 1 in 53% of patients. No patient had a score greater than 2. Arterial pressure was decreased in 2 (11%) patients without any hemodynamic decompensation. The rate of hypotension was significantly higher in the TT group than in the UFH (4 (44%) vs. 2 (22%), p=0.07). Symptoms presented in two groups had a similar range (tab. 1).

Table 1. Clinical characteristics of studied patients in both groups

	TT (n=9)	UFH (n=9)	P value
Age	69±14	63±16	0.085
Gender, n (%)			0.107
Masculin	5 (55)	3 (33)	
Feminin	4 (45)	6 (67)	
SBP (mm Hg)	126±23	124±17	0.9
DBP (mm Hg)	77±13	80±11	0.758
Breathing (per minute)	26±2.9	24±3.2	0.6
Oxygen level in breathing air	88±4.8	91±4.8	0.19
Pa O_2 (mm Hg)	62±5.8	70±6.2	0.32
Troponins (g/mL)	2.60±0.4	2.48±0.4	0.685
NT – proBNP	986±120	783±230	0.78
Risk factors, n (%)			
Imobilisation	3 (33)	2 (22)	0.338
Malignancy	2 (22)	1 (11)	0.560
Previous DVT	2 (22)	1 (11)	0.564
Previous PE	1 (11)	1 (11)	1.000
BMI (kg/m^2)	28 (25.73–32.0)	26.95 (25.32–29.9)	0.365
Obesity (BMI >30 kg/m^2)	2 (22)	1 (11)	0.329
Arterial hypertention	6 (66)	5 (55)	0.518
Diabetus mellitus	2 (22.0)	3 (33.3)	0.247
COPD	2 (22)	0	0.335
Surgical procedure (in the last 45 days)	1 (11)	2 (22)	0.787
Trauma (in the last 45 days)	1 (11)	0 (0)	0.358
Clinical presentation, n (%)			
Dyspnea	8 (89)	9 (100)	0.909
Thoracic pain	5 (55)	6 (67)	0.476
Cough	3 (33)	4 (44)	0.480
Hemoptysis	3 (33)	3 (33)	1.000
Syncope	1 (11.1)	0 (0)	0.9
Onset (days)	3.6	4.86	0.09
HASBLED score			
1	5 (56)	4 (44.8)	0.746
2	4 (44)	5 (55.2)	0.588
PESI score	112 (108–121)	111.5 (105–120)	0.565
PESI class			
3	3 (33.3)	5 (55.5)	0.488
4	6 (66.6)	4 (44.4)	

Note: BMI, body mass index; DBP, diastolic blood pressure; DVT, deep vein thrombosis; PaO2, partial pressure of arterial oxygen; rt-PA, tissue-type plasminogen activator; SBP, Systolic blood pressure; NT – proBNP, N-terminal pro–B-type natriuretic peptide; PE, pulmonary embolism; PESI, pulmonary embolism severity index; COPD, chronic obstructive pulmonary disease. There was no statistical difference between the two groups in ECG and chest X-ray results.

Immobilization, trauma, hospitalization, malignancy and known history of VTE (DVT and/or PE) were predisposing factors in the studied groups (tab. 1). The influence of hormonal therapy, oral contraceptive drugs, hormone replacement treatments during menopause and hormonal drugs used for malignant diseases were evaluated. Only one person had VTE during hormonal therapy in breast cancer.

In this study, pulmonary CT angiography (CTPA) and echocardiography (ECHOCG) were used as the main diagnostic method. However, ECHOCG and perfusion scintigraphy were used to diagnose a high probability PE in 3 patients in which a high level of creatinine was found. The PACTOIR index on CTPA was 75% in both groups. PACTOIR was 100% in 3 patients in the half-dose rt-PA group and in 2 patients in the UFH group. The same index was not significantly different between the two groups (p=0.505). The mean RV/LV ratio on CTPA was 1.2 in both groups. As the location of the embolic thrombus, the main pulmonary artery was more frequently found in the TT group, although there was no statistically significant (44.4 vs. 11.1%, p=0.095). The CTPA findings are summarized in tab. 2, and the CTPA images of 3 cases are shown in figures 1 and 2.

Echocardiography revealed ventricular septal displacement in 15 patients (8 patients (88%) in the half-dose rt-PA group and 7 patients (77%) in the UFH group. Pulmonary arterial pressure had a mean of 55 mm Hg in both groups. Right ventricular enlargement or RV hypokinesis was detected in all patients in the half-dose rt-PA group (100%) and in 8 (88%) patients in the UFH group. There was no significant difference in echocardiographic parameters in both groups except systolic PAP. When the TT and UFH groups were compared in terms of systolic PAP, the TT group had a higher prevalence of elevated PAP (53 [43–60] vs. 41.5 [37–45] mmHg, P<0.05). Imaging data are summarized in tab. 2.

Table 2. Results of imaging investigations (CTPA and ECHOCG) in 18 patients with submassive PE (intermediate risk)

	TT n=9 (%)	UFH n=9 (%)	P valoare
PACTOIR	73 (12.5–100)	75 (25–100)	0.505
RV/LV	1.2 (1.0–1.7)	1.2 (0.9–2.0)	0.888
Pleural effusion	2 (25)	1 (14.2)	0.176
Hampton's sign	2(25)	1 (14.2)	0.127
Diffuse hypoperfusion	3 (37.5)	2 (28.4)	0.491
Embolus localization			
Bilateral	6 (75)	6 (85.7)	0.243
Bilateral basal	1 (12,5)	1 (14.3)	0.615
Main AP	1 (12.5)	0 (0)	0.95
ECHOCG features			
LVEF (%)	60 (58–60)		0.509
RV/LV	1.2 (1.0–1.2)	1.13 (1.0–1.2)	0.625
TAPSE (cm)	1.6 (1.5–1.7)	1.6 (1.5–1.7)	0.585
Systolic PAP (mmHg)	53 (43–60)	41.5 (37–45)	<0.05

Note: UFH, unfractioned heparin; PACTOIR, pulmonary Arterial Obstruction CT Index Rate;
CTPA, computed tomography pulmonary angiogram; LV, left ventricle; RV, right ventricle; Systolic PAP, pulmonary artery systolic pressure; TT, thrombolytic therapy; TAPSE, tricuspid annular plane systolic excursion.

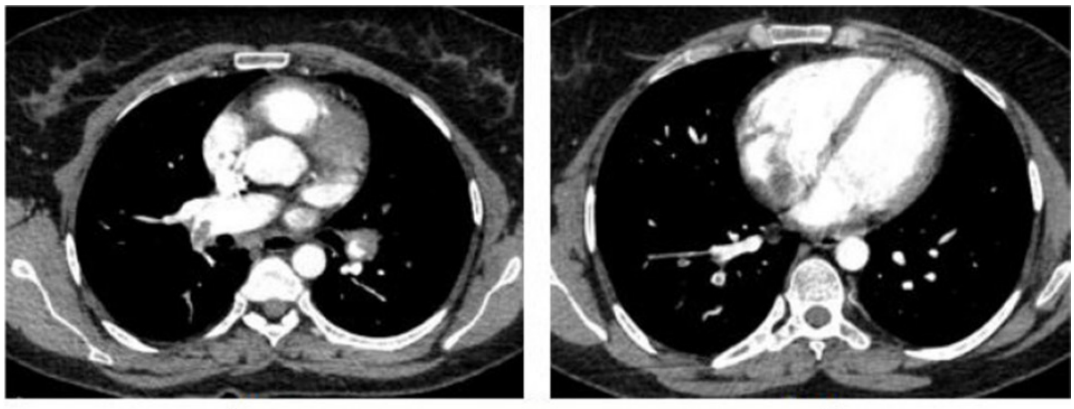

Fig. 1. This is a patient in the UFH group who was immobilized due to surgery and had bilateral total pulmonary artery occlusion (A) with a right-left ventricular ratio of 1.07 (B) on pulmonary CT angiography.
Pulse rate, respiratory rate, blood pressure, sPESI, and PASP on echocardiography at presentation were 121 per minute, 24 per minute, 120/70 mm Hg, 2, and 65 mm Hg, respectively. Half rt-PA escalation dose was used due to hemodynamic decompensation and severe dyspnea. He had mild dyspnea at 6 months with a PASP of 55 mmHg on echocardiography. UFH, unfractioned heparine; CT, computed tomography; sPESI, simplified pulmonary embolism severity index; PASP, pulmonary artery systolic pressure; rt-PA, tissue plasminogen activator.

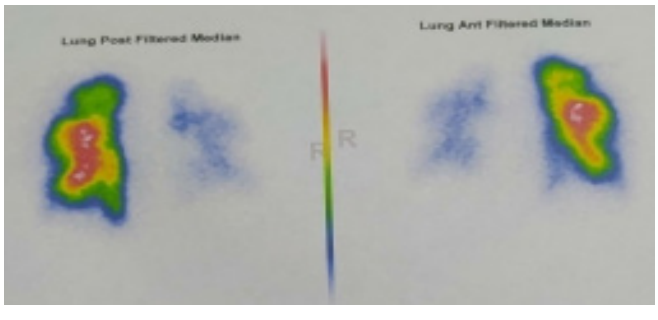

Fig. 2. Perfusion scintigraphy shows in a patient persistent filling defects of the pulmonary arterial bed

Efficacy and safety results

A summarized data of pre– and post-treatment echocardiographic and clinical parameters for both groups is specified in table 3. Oxygen saturation in arterial blood and TAPSE increased in the post-TT period, while heart rate, RV/LV ratio and systolic PAP decreased significantly in both groups (tab. 3). Low-dose TT has been used successfully in all patients. The number of hypotension significantly decreased after TT (22 vs. 0%, P<0.5), but did not decrease after UFH (2.4 vs. 7.1%, p=0.625).

The rates of primary and secondary outcomes in the first 3 months are shown in tab. 4. The proportion of primary outcomes at 3 months was not different between the two groups (2.4 vs. 11.9%, p=0.106). However, hemodynamic decompensation was significantly lower in the TT group (11.1 vs. 33.3%, p=0.05). The rate of secondary endpoints was significantly higher in the UFH group (Table 4). The prevalence of pulmonary hypertension was significantly higher in the UFH group (0 vs. 19%, p=0.003). Although all-cause mortality (0 vs. 11.1%, p=0.253) and recurrent PE (0 vs. 11.1%, p=0.253) were not significantly different, they were numerically higher in the group with UFH at 3-month follow-up.

Table 3. Comparison of pre– and post-treatment echocardiographic and clinical parameters according to treatment strategy

	TT group			UFH group		
	Initial level	At 3 months	p	Initial level	At 3 months	p
HR (beats/min)	111 (109–118)	80 (77.5–86)	<0.05	110.5 (109–117)	80 (75–82.5)	<0.05
O_2 saturation	87 (86–88)	96 (95–96)	<0.05	87 (85.75–88)	94 (92–95)	<0.05
High respiratory rate, n (%)	4 (44.4)	0	<0.01	3 (33.3)	1 (11.1)	0.013
Hypotension, n (%)	2(22.0)	0	<0.05	1 (11.1)	1 (11.1)	0.825
RV/LV	1.2 (1.0–1.2)	0.66 (0.63–0.70)	<0.05	1.1 (1.0–1.2)	0.71 (0.65–0.84)	<0.05
Systolic PAP (mmHg)	53 (43–60)	24 (23–25)	<0.05	41.5 (37–45)	32 (29.5–37.25)	<0.05
TAPSE (mm)	16 (15–17)	24 (21.5–25)	<0.05	16 (15–17.1)	20.5 (19–24)	<0.05

Note: HR, heart rate; LV, left ventricle; RV, right ventricle; systolic PAP, pulmonary artery systolic pressure; TAPSE, tricuspid annular plane systolic excursion; TT, thrombolytic therapy; UFH, unfractionated heparin.

Table 4. Three-month clinical outcomes in both groups

Parameters	TT group (n=9), n (%)	UFH group (n=9), n (%)	p
Primary end-point	1 (11.1)	3 (33.3)	0.06
All cause mortality	0	0	1.00
Hemodynamic decompensation	1	3 (33.3)	0.059
Severe blood loss	1 (11,1)	0	0.54
Secondary end-point	0	1 (11.1)	0.16
PE recurrence	0	1 (11.1)	0.553
Pulmonary hypertension (Systolic PAP ≥40 mmHg at ECHOCG)	1(11,1)	4 (44.4)	0.39
Moderate blood loss	1 (11,1)	1(11.1)	0.494
Minor blood loss	3 (33.3)	2 (22.2)	0.615
Mean hospital lenght of stay in IT department (days)	3.8±1.8	5.2±1.2	0,05
Total lenght of stay (days)	7.4±2.1	8.8±2.9	0.05
Hospitalization cost	13237±2341	13421±1673	0.06

Note: TT, thrombolytic therapy; UFH, unfractionated hepatin; PE, pulmonary embolism; PAP, pulmonary artery pressure; ICU, intensive care unit; ECHOCG, echocardioglaphy

There was 1 patient with relatively severe bleeding in the TT group (massive subcutaneous ecchymosis at the jugular puncture site that was associated with a decrease in hemoglobin by 40 units and required the transfusion of 1 bag of erythrocyte mass) (tab. 4). Moderate hemorrhages – persistent epistaxis and menorrhagia were presented in 1 patient from both research groups; menor hemorrahges as slight ecchymoses and gingival hemorrhages, episodic epistaxis, profuse mensis, hemorrhoidal bleeding were presented in 3 patients from the TT group and 2 patients from the UFH group.

The hospitalization length of stay was significantly shorter in the TT group both in the ICU and in the recovery department. The costs presented a different structure, without significant changes in both groups. In the TT group it was lower due to the reduction in the duration of hospitalization, but at least without significant difference because of increased cost of thrombolysis agent and laboratory tests perceived more frequently.

There was no in-hospital mortality from any cause or serious hemorrhagic complications requiring additional medical implications in both research arms.

To conclude, half-dose thrombolytic therapy in patients diagnosed with submassive PE (intermediate risk) significantly reduced death and hemodynamic decompensation in the first 7 days compared to anticoagulant therapy only. There was no significant difference in both treatment groups regarding bleeding complications and none of the patients had serious hemorrhagic complications. The obtained results show the benefit of the use of low-dose rt-PA in cases of normotensive PE with right ventricular dysfunction diagnosed on echocardiography and/or high right-to-left ventricular ratio on CTPA, especially in cases with PACTOIR and/or high PASP on echocardiography associated with biomarkers (elevated troponin and/or NT-proBNP). Thus, patients with high-intermediate risk PE could benefit from reduced-dose TT with higher minor bleeding complications compared to general low-intermediate-risk group.

Importance and practical meaning of the research

What is already known about this topic?

– Patients in the intermediate-risk group are more controversial for thrombolytic therapy.

– Although thrombolytic drugs have been used as a life-saving agent in massive pulmonary embolism in high-risk group with persistent hypotension, they have not been used in the non-massive (low-risk) group due to the high frequency of fatal intracranial hemorrhage.

– In the PEITHO trial, was observed a higher incidence of stroke and major non-intracranial bleeding in the thrombolytic group, with controversial issue for intermediate-risk pulmonary embolism.

– The use of thrombolysis in PE with intermediate risk was not sufficiently studied in the PEITHO trial.

What are the new findings?

– The main objectives in the treatment of patients with pulmonary thromboembolism are the prevention of mortality without causing bleeding in the acute situation, the prevention of recurrence and the development of pulmonary hypertension as long-term outcomes.

– The present study aimed to answer the same question (clinical use and applicability of reduced doses thrombolytics in PE patients with intermediate risk), comparing half-dose thrombolytics with standard anticoagulation.

– This study revealed that half-dose tissue plasminogen activator (rt-PA) prevents death or hemodynamic decompensation in the first 7 days in patients with submassive PE without increasing major bleeding complications.

How might these results change the course of further research or clinical practice (practical applicability)?

– Pulmonary embolism at intermediate risk treated by half-dose thrombolytic therapy reduced patient's death or hemodynamic decompensation in the first 7 days compared with anticoagulant therapy only.

– There was no significant difference in both treatment groups regarding bleeding complications and none of the patients had intracranial bleeding.

These results support the use of low-dose rt-PA in cases of normotensive PE with right ventricular dysfunction and/or a high right ventricular to left ventricular ratio on pulmonary CT angiogram. Moreover, it showed the benefit for patients with a high CT index for pulmonary artery obstruction and/or very high pulmonary artery Systolic pressure on echocardiography, in association with markers of myocardial injury and positive cardiac overload.

Discussion

This research revealed the benefit of half-dose rt-PA that supposingly prevents death/hemodynamic decompensation within the first 7 days in intermediate-risk PE patients without increasing the rate of bleeding complications. The given this study is one of the few prospective

studies that have compared half-dose rt-PA and unfractioned heparin [31]. In this study, the two therapies had similar effects on PE recurrence and development of pulmonary hypertension at 3 months. The main objectives in the treatment of patients with pulmonary thromboembolism are the prevention of mortality without causing bleeding in the acute situation, the prevention of recurrence and the development of pulmonary hypertension as long-term outcomes. This study met the first objective, with no deaths in either group. Moreover, both therapies also showed similar effects on thromboembolic event recurrence and development of pulmonary hypertension, compared with previous studies [32, 33].

Efficacy of thrombolytic therapy

Although the efficacy of thrombolytics is indisputable in massive (high life-risk) PE, the management of intermediate-risk PE remains controversial. As the risk of mortality being very high due to acute hemodynamic deterioration in massive pulmonary thromboembolism, clinicians need to make decisions based only on their clinical judgment. A trial comparing thrombolytic therapy with heparin in patients with massive PE was stopped early because all patients in the heparin group died [34]. Based on the evidence, thrombolytic therapy has been accepted as standard treatment in this specific group [14, 29]. Thrombolytic therapy leads to a more rapid improvement in the pulmonary circulation, reducing pulmonary obstruction, pulmonary arterial pressure, pulmonary vascular resistance, with reduction of right ventricular failure in patients with massive PE compared with unfractionated heparin (UFH) alone [34-37]. The earlier starts treatment, the greater is the benefit. However, thrombolytic therapy may still be used only in patients with the beginning of symptoms no more than 14 days before [38]. Normotensive patients with PE may be at increased risk of early death if they have right ventricular dysfunction or myocardial injury secondary to acute pressure overload [39, 40]. However, clinical guidelines do not recommend the routine use of thrombolytics in cases of intermediate-risk PE due to insufficient research data. Rescue thrombolytic therapy is, instead, recommended for patients with hemodynamic deterioration under anticoagulant treatment [14, 29].

There are some randomized clinical trials showing the efficacy of thrombolytic therapy in submassive PE, but with controversial results regarding bleeding, especially ICH. The Management Strategies and Prognosis of Pulmonary Embolism-3 (MAPPET-3) study investigated the use of heparin in addition to 100 mg rt-PA in 118 patients with 138 who received heparin plus placebo [23]. The primary end-point was met, with in-hospital mortality or clinical deterioration significantly higher in the heparin/placebo group (p=0.006) and the 30-day survival and event-free being higher in the rt-PA plus heparin group (p= 0.005). The bleeding rate was higher in rt-PA group, without any fatal bleeding or ICH. The randomized, double-blind, PEITHO9 study was a pivotal trial in acute PE comparing full-dose tenecteplase plus heparin with heparin plus placebo in 1006 intermediate-risk PE patients. The primary end-point (death from any cause, hemodynamic decompensation or collapse within 7 days of randomization) occurred in 13 of 506 (2.6%) patients in the thrombolytic group compared with 28 of 449 (5.6%) patients in the placebo group (p. =0.02). Intracranial hemorrhage occurred in 10 (2%) patients from the tenecteplase group and in only 1 (0.2%) patient from the placebo group. Similar to the current study, this one revealed that thrombolytic therapy prevents hemodynamic decompensation. Although, there was observed an increased risk of major hemorrhage and stroke, this data is different from the data of the given research. The PEITHO trial influenced guidelines' recommendations, in which it is not recommended to use as routine thrombolytic therapy for intermediate-risk PE cases, although there are some small trials that have shown no difference in bleeding complications between groups with full-dose thrombolytics and heparin alone [18, 41, 42].

High rates of ICH in the PEITHO trial led researchers to start new studies, lowering the usual dose of thrombolytics to achieve positive result with minor complications. Although guidelines recommended rt-PA 100 mg in 2-hour infusion in high-risk PE treatment, there aren't defined clearly optimal doses in specific situations. Even there is no strong evidence, in the "real world", many clinicians choose to use lower doses rt-PA, being cautious because of major bleeding. Lower doses of thrombolytics may be of particular interest in the elderly, body mass less than 65 kg and pregnant women, as well as those with relative contraindications [43]. The currently standard dose of rt-PA is based on experience and studies in cardiology [44]. However, if it is considered that the lungs receive the entire cardiac output compared to the coronary arteries which receive only a fraction of it, reducing the dose of thrombolytics in the treatment of PE could be a logical approach.

A randomized trial of 118 patients with high or intermediate-risk PE established that using half-dose rt-PA resulted in fewer bleeding complications than full-dose rt-PA. Moreover, it had similar effects on improving right ventricular diameter at echocardiography, as well as the reduction of perfusion defects and obstruction of the pulmonary arteries in CTPA [32].

Subsequently, a prospective, randomized trial on intermediate-risk pulmonary embolism treated by thrombolysis (MOPETT) compared low-dose rt-PA versus UFH or enoxaparin in 121 cases [31]. The all-cause mortality was similar in both groups and the progression of pulmonary hypertension was statistically decreased in the low-dose thrombolytic group. During the 12-month follow-up, pulmonary hypertension and recurrent PE developed in 1 (11%) patient in the low-dose thrombolytic group and in 3 (33%) in the heparin-only group. However, no major or minor bleeding reported in either group was specifically described, being an unusual fact in a study in which 61 patients received systemic thrombolysis.

Other study comparing half-dose (50 mg/2 h) and full-dose (100 mg/2 h) rt-PA treatment strategies in massive (high-risk) pulmonary thromboembolism, showed efficacy and hemodynamic stability in both groups, although in

the low-dose group had been fewer bleeding complications in patients with body mass less than 65 kg [32]. Recurrent pulmonary thromboembolism was described in none of the studied groups. Therefore, according to this study, when the half-dose was compared with the full-dose, in pulmonary thromboembolism, the similar efficacy of the half-dose regimen provided more protection in terms of bleeding complications (in separate patient groups) [32]. Half-dose alteplase is used as an initial treatment strategy versus full-dose alteplase in approximately 19% of ICU patients with acute pulmonary embolism, being used more often from 2010 to 2014. Full-dose alteplase therapy is preferred by clinicians in high-risk pulmonary embolism patients. Less than a quarter of patients in the half-dose group received vasopressors at the time of thrombolysis. In addition, at the time of alteplase administration, patients treated with half-dose therapy were less likely to receive mechanical ventilation and less frequently evaluated by cardiac functional tests. However, patients receiving half-dose alteplase were more likely to receive positive outcome and additional care in the management of acute pulmonary embolism.

Zhang et. al. compared low-dose thrombolytic therapy (30 mg/rt-PA) and LMWH treatment in patients diagnosed with intermediate-risk pulmonary thromboembolism [33]. It was observed that in contrast with the LMWH group, there was a significant decrease in blood pressure and an important decrease in symptom intensity in the rt-PA group. After 90 days, there were no differences in mortality, recurrent VTE and major bleeding between groups. However, in the rt-PA group, there was increased minor bleeding and reduced hemodynamic decompensation. As a conclusion to this study, low-dose thrombolytic treatment could be recommended as a protective and effective treatment for patients diagnosed with intermediate-risk pulmonary thromboembolism [33].

According to the guidelines of the European Society of Cardiology/European Respiratory Society (ESC/ERS)[29] risk stratification is done by evaluating the pulmonary embolism severity index (PESI) (or the simplified pulmonary embolism severity index (sPESI)), a right ventricular function on echocardiography and cardiac troponin testing after a diagnosis of PE has been confirmed. The intermediate-risk group is further divided into intermediate-high and intermediate-low risk groups. There was mentioned a small difference in hemodynamic decompensation according to the classification of intermediate-high and intermediate-low risk, observing a more frequent need for treatment update among patients in the intermediate-high risk group (3 vs 1 patient). Four patients in the intermediate-low risk group presented a faster compensation and a shorter length of hospitalization.

Several studies have shown that low-dose thrombolytics decrease systolic PAP during the first week of treatment [45]. However, no difference in systolic PAP on echocardiography was observed in low-dose thrombolytics compared with long-term heparin anticoagulation alone, except in the MOPETT trial [37]. In the MOPETT trial, systolic PAP measurements on dynamic echocardiography after 28 months were statistically higher in patients receiving anticoagulant therapy alone compared with half-dose rt-PA therapy [31]. However, chronic thromboembolic pulmonary hypertension was not confirmed with right heart catheterization in either study. Of the 16 patients who developed long-term pulmonary hypertension, 11 were in the intermediate-high risk group and 5 in the medium-low risk group according to the ESC/ERS4 guidelines. In this study, 1 patient from the TT group and 4 from the UFH at 3 months had presented with PH, and at 12 months of follow-up only 2 of the 4 still had systolic PAP above 40 mmHG.

It should be noted that according to the results of the conducted study, hemodynamic decompensation and pulmonary hypertension can develop in both subgroups of PE with intermediate risk.

Adverse events

In the PEITHO trial, extracranial and intracranial bleeding complications were significantly higher in the tenecteplase group compared with the placebo plus anticoagulant group [16]. In another study that compared tenecteplase plus UFH with placebo plus UFH groups, there was no difference in major bleeding complications, whereas minor bleeding was more frequent in tenecteplase patients [41]. In the MAPPET [2, 3] trial, no significant difference in bleeding complications was found between rt-PA plus UFH and placebo plus UFH treatment. In the meta-analysis made by Nakamura et al., the risk of major bleeding and ICH was found to be more frequent in the thrombolytic group, but it was not statistically significant [46]. In the MOPETT trial, no significant bleeding was observed in any of the patients receiving half-dose rt-PA plus anticoagulant and anticoagulant alone [31]. In a systematic review by Zhang et al., it was observed that bleeding complications were reduced in half-dose thrombolytic treatment compared with full-dose [45]. In the given study, there was no statistically significant difference between the half-dose rt-PA group and the UFH group in terms of bleeding complications (p=0.254), but minor bleeding was more frequent among patients with thrombolysis. No ICH was observed in this study. Increasing age and comorbidities have been shown to be associated with a higher risk of bleeding complications in full-dose thrombolytic trials [16, 45]. Using half-dose rt-PA, we observed no difference in bleeding complication including ICH between patients who were older than 75 years or younger. The obtained findings, combined with those of another controlled trial of thrombolysis in submassive PE, support the use of half-dose rt-PA instead of UFH/LMWH in the prevention of hemodynamic decompensation and the use of secondary thrombolytic therapy [31].

Clinical trials in progress

The PEITHO-3 trial is a global randomized, placebo-controlled, double-blind, multicenter trial with long-term follow-up (ClinicalTrials.gov Identifier: NCT04430569) [47]. Researchers are studying the safety and efficacy of low-dose alteplase therapy with conventional heparin an-

ticoagulation. However, they give a weight-adjusted dose of 0.6 mg/kg, up to a total of 50 mg over 15 minutes. If PEITHO-3's theory proves to be correct International clinical practice guidelines would revise their recommendations to include reperfusion and, in particular, low-dose systemic thrombolysis as first-line treatment in intermediate-risk PE. If the hypothesis is rejected, catheter-directed treatment may become the only option to improve prognosis of intermediate-high-risk PE patients.

Study limits

– This is a single-center study, this being the reason of a small number of patients to study. It limits from obtaining statistically stronger results, especially regarding the mortality rate.

– This study group included some different values, such as respiratory rate, oxygen saturation, and partial pressure of arterial oxygen (PaO2), indicating that the low-dose thrombolytic group has more severe cases, although there was no difference between groups regarding risk classification. Although it is believed this difference to be coincidental, this unintentional situation increases the credibility of the study.

– Another limitation was a non-blinded approach, as physicians responsible for the treatment and follow-up of the patients were aware what treatment each patient had in acute phase. However, very strict criteria were defined for hemodynamic decompensation and for further escalation of treatment to thrombolysis.

– Decision making in case of using thrombolysis is variable because of the bleeding risk fear. It is easy to associate patient's mean HAS-BLED score in the TT group with the precaution use of systemic alteplase.

– A limited or delayed access to CTPA is an important factor influencing the safe use of thrombolytic therapy and the enrollment of patients to the study. At the same time, the lack of other imagistic tools, such as ventilation/perfusion scintigraphy does not allow the enrollment of patients with contraindications to the contrast injection.

– Delayed addressability at a specialized medical institution in the acute PE (more often over 14 days), limits the enrollment of patients to the thrombolysis group, and reduces the results of complete thrombus lysis regardless to the treatment's options.

In conclusion, more studies of high methodological quality are needed to assess safety and cost effectiveness of thrombolytic therapy in intermediate-risk pulmonary embolism.

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Authors' contributions. ND conceptualized the idea; IC, GS, TC, and SRI conducted literature review, and wrote the manuscript; AG revised critically the manuscript. All the authors approved the final version of the manuscript.

Funding. This research was part of the project No 20.80009.8007.28 "Strategic approach to pulmonary embolism in the pre– and post-hospitalization period in the Republic of Moldova", within the State Program of the Republic of Moldova (2020-2023).

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

Conflict of interests. The authors have no conflict of interests to declare.

https://doi.org/10.52418/moldovan-med-j.66-2.23.10
UDC: 616.857:616.133.33

Cephalalgic syndrome in autosomal dominant cerebral arteriopathy with subcortical infarctions and leucoencephalopathy

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Manuscript received November 3, 2023; revised manuscript December 5, 2023; published online December 27, 2023

Abstract

Background: Autosomal Dominant Cerebral Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL) is caused by mutations in NOTCH3 gene, classic symptoms include migraine with aura, ischemic strokes, apathy, depression and dementia. Headache is usually the first symptom, characterized by recurrent attacks of migraine with typical, hemiplegic or prolonged aura with unusual frequency.
Material and methods: All the data were picked from the patient's medical recordings. The patient had undergone a complete clinical exam, a contrast enhanced MRI-scan and a genetic test. Then a literature review was done based on the peculiarities of the case.
Results: A 43-year-old woman presented with pulsatile, alternating, severe headache, accompanied by phono, and photophobia, nausea and vomiting, with an onset at 35 years and a frequency of 12/30, triggered by menstruation and stress, preceded by a day by a visual aura lasting 5-6 minutes. Family history revealed cases of stroke and migraine. Neurologic examination was normal, but a contrast enhanced MRI showed diffuse polymorph confluent subcortical white matter lesions, involving external capsule and anterior poles of the temporal lobes. NOTCH3 gene sequencing revealed the presence of a heterozygote missense c.421C>T mutation, localized in the 4thexone. After establishing the diagnosis, the patient was prescribed a symptomatic treatment.
Conclusions: Headache in CADASIL patients has well-defined diagnostic criteria in the International Classification of Headache Disorders, is being considered a secondary headache which may resemble or not migraine with aura. The patient presented a migraine-with-aura-like headache but with some peculiarities.
Key words: CADASIL, NOTCH3, migraine with aura.

Cite this article
Gutu C, Grosu O, Rotaru L, Odobescu S, Moldovanu I. Cephalalgic syndrome in autosomal dominant cerebral arteriopathy with subcortical infarctions and leucoencephalopathy. *Mold Med J*. 2023;66(2):66-70. https://doi.org/10.52418/moldovan-med-j.66-2.23.10.

Introduction

This article presents a rare clinical case of secondary migraine-like headache attributed to Autosomal Dominant Cerebral Arteriopathy and Leukoencephalopathy. This type of headache is described in the International Classification of Headache Disorders, 3rd ed., section "Headache attributed to cerebral and/or cervical vascular diseases", subchapter 6.8 "Headache and/or migraine-like aura attributed to chronic intracranial vasculopathy", form 6.8.1 "Headache attributed to CADASIL" [1].

Headache is one of the 5 major symptoms of Autosomal Dominant Cerebral Arteriopathy with Subcortical Infarcts and Leukoencephalopathy. The prevalence of headache in CADASIL patients according to the data of different studies, ranges between 14% and 72% [2-6], being higher in women than in men. The pattern of migraine differs from that observed in the general population, with migraine with aura being dominant [7]. The vast majority of patients report the presence of migraine with aura (80-90%) [3, 8]. The aura is typical in 44%, while 56% of patients show atypical aura – aura without headache, hemiplegic aura, basilar or prolonged aura [5, 9]. Imaging changes do not differ between patients with migraine with aura and those without aura [9].

Autosomal dominant cerebral arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is the most common cause of hereditary cerebral infarcts in adults [10, 11]. Clinically, it is manifested by migraine with aura, depression, apathy, dementia and recurrent ischemic strokes [12]. CADASIL is caused by mutations in the NOTCH3 gene on chromosome 19q12 [13], a transmembrane receptor located on cerebral vascular smooth myocytes, the mutation of which produces thickening and fibrosis of the vascular wall, changes responsible for the production of subcortical cerebral infarcts [10].

According to the International Classification of Headache Disorders, headache associated with CADASIL has the following criteria [1]:

A. Recurrent migraine attacks with typical aura, hemiplegic, or prolonged aura, fulfilling criterion C.

B. The existence of CADASIL has been demonstrated[1].

C. One or both of the following:

1. Migraine with aura is the earliest manifestation of CADASIL

2. Migraine attacks with aura improve with the appearance of other manifestations of CADASIL (ischemic strokes, affective disorders or cognitive disorders)

D. Not explained by another diagnosis from the International Classification of Headache Disorders.

Note:

1. Diagnosis is made by screening for the NOTCH3 mutation, by a simple skin biopsy with anti-NOTCH3 antibody immunofixation, or by electron microscopy to assess osmophilic granular extracellular material (GON) in the medial arterial wall.

The results of studies on the epidemiology of headache in CADASIL are controversial [6, 14-21]. Moreover, little is known about what the correct therapeutic approach would be and whether its management should be different from the management of migraine in the general population [22-26]. Although migraine with aura has been shown to be a risk factor for stroke in the general population [26-30], it is unclear whether migraine with aura in CADASIL is a predictive factor for infarction cerebral or for a more aggressive phenotype.

The purpose of the research was to analyze the characteristics of headache in CADASIL patient.

Material and methods

The case is reported of a patient diagnosed with CADASIL, who phenotypically presented migraine-like headaches, but with some particularities, and the literature review is presented regarding the particularities of the headache syndrome in CADASIL. The patient's medical data were extracted from the medical record with her consent. The creation of the literature review was carried out in accordance with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) criteria. 380 articles in the PubMed database published between 1993 and 2023 dedicated to the headache syndrome in CADASIL were identified. Keywords to identify references were: *CADASIL, headache, migraine*. Articles reporting clinical cases and articles referring to basic research were excluded, as a result, 14 studies on the epidemiological, clinical and treatment features of headache syndrome in CADASIL were included.

Results

A 42-year-old female patient presents with generalized, pulsatile alternating hemicrania that gradually increases in intensity to 9/10 SVA on the first 2 days, then 8/10 SVA on the 3rd day, then 5-6/10 SVA on the 4th day, with a frequency of 12/30 days, duration – 72 hours, triggered by menstruation and psycho-emotional stress, relieved by the administration of analgetics, which started at 35 years old, accompanied by nausea, vomiting, phono- and photophobia, visual aura lasting 5-6 minutes, which appears a day before the headache and sensitive aura – numbness in the left hand, history of eredo-collaterals – paternal aunt suffers from migraines. Neurological examination was unremarkable.

When collecting the anamnesis, the patient revealed that the paternal grandmother suffered a stroke, as a result of which she was paralyzed, the patient's father died of a cerebral infarction at the age of 49, and the paternal aunt suffers from migraines. Figure 1 shows the genealogical tree of the family, which outlines a pathology with autosomal dominant transmission.

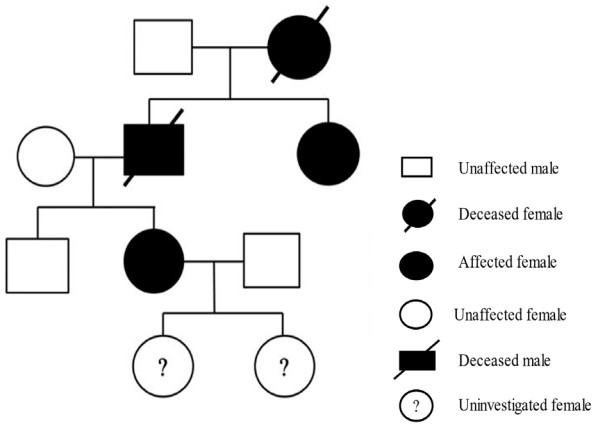

Fig. 1. The genealogical tree of the family
Family history showed that her paternal grandmother and her father, aged 49, died both from a stroke, whilst her paternal aunt has migraines. The family pedigree revealed an autosomal dominant type inheritance.

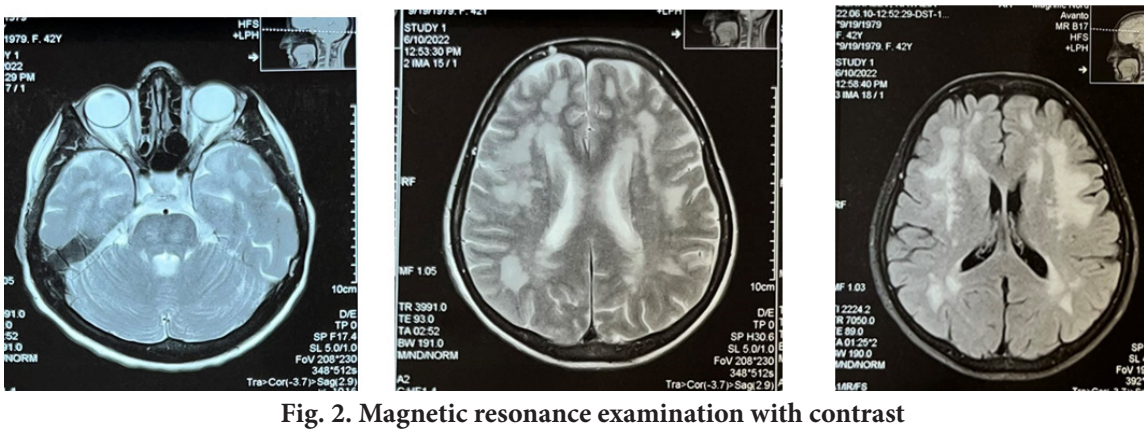

Fig. 2. Magnetic resonance examination with contrast

Figure 2 shows images of the 1.5T cerebral Magnetic Resonance examination with contrast which revealed the existence of multiple confluent polymorphous lesions in the subcortical, bihemispherical periventricular and infratentorial cerebral white matter at the level of the pons of Varolio bilaterally, the anterior poles of the temporal lobes bilaterally, the external capsule. Lacunar focus <4mm in the frontal subcortical white matter on the right.

Taking into account the presence of the clinical picture of migraine with atypical aura and the suggestive imaging changes, the patient was recommended to perform the genetic test that identified the mutation c.421C>T (p. Arg141Cys) at the level of exon 4 of the NOTCH3 gene in a heterozygous state. This change is reported in the specialized literature as having clinical pathological significance. Interpretation: the patient is affected by CADASIL syndrome as a result of the heterozygous c.421C>T mutation in exon 4 of the NOTCH3 gene. The clinical diagnosis was established – Autosomal dominant cerebral arteriopathy with subcortical infarcts and leukoencephalopathy. Over 3 months, the patient reported a decrease in both headache intensity to 4/10 SAV and frequency – 4/30.

Discussion

The diagnosis of primary headache is established only on the basis of clinical criteria, according to the International Classification of Headache Disorders, 3rd edition, but it is imperative to initially exclude the causes of secondary headaches, especially treatable ones.

This case meets the criteria of the International Classification of Cephalalgic Disorders. Criterion A – the patient suffers from migraine attacks with atypical aura. Criterion B – the existence of CADASIL has been demonstrated. Criterion C – Migraine with aura is the earliest clinical manifestation of CADASIL.

In the case of this patient, the first clinical symptom was a migraine-like headache, which is, by the way, the most frequent and typical manifestation, but cases with atypical onset are described in the literature, such as migrainous status [7], hemiplegic migraine sporadic [8], acute confusional migraine [9], progressive asymmetric parkinsonism [10], generalized epileptic seizures [11] or recurrent intracerebral hemorrhage [12].

Headache in CADASIL is not a constant symptom, studies report different results, thus the study by Guey et al., which included 378 patients, reported a migraine incidence of 54.4%, of which with aura – 45.8% [6], Bianchi et al. (229 patients) report a migraine incidence of 42%, migraine with aura – about half [14], Paraskevas et al. (54 patients) report the incidence of migraine with aura – 39% [15], Tan et al. (52 patients), of which 39.58% suffer from migraine [16], Liao et al. (112 patients) report a very low incidence of migraine, only 2.7% [17], Chen et al. (169 patients) – 32.2% of patients have migraine [21], in the study by Ince et al. (25 patients) – 52% have migraine [20], Hawkes et al. (13 patients) report an incidence of 38.5% [19], and Nogueira et al. in the study that included 26 patients, 1/3 patients suffer from migraine [18].

Although CADASIL has diverse clinical presentation, even within the same family, 5 core symptoms are characteristic: migraine with aura, subcortical ischemia, affective disturbances, apathy, and cognitive impairment. These symptoms vary in frequency depending on age and duration of illness [3, 12, 31, 32].

There are several theories regarding the pathogenetic mechanisms of the headache syndrome in CADASIL. The first theory suggests that migraine with aura is the result of episodic ischemia caused by decreased blood circulation and cerebrovascular reactivity, which corresponds to the classical vascular theory of migraine [5]. The second theory claims that in CADASIL there is an increased susceptibility for the expansion of cerebral depression, which is caused by chronic vascular cerebral lesions or acute episodes of hypoperfusion [33], a mechanism already demonstrated in animal models [5, 34, 35]. Functional MRI studies have shown that in migraineurs with aura, areas located in the dorsal pons are excessively activated [36-39], at the same time, pontine lesions are often observed in patients with CADASIL [39, 40], thus, hypothetically these lesions could lead directly or indirectly to the occurrence of migraine with aura [5].

The most used preparations for the acute treatment of headache belong to the following groups: non-steroidal anti-inflammatory drugs, opioids, triptans, and for prophylactic treatment: calcium channel blockers, beta-blockers, anticonvulsants, and from other groups. Beta-blockers, although effective for the prophylactic treatment of migraine, in CADASIL headache patients report frequent side effects (fatigue and nightmares) [41]. Several small studies demonstrate that acetazolamide may be effective as a prophylactic treatment for CADASIL headache [22-24]. CGRP inhibitors would be potential drugs for the prophylactic treatment of CADASIL headache [42], but other authors argue that their use should be limited due to adverse effects (exacerbation of ischemia and psychiatric effects) [43].

Conclusions

1. Headache in CADASIL has well-defined criteria in the International Classification of Headache Disorders, being similar or not to migraine with aura.

2. The given patient presented a headache similar to migraine with aura, but with certain peculiarities (onset at 35 years, atypical visual aura and family history), suggesting that it is a secondary headache.

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Funding
This study was supported by the grant from the National Agency for Research and Development of the Republic of Moldova, Project code 20.80009.8007.01. The authors take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The research protocol was approved by the Research Ethic Board of the *Diomid Gherman* Institute of Neurology and Neurosurgery and the tests have been done according to the contemporary principles in biological standardization of experiences and Declaration of Helsinki with further amendments (Somerset West Amendment, 1996).

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.11
UDC: 618.36-008.64:618.344

Ang2 immunoexpression vs vascular profile in chorio-villous germinative status in early-term compromised pregnancies

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Manuscript received September 29, 2023; revised manuscript November 22, 2023; published online December 27, 2023

Abstract

Background: The angiopoietin/TIE system is one of the signalling pathways that regulate vascular development and remodelling during morphogenesis of the placental complex. Deregulation of the vascularization process is associated with primary placental insufficiency through destabilization of the maternal-fetal functional system. Disruption of expression of pro-angiogenic factors is associated with reduction of the microcirculatory bed and installation of circulatory dysfunction.
The aim: Evaluation of Ang2 immunoexpression in early term compromised uterine pregnancies in the context of chorio-villous circulatory dysfunction in primary placental insufficiency.
Material and methods: Abortion product from 67 patients with early term compromised pregnancies (stagnant pregnancies – 43 cases, early miscarriages – 11 cases, control batch that included 13 cases of pregnancies solved on social indications) were immunohistochemically evaluated with the marker for anti-Ang2 and anti-CD31.
Results: The majority of sites analyzed were Ang2 negative, with the exception of syncytiotrophoblast, that reached the highest score (+3) in most cases. A maximum placental vascularisation index was achieved in the control group. The stagnant pregnacies batch has registered the lowest vascular density mean.
Conclusions: The placentation period is characterized by a weak Ang2 cellular environment in the chorio-villous germinal site in the group of short-term compromised pregnancies, except a highly positive syncytiotrophoblast. The immunoexpression profile in vascular endothelium correlates statistically significantly with placental vascularization index and chorio-villous vascular density in stagnant pregnancies.
Key words: Ang2, angiogenesis, fetal conceptus, compromised pregnancies, primary placental insufficiency.

Cite this article
Carpenco E, Fulga V, Saptefrati L, Sinitina L, David V. Ang2 immunoexpression vs vascular profile in chorio-villous germinative status in early term compromised pregnancies. Mold Med J. 2023;66(2):71-77. https://doi.org/10.52418/moldovan-med-j.66-2.23.11.

Introduction

Placental insufficiency is a syndrome caused by morpho-functional changes that lead to the incompetence of the placenta to maintain adequate metabolism between mother and fetus. The primary form of placental insufficiency is one of the types described and it occurs in the first trimester [1]. The first trimester of gestation is characterized by important successive phenomena on which the subsequent course of pregnancy will depend, namely: the first phase of cytotrophoblast invasion, implantation, placentation [2]. This period is of particular importance because any dysregulation of the above-mentioned phenomena under the influence of different factors (infectious, endocrine, genetic, etc.) contributes to the establishment of primary placental insufficiency, which is the main cause of spontaneous abortion and stagnant pregnancies [3].

The vascularization of chorionic villi is an important morphological indicator of the functional state of the placenta. It reflects the expression of metabolic processes between mother and fetus [4], and the physiological formation of the placental vascular network occurs under the action of various angiogenic factors.

The formation of such a competent vascular network in the early period is achieved by two successive processes: vasculogenesis (formation of vessels from endothelial progenitor cells) and angiogenesis (formation of vessels from pre-existing vessels) [5], the destabilization of which contributes to the onset of placental dyscirculatory failure.

The angiopoietin/TIE system is one of the signaling pathways that regulate vascular development and remodeling [6]. According to the molecular profile, 4 ligands (Ang1, Ang2, Ang3, Ang4) are elucidated. The first 2, as well as their tyrosine kinase-like receptors (TIE-1 and TIE-2), have a significant role in vascular morphogenesis of the placentation period [7]. The Ang1/ TIE-2 pathway promotes endothelial cell survival, endothelial integrity, anti-inflammatory and anti-apoptotic responses, which togeth-

er support reduced vascular permeability [8, 9]. Ang2 is an antagonist that competes with Ang1 in binding to TIE-2. The Ang2/ TIE-2 pathway reduces vessel stability and enhances vascular remodeling [10]. In experimental conditions, the effect of Ang2 in promoting endothelial cell survival, sprouting and migration has been demonstrated in a temporal and concentration-dependent manner [11-13]. The aforementioned ascribe to Ang2 a TIE-2-dependent agonist or antagonist effect [10, 14]. Angiopoietin 2 represents an endogenous ligand of the vascular endothelium-specific receptor tyrosine kinase Tie-2, which is expressed as early as the early period of placentogenesis with endo- and non-endothelial effects [15].

Vascular disturbances that could appear in the placentation period are frequently the cause of intrauterine developmental restriction [16, 17]. When assessing Ang2 levels in the first trimester of gestation, an association with risk of early-term miscarriage has been found [18]. Decreased expression of angiopoietin-2 in developmental restriction contributes to angiogenesis disturbances, mainly in the intermediate and terminal villi [19].

Deregulation of secretion and expression of pro-angiogenic factors during placentation is associated with disruption of vascular morphogenesis in the chorio-villous compartment by reduction of the chorio-villous fetal vascular bed, which makes this study of a great topicality.

Thus, the aim of the current study was to evaluate Ang2 immunoexpression in early-term compromised uterine pregnancies in the context of chorio-villous circulatory dysfunction in primary placental insufficiency.

Material and methods

Tissue samples obtained by uterine aspirate from 62 patients with early compromised pregnancies (3-12 weeks) were used. The specimens were collected at the Level III Perinatal Center, Institute of Mother and Child, during 2020. All patients were examined by ultrasonographic investigation and the gestational term was determined based on the first day of the last menstruation. The cases were grouped as follows: stagnant pregnancies (SP) – 43 cases, early miscarriages (EM) – 11 cases and pregnancies solved on social indication (SI) – 13 cases, last one being the control group. The age of the patients ranged from 22 to 40 years (mean±std. dev. being 30.5±5.6 years).

Clinical data were obtained from the medical records of each patient. The current research is part of a larger study of early-term compromised pregnancies within the state program "Morphological approach by conventional, histo- and immunohistochemical methods of the peculiarities of the pathological profile of early placentogenesis in early-term compromised pregnancies", code 20.80009.8007.17 P1P2 0750.

Cases were selected according to the inclusion and exclusion criteria:

Inclusion criteria: terminated pregnancies with gestational term from 3 to 12 weeks (clinically confirmed by ultrasound and terminated in the Institute of Mother and Child); pregnancies with pathological evolution: stagnant, early miscarriage; pregnancies with abortion at social indications/ desire; quality and volume of the aspirate: chorionic villi and decidual plates of a sufficient volume in standard paraffin blocks (1.0x1.0x0.5cm); monofetal pregnancies; no age threshold.

Exclusion criteria: serious somatic pathology; multiple pregnancies; pregnancies terminated on medical indication; lack of clinical-anamnestic data in medical records; lack of gestational term specification and ultrasound confirmation of pregnancy status.

The examination included histoprocessing of tissue samples, application of the usual histological method (haematoxylin-eosin), immunohistochemical method (anti-CD31; anti-Ang2) with evaluation of histopathological features and immunoexpression, and statistical analysis.

Primary processing. Tissue material of the conception product was collected in a short time in obstetric department with rapid fixation in 10% formalin, pH 7.2-7.4, to reduce the risk of early lysis of tissue material and bacterial flora growth. The fixation period in 10% buffered formalin solution was 24 hours. The paraffin embedding system was DP500/CIT2002 (Bio-Optica, Italy). Histochemical and histological processing of samples was performed on the histoprocessor "TISSUE-TEK, VIP 6AI" (Sakura, Japan), sectioning on the HM325 microtome (Thermoscientific) (USA). 3.5 μm thick sections were placed on positively charged slides (APTACA, Italy).

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

Immunohistochemical method. Immunohistochemical assays included manually adopted operational procedures for anti-Ang2 (ab5630) antibodies with the application of the Novolink™MaxPolimer detection system, Leika (RE7280-K) [20] and anti-CD31 (JC70A) with the application of the EnVision™FLEX detection system, high pH (K8000) [21]. The conventional immunohistochemical method was applied (Table 1). Deparaffinization was performed in two toluene baths (code UN1294, Sigma-Oldrich), the first bath for 60 min at 59°C in thermostat, followed by the second bath for 5 min at room temperature. Slides were then placed in a mixed bath of toluene and 96% alcohol for 5 min, then – 2 baths of 96% alcohol with 2 rehydrations of 10 min each in distilled water. For the purpose of epitope unmasking, sections intended for application of Ang2 and CD31 antibody were exposed to dissolved Target Flex solution (1ml Target: 49 ml distilled water) at high pH, 20 minutes exposure time at 95°C–96°C with a total pretreatment and posttreatment time of 60 minutes.

Table 1. Antibodies used: source, dilution, unmasking system, detection system, incubation time

Antibody / clone	Sourse/ incubation time/ dilution	Retrieval system / time	Detection system / time
CD31 JC70A	Abcam, Cambridge, UK/ 20 min/ ready- to-use	Solution Target Flex, high pH/ Water bath at 95°C - 96°C / 20 min	EnVision™FLEX, high pH
Ang2 ab56301	Abcam, Cambridge, UK/ 12 hours/ 1:1000	Solution Target Flex, high pH/ Water bath at 95°C - 96°C / 20 min	Novolink™MaxPolimer, Leika / 30 min

Neutralization of endogenous peroxidase was performed with peroxidase block for 7 minutes followed by incubation with Novocastra Protein Block for 5 minutes. Next step was incubation with primary antibody (anti-Ang2) for 12 hours at +4°C, 1:1000 dilution, including 5 minutes in thermostat at 59°C. In the case of anti-CD31 antibody, incubation lasted for 20 minutes at room temperature. After incubation with the primary antibody, neutralization of endogenous peroxidase with peroxidase block was performed for 5 minutes, followed by application of the secondary antibody (HRP) for 20 minutes and DAB (3,3'-diaminobenzidine) applied as a chromogenic substrate for 5 minutes. Counterstaining of nuclei was performed with Leica haematoxylin (RE7164) when using the Novolink™MaxPolimer detection system and with Mayer's haematoxylin (HEMM-36/21, BIOGNOST, Slovenia) when using the EnVision™FLEX system. The final product of the reaction was stained brown with cytoplasmic patern for Ang1 and membranous patern for CD31. Then, the histological slide panel was subjected to the dehydration and clarification procedure using two absolute alcohol baths, one alcohol and toluene mixed bath and three toluene exposures, each exposure being 5 minutes. The final procedure consisted of mounting the slides with BMC-100 solution. In the manual immunohistochemical staining procedure, Sequenza™ Immunostaining Center was applied using Thermo Shandon Coverplat.

Microscopic evaluation. The CD31 protein (endothelial cell adhesion receptor) was detected at the membrane level, manifested by the presence of brownish colour in the tissue studied. In all sections, blood vessels were quantified by the hot-spot method, the technique having been described in detail in a previous study. For the assessment of Ang2 immunoexpression, initially, areas with the highest density of chorionic villi were determined at ×100 magnification. Immunoexpression was assessed at ×200 magnification using the semiquantitative hot-spot method applied to three representative areas of the germinal site corresponding to chorionic villi (vascularized and avascularized). For the evaluation of anti-Ang1 immunoexpression, the scoring system based on the intensity of immunoreactivity was applied. The reaction was considered to be positive in the presence of brown color in the tissue studied according to the specificity of each antibody. The following score was applied: 0 (no staining); +1 (weak but detectable staining); +2 (moderate or distinct staining); +3 (strong or pronounced staining). The reaction for anti-Ang2 was analyzed in the following areas: trophoblast, mesenchymal stromal cells, angiogenic/ endothelial vascular cells.

The above-mentioned structures were counted in each of the 3 study groups (SP, EM, SI), grouped according to gestational term into the following groups: 3-5 weeks, 6-9 weeks and 10-12 weeks. Quantification of positive cells was performed on the Axio Imager A2 microscope (Carl Zeiss, Germany) equipped with the AXIOCam MRc5 recording camera.

Data analysis. Statistical procedures (Winstat 2012.1, R. Fitch Software, Bad Krozingen, Germany) included determination of the Spearman's rank correlation coefficient (Spearman (rs)) and differences between groups and subgroups (Mann-Whitney U test). Results were considered statistically significant at $p<0.05$.

Results

The study was carried out on a group of 67 cases of early compromised pregnancies, divided into: stagnant pregnancies – 43 cases (64.17%), early miscarriages – 11 cases (16.41%) and the control batch that included 13 cases of pregnancies solved on social indications/ desire (SI) (19.4%).

The intensity of Ang2 immunoexpression varied from 0 to +3 and can be analyzed in the tables 2, 3 and 4.

The majority of sites analyzed in the control group were

Table 2. Immunoexpression intensity of anti-Ang 2 (control batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	+3	0	+1	+2	+3	0	+1	+2	+3	0	+1	+2	+3
3-5	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6-9	6	0	0	0	0	0	0	**6**	**6**	0	0	0	**6**	0	0	0	**6**	0	0	**0**
10-12	7	0	0	0	0	0	0	**7**	**7**	0	0	0	**7**	0	0		**7**	0	0	**0**
Total	**13**	0	0	0	0	0	0	**13**	**13**	0	0	0	**13**	0	0	0	**13**	0	0	0

Note: GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

Table 3. Immunoexpression intensity of anti-Ang 2 (SP batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**
3-5	**8**	0	0	0	0	0	2	**6**	**4**	0	1	3	**8**	0	0	0	**8**	0	0	0
6-9	**26**	0	0	0	0	0	1	**25**	**10**	8	4	3	**20**	0	2	0	**25**	0	1	0
10-12	**9**	0	0	0	1	0	0	**8**	**4**	4	0	0	**3**	3	2	0	**5**	3	0	1
Total	**43**	0	0	0	1	0	3	**39**	**18**	12	5	6	**31**	3	2	0	**38**	3	1	1

Note: SP – stagnant pregnancies, GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

Table 4. Immunoexpression intensity of anti-Ang 2 (EM batch)

GT	CV-cyto-tropho				CV-syn-tropho				CV-vasc-endot				CV-hofbauer				CV-stroma			
	0	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**	**0**	**+1**	**+2**	**+3**
3-5	**2**	0	0	0	0	0	1	**1**	**1**	1	0	0	**1**	1	0	0	**2**	0	0	0
6-9	**7**	0	0	0	0	0	0	**7**	**6**	0	1	0	**5**	0	0	0	**7**	0	0	0
10-12	**2**	0	0	0	0	0	0	**2**	0	**2**	0	0	**1**	1	0	0	**1**	0	1	0
Total	**11**	0	0	0	0	0	1	**10**	**7**	3	1	0	**7**	2	0	0	**10**	0	1	0

Note: EM – early miscarriages, GT – gestational term, CV-cyto-tropho – chorionic villi cytotrophoblast, CV-syn-tropho – chorionic villi syncytiotrophoblast, CV-vasc-endot – chorionic villi vascular endothelium, CV-hofbauer – chorionic villi Hofbauer cells, CV-stroma – chorionic villi stroma. Immunoexpression intensity was rated as follows: 0 (absent); +1 (weak); +2 (moderate); +3 (pronounced). The highest values were marked as **bold**.

Ang2 negative, with the exception of syncytiotrophoblast, that reached the highest score (+3) in 100% cases. Cytotrophoblast was totally Ang2 negative in SP and EM groups. The Hofbauer cells and the stromal ones showed different scores (SP: 70.4/ EM: 63.6%; SP: 86.3/ EM: 90.9%, respectively) (tab. 3, 4). Endothelial cells were Ang2 positive in 56.1% of SP cases and in 63.6% of EM cases. The syncytiotrophoblast was Ang2 positive in most of cases of all groups studied: 90.6% in SP batch and 90.9% in EM batch (fig. 1).

Then was analyzed the different Ang2 immunoreactivity between batches and inside batches by applying the Mann-Whitney U test. Thus, were obtained the following statistically significant differences of endothelial cells immunoreactivity between groups: SP 6-9 weeks vs ASI 6-9 weeks (p=0.01); SP 10-12 weeks vs ASI 10-12 weeks (p=0.04); ASI overall cases vs SP overall cases (p=0.001) and overall EM cases vs ASI overall cases (p=0.025). At the same time, differences were attested in the Hofbauer cell compartment: SP 10-12 weeks and ASI 10-12 weeks (p=0.02); SP 10-12 weeks and SP 6-9 weeks (p=0.005). Were also identified variations in the stromal areas: SP 10-12 weeks vs SP 6-9 weeks (p=0.003). Statistically significant differences in the ASI batch were not attested.

Subsequently, to assess the involvement of Ang2 expression in the morphogenesis of the chorio-villous vascular network, placental vascularization index (PVI, %) and chorio-villous vascular density (VD, %) were determined with the application of anti-CD31 antibody. According to the results obtained, anti-CD31 immunoexpression in the

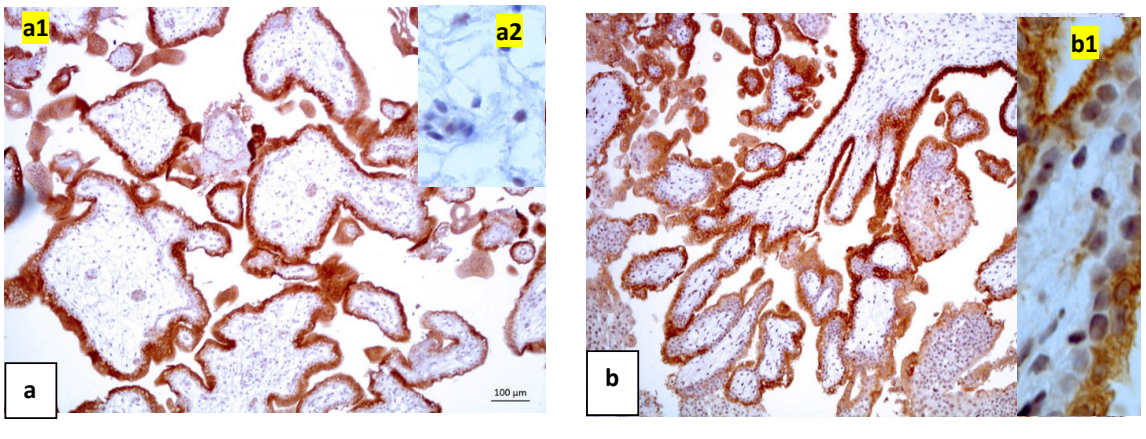

Fig. 1. Differential Ang2 immunoexpression in choriovillar cell profile in early compromised pregnancies: a) SI - a1 (syncytiotrophoblast - score +3, cytotrophoblast - 0) and a2 (stroma - 0); b) EM – b1 (cytotrophoblast - 0; syncytiotrophoblast - score +3; endothelium - score +1). Immunoreaction for anti-Ang2, DAB; a1, b1 ×100; a2, b2 ×400.

Table 5. Descriptive statistical analysis of placental vascular density

Vascular profile	ASI batch (M±SD), weeks			EM batch (M±SD), weeks			SP batch (M±SD), weeks		
	3-5	6-9	10-12	3-5	6-9	10-12	3-5	6-9	10-12
PVI%	-	92.08 ±7.67	90.52 ±12.08	97.18 ±3.98	73.39 ±12.98	78.58 ±23.7	64.12 ±33.58	51.72 ±27.24	55.44 ±32.24
PVI_{total} %	-	91.3±9.87		83.05±13.5			57.09±31.02		
VD%	-	7.15 ±1.38	11.91 ±9.47	7.23 ±0.43	7.26 ±3.1	16.91 ±2.24	4.87 ±1.77	5.53 ±2.99	6.52 ±5.8

Note: M – mean; SD – std. Dev.; GT – gestational term, weeks; ASI – abortion on social indications; EM – early miscarriage; SP – stagnant pregnancies; PVI – placental vascularization index; VD – chorio-villous vascular density.

chorionic villous stroma determined a maximum IVP in the control group (ASI) (91.3±9.87). The values were lower in EM and SP batches (83.05±13.5 and 57.09±31.02, respectively). The maximum VD mean values were determined in the EM and ASI groups (10.46±1.92 and 9.53±5.42, respectively). The SP batch has registered the lowest VD mean (5.64±3.52) (tab. 5, fig. 2).

Next step was to analyze the VD and PVI differencies between batches, grouped by gestational term. The correlations can be seen in table 6.

Table 6. Mann-Whitney U test results, intergroup analysis

GT, weeks	Batch	Correlations obtained for the VD	Correlations obtained for PVI
6-9 n=38	ASI vs EM	p= 0.668	**p=0.01**
	ASI vs SP	**p= 0.016**	**p=0.001**
	EM vs SP	p=0.116	p=0.059
10-12 n=20	ASI vs EM	p=0.076	p=0.236
	ASI vs SP	p=0.189	**p=0.006**
	EM vs SP	p=0.143	p=0.242
3-5 n=9	EM vs SP	p=0.142	p=0.106

Note: GT – gestational term, weeks;ASI – abortion on social indications; EM – early miscarriage; SP – stagnant pregnancies; PVI – placental vascularization index; VD – chorio-villous vascular density. Statistically significant correlations were marked as **bold**.

The CD31 immunoreactivity of endothelial cells was directly dependent on the PVI in the SP group (rs=0.67, p=0.04). Statistical analysis of data also showed some statistically significant correlations between VD and the CD31 expression by endothelial cells (SP batch, 3-5 weeks and 10-12 weeks) (rs=0.85, p=0.003 and rs=0.65, p=0.04, respectively). VD was also dependent on the immunoreactivity of Hofbauer cells and stromal cell of chorionic villi (SP batch) (rs=0.36, p=0.01 and rs=0.31, p=0.02, respectively). The SI and EM batches did not show any statistically significant correlations.

Discussion

The first trimester of gestation is an important one due to the formation of the vascular capillary network and the large vessels, arterioles and venules, which are of particular importance in the establishment of the blood supply to ensure the requirements of the growing embryo [22, 23]. Thus, the formation of blood vessels in the chorionic villi, which happens in the first trimester of gestation, is fundamental in the establishment of full metabolic processes between mother and embryo/fetus, which favors the physiological progress of pregnancy.

The vascularization of the chorionic villi is an important morphological indicator of the functional state of the placenta in achieving this connection [4], and the physiological formation of the placental vascular network oc-

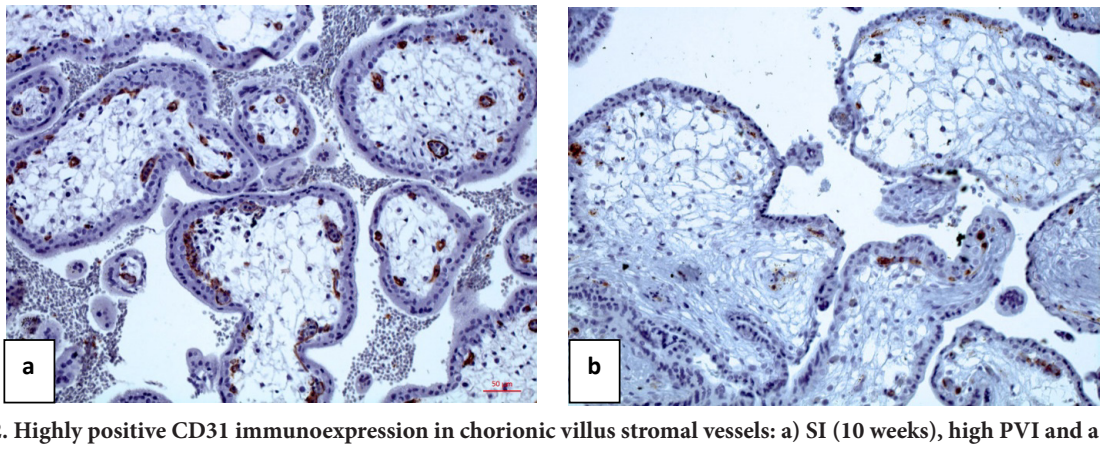

Fig. 2. Highly positive CD31 immunoexpression in chorionic villus stromal vessels: a) SI (10 weeks), high PVI and a reach blood network and b) SP (12 weeks), low PVI, hypovascularized villi. Immunoreaction for anti-CD31, DAB; a, b x100.

curs under the action of various angiogenic factors, among which an important role is attributed to the angiopoietins family.

Angiopoietins are a family of extracellular ligands, integral proteins involved in angiogenesis and vascular remodelling. The angiopoietin/TIE system is one of the signaling pathways that regulate vascular development and remodeling [6]. According to the molecular profile, 4 ligands (Ang1, Ang2, Ang3, Ang4) are elucidated, the first two, as well as their receptors (TIE-1 and TIE-2) having significance in the vascular morphogenesis of the placental period. [7].

Ang2 contains 496 aminoacids and 60% of it is homologous to Ang1 [24]. Both ligands have a similar structure with different biological functions and act through the common tyrosine kinase receptor (TIE-2) [10, 14]. The effect of Ang2 is contradictory and depends on the presence or absence of VEGF, which may convert its anti-angiogenic role into a proangiogenic one. Thus, Ang2 is considered to be an antagonist for Ang-1 when binding to the TIE-2 receptor that leads to a reduced vascular stability through destabilization with subsequent restructuring [10].

Therefore, Ang2 immunoexpression in chorio-villous germinal status was assessed by immunohistochemical investigation with determination of angiogenic profile. Were evaluated the localization and degree of immunoexpression (tab. 2, 3, 4). According to the results obtained, a differentiated immunoexpression was established both based on the cellular profile and in relation to the batch of early compromised pregnancies. Were found out some statistically significant differences, with a predominance of negative immunoexpression and a highly positive (score +3) syncytiotrophoblast.

In order to assess the proangiogenic effect in the early period of vascular capillary bed formation in the stroma of developing chorionic villi, cellular immunoexpression data were correlated with the placental vascularization index. Statistically significant positive correlation was attested only in the SP group, 3-5 weeks of gestation, in the endothelial cell compartment (rs=0.67, p=0.04). The batch of SI/D and the EM one showed no intragroup correlations. Thus, differential immunoexpression in the cellular compartment denotes early-term Ang2 involvement in reducing placental vascular index.

As a confirmation of this hypothesis, when analysing immunoexpression in the research sites vs chorio-villous vascular density, statistically significant correlations were found in the SP group, 3-5 and 10-12 weeks, between endothelial cell and stromal/mesenchymal cell components as well as Hofbauer cells for the SStotal group.

The Ang2 expression is different during pregnancy, i. e., in the early period it is high, whereas later it decreases. This was proved by assessing serum levels of these proteins in normal pregnancies, including mRNA determination [15]. According to one study, the authors have noticed the presence of Ang2 in the early period of placentalogenesis (4th week) in the trophoblast, particularly in the syncytiotrophoblast [25]. At the same time, various studies are presented reporting a variety of Ang2 expression and its involvement in various pathologies, such as growth restriction, pre-eclampsia etc. [19], which are not the aim of investigation in this paper. The results are consistent with the authors' data supporting differential cellular immunoexpression of Ang2 in the placental parenchyma, including diversity in the degree of expression in early pregnancies [25].

Conclusions

The placentation period is characterized by a weak Ang-2 cellular environment in the chorio-villous germinal site in the group of short-term compromised pregnancies, except a highly positive syncytiotrophoblast. The immunoexpression profile in vascular endothelium correlates statistically significantly with placental vascularization index and chorio-villous vascular density in stagnant pregnancies.

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

Funding
This work was carried out within the framework of the state project "Morphological approach by conventional, histo- and immunohistochemical methods of the peculiarities of the pathological profile of early placentogenesis in early term compromised pregnancies", Project code 20.80009.8007.17 P1P2 0750.

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

Conflict of interests
No competing interests were disclosed.

REVIEW ARTICLE

https://doi.org/10.52418/moldovan-med-j.66-2.23.12
UDC: 616.98:578.834.1:616.12-008.331.1

Covid-19 infection and arterial hypertension: the relationship between the two entities

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Manuscript received March 17, 2023; revised manuscript May 22, 2023; published online December 27, 2023

Abstract

Background: The aim of the study was to breakdown the relationship and its nature between the two pathological entities arterial hypertension (AHT) and SARS-CoV-2 infection. The analysis of existing literature on both topics has been conducted. Sources including journals, books, existing publications and online platforms like Google Academic, PubMed, HINARI were used as search engines. Additionally, guidelines and circulars from European Society of Cardiology, American Heart Association and other respective bodies were also referred to. Data from large meta-analyses and clinical studies were included to bring out the relationship study. Pathophysiological breakdown of the two entities, contradicting proposals regarding treatment, new treatment modalities and *de novo* onset of AHT in post-Covid infection were included to delineate the relationship between the two pathological entities. **Conclusions:** Findings emphasize that the role of arterial hypertension in SARS-CoV-2 infection is mediated through its effect on the regulation of Renin Angiotensin Aldosteron System (RAAS), inflammation and immune responses. *De-novo* arterial hypertension was also reported in post SARS-CoV-2 infection patients. Though some initial studies hypothesized that RAAS inhibitors may add to clinical adversities, most studies afterward disproved the same and in fact revealed a protective role of the same. Angiotensin Converting Enzyme 2 (ACE2) is proposed as a treatment option in SARS-CoV-2 infection.
Key words: SARS-CoV-2 infection, arterial hypertension, RAAS, RAAS inhibitors.

Cite this article
Gavriliuc S, Sanalkumar A, Jeevan S, Calin G, Sasu D. Covid-19 infection and arterial hypertension: the relationship between the two entities. *Mold Med J*. 2023;66(2):78-82. https://doi.org/10.52418/moldovan-med-j.66-2.23.12.

Introduction

Arterial Hypertension (AHT) is one of the important and major established cardiovascular risk factors and has been an integral part of the cardiovascular risk stratification systems including the SCORE chart for quite long. The proportion of hypertension in the global burden of diseases has increased from about 4.5% (0.9 billion adults) in 2000, to 7% in 2010 and was projected to rise to 1.6 billion adults in 2025 [1, 2]. With the emergence of the severe acute respiratory syndrome (SARS)-Coronavirus II infection, hypertension as an entity gained spotlight than ever before. The SARS-Covid 2 infection or simply referred to as 'COVID-19 infection' was first reported in Wuhan, Province of Hubei, China on December 31 2019, which later evolved into a global pandemic affecting the entire healthcare system and raised an entire arsenal of questions including those directed to its spread, epidemiology, pathogenic mechanisms, prevention and above all treatment and prophylaxis [3]. AHT has been identified as the most prevalent cardiovascular comorbidity in patients with SARS-CoV-2 infection that demonstrably increases the risk of hospital admissions and death. Worser outcomes like profuse lung injury, higher severity and mortality were associated mostly with AHT (30%) followed by diabetes (19%), and coronary heart disease (8%) [4]. The importance of the relationship study was further catalyzed by the pathogenic linkage between the two entities. *The aim* of the study was to delineate the relationship between Covid-19 infection and AHT and to shine light on other important and relevant areas within the relationship spectrum itself, such as antihypertensive therapy in SARS-CoV-2 infection. *The purpose* is to study and refer to the vast array of literature, and understand the intricacies of the relationship. The study has *the objective* of giving forth some clarity on the relationship between the two pathological entities and addressing some concerns linked to the same, such as antihypertensives. Analysis of this relationship is rendered complex by many factors. For instance, AHT is more common among the elderly. At the same time, the elderly was associated with a higher risk of protracting SARS-CoV-2 infection and having a worser outcome in comparison to

the general population. In addition, many elderlies have other systemic comorbidities like Type 2 Diabetes Mellitus (T2DM) and dyslipidemia. They also receive polychemotherapy which along with the above-mentioned comorbidities hinders in clearly delineating the relationship.

Material and methods

This research was conducted on the basis of vast literature on related topics and pathophysiological breakdown of both entities using journals, books, online platforms like 'Google Academic', 'PubMed', 'HINARI', respective guidelines and circulars from authorities like European Society of Cardiology (ESCAR) and American Heart Association (AHA). The literature also reviewed advancements over time and contrasting findings. An example of this would be the usage of antihypertensives. Contradicting hypotheses were put forth and intense debates for discontinuation of certain medications were found to be present initially. The need for switching from Renin Angiotensin Aldosterone System Inhibitors (RAAS inhibitors) was also put forward. For debunking the very idea of an elderly hypertensive patient acquiring SARS-CoV-2 infection being a mere co-incidence, accumulating evidence of not only their prevalence but also worser outcome, severity and lethality can be pointed out by the study [5-8]. According to these, most elderly patients are at risk for both AHT and SARS-CoV-2 infection. However, this does not mean that co-existence of both entities in an elderly patient is a mere co-incidence. AHT as a pathological entity itself has implications in SARS-CoV-2 infection affecting disease course, morbidity, outcome etc. Age-related changes have effects on individual organs and organ systems that collectively increase the susceptibility to many pathologies. There may also be other risk factors that may be prevalent in adult and adult elderly populations which complicate their relationships.

Results

The findings from a large meta-analysis that included 76993 patients with SARS-CoV-2 infection found that the pooled prevalence of AHT, cardiovascular disease, smoking history, and diabetes was 16.37%, 12.11%, 7.63%, and 7.87%, respectively [9]. It was also demonstrated that patients with severe SARS-CoV-2 infection who required ICU-admission, mechanical ventilation and death attributed to the same were found to have a significantly higher percentage of AHT, diabetes, coronary artery disease, cerebro-vascular disease, chronic obstructive pulmonary diesis (COPD), chronic renal disease, and cancer [10]. These reveal that AHT independently contributes in disease protraction risk, severity and outcome. For a better understanding of their relationship, it is necessary to review the pathogenesis. The root of this pathogenetic linkage lies in the Renin Angiotensin Aldosterone System (RAAS) and its two main regulatory axes. Recent findings demonstrated that AHT plays significant and important role in the regulation of RAAS, inflammation, immune responses, and the gastrointestinal tract [11]. SARS-CoV-2 infection itself is considered to have systemic implications on inflammatory and immune areas as well. SARS-CoV-2 pathogen enters human cells by binding its spike protein to the membrane receptor angiotensin converting enzyme 2 (ACE2) and interacting with the transmembrane serine protease 2 (TMPRSS2, widely expressed in epithelial cells at the respiratory, gastrointestinal and urogenital levels), leading to unrestrained ACE2 downregulation [12, 13]. While SARS CoV-2 infection uses ACE2 and its axes to interact with RAAS system; AHT pathophysiology and treatment pharmacology is mostly linked to the ACE/Angiotensin II/Angiotensin II receptor type 1 axis; the other axes involved in RAAS. This axis is associated with positive regulation of RAAS and hence an increase in systemic arterial blood pressure, ACE and its products (e.g. -Ang II), aldosterone etc. will be the result of its activation which translates to systemic vascular and cardiac remodeling effects. This is consistent with effects seen in long-term hypertensive patients. This axis contributes to cardio-renal remodeling by inducing prooxidative, proinflammatory, and profibrotic changes [14-16]. Conversely, the other axis ACE2/Angiotensin (1-7), Angiotensin (1-9)/Angiotensin receptor type 2 is associated with vasodilation and decreasing blood pressure. Additionally, MAS receptor plays protective roles in a variety of human target organs by reducing cardiac hypertrophy and pathological cardiac remodeling and preventing the occurrence of heart failure after myocardial infarction [17, 18]. ACE axis activation or ACE2 axis downregulation hence results in target organ damage, as one works opposite to the effects of the other. Additionally, SARS-CoV-2 infection was found to downregulate the ACE2 pathway [19]. This leads to an elevation or increase in angiotensin II through ACE pathway. This is brought upon by the decrease in ACE2 which implies a significant reduction in the conversion of angiotensin to angiotensin (1-7) [4, 19-20]. The ACE 2 fall will also lead to decreased degradation of Ang I and Ang II leading to their increased levels in plasma, which brings along with it the prooxidative, pro-inflammatory and profibrotic changes associated with it [21-25]. This also means that there will be higher aldosterone production which will lead to K+ excretion via the urine, sodium (Na) retention and inflammation. Potassium (K+) excretion over time will lead to hypokalemia which was demonstrated to be biological marker or predictor of worsening outcome of the disease [26]. Hypokalemia, a clinical marker of this complex interaction indicates sodium retention, raising ACE, both of which translates to prohypertensive, pro-inflammatory effect in SARS CoV-2 infection and signifies its indulgence in AHT pathophysiology. In AHT the role of RAAS is mostly implicated, as it is the major established regulator of blood pressure in the human body. Increased ACE and upregulated ACE/Ang-II/AT1R is mostly responsible for hypertension. A genome wide association study of Korea and other countries in-

ferred that ACE among the RAAS components has the strongest association with AHT after an adjustment for sex, age and weight [27]. Since the pathogenetic link between the two entities resides in RAAS, antihypertensive drugs became a hot topic. This was because a large proportion of patients used RAAS inhibitors for their treatment. Two of the most commonly employed groups of drugs are ACEIs which reduce the generation of Ang II by inhibiting ACE and ARBs which reduce blood pressure by blocking the binding of Ang II with AT1R. Both of them also increase the level of ACE2 and have been extensively used in patients with AHT and other cardiovascular diseases to maintain the stability of blood pressure and reduce the risk of adverse events in cardiocerebrovascular system and kidney [28, 29]. The argument was that these drugs may contribute to higher disease protraction or worser outcome and extensive lung injury as more ACE2 meant more susceptible to pathogenic entry. The question arose to whether discontinuation of these drugs was necessary in SARS-CoV-2 infection patients. Many early studies hypothesized that in fact these drugs may bring potential harm. Later extensive studies showed a protective effect of these groups of medications against lung injury. ACE2 independently demonstrated the ability to reduce lung tissue damage, associated inflammation and severe acute lung failure [30]. To correlate this information, experimental studies were conducted which also revealed protective role of these drugs against lung injury [31]. An Intensive Care Unit team demonstrated and showed that an increase in angiotensin 1 to 10 and a decrease in angiotensin 1 to 9 (its ACE2 processing product) was correlated with a poor prognosis in ARDS [20]. In response to the proposed adverse effects of ARBs or ACE inhibitors in risk and poorer outcome, many studies were done which found no adverse role and even protective role of the same drugs. Other correlation studies also revealed no increase in risk or mortality [32-34]. The European Society of Cardiology (ESCAR) and other medical associations also advised not to change the treatment regimen of ACEIs/ARBs for patients with hypertension during the COVID-19 pandemic, unless supported by definitive clinical evidence. For patients who are currently receiving RAAS antagonists for conditions for which they are known to be advantageous, such as hypertension, heart failure or ischemic heart disease, the ACC, HFSA and AHA advise continuing these medications. It was further stated that specific treatment decisions should be made based on each patient's hemodynamic situation and clinical presentation in the event that individuals with cardiovascular illness are identified with SARS-CoV-2 infection. In fact, by preventing the pro-inflammatory effects of Ang II and by promoting the anti-inflammatory, anti-organ remodeling and tissue protective effects demonstrated in the lungs, these drugs can even have a protective role. Increased soluble ACE2 in the circulation could also serve as a binder of the SARS-CoV-2, thereby protecting other ACE2 bearing organs but most importantly the lungs themselves. In this aspect recombinant ACE2 is also being extensively studied [35]. It was proposed that high-affinity variants of sACE2 can be engineered using mutagenesis, which may serve as decoy receptors for the pathogen. These variants by interacting with the spike proteins can serve as a competitor for native ACE2 in SARS-CoV-2 infection. This can prevent the ACE2 downregulation as more native ACE2 will be available to convert angiotensin to Ang (1-7) and Ang (1-9) which can reduce inflammation potential [36-38]. In search of other treatment options in SARS-CoV-2 infection, the medical research community focused on viral entry mechanism to find loop holes. In light of this direction, it was also demonstrated that pharmacological inhibition of proteases like TMPRSS2 or CatB/L reduced SARS-CoV-2 S-pseudo typed vesicular stomatitis virus or lentivirus entry [13, 39-40]. Future treatment aspects also pose a need to further analyze host entry co-factors like Neuropilin-1, CD147, phosphatidylserine receptors, heparan sulfate proteoglycans, sialic acids, and C-type lectins. Another interesting but understudied area is the *de novo* onset of hypertension after SARS-CoV-2 infection. SARS-CoV-2 infection can cause *de-novo* hypertension or worsen existing hypertension by its effect on RAAS or endothelium [41, 42]. A retrospective study in a tertiary care center summarized that there is a real possibility that more than 10% of the general population are going to be affected by AHT post-SARS-CoV-2 infection, many of them undetected, especially among patients with no prior conditions in their medical histories [43]. Among patients, 32 of them (16.08%) had either new onset arterial hypertension (15 patients) or a worsening of an existing hypertensive condition (17 patients) related to COVID-19. Another study found out new onset hypertension in 18 patients (12%), while diabetes mellitus, coronary artery disease, and COPD were not significantly different between admission and post-covid infection period [44].

Discussion

Rodriguez et al. earlier pointed out that SARS-CoV-2 is oftentimes underestimated as an infectious disease and gave insight to its effect on immune dysregulation caused by it [45]. The same inference can be obtained while analyzing the entity for connecting the dots between AHT and SARS-CoV-2 infection. Immune and inflammatory dysregulations are caused by both AHT and SARS-CoV-2 infection. It is fascinating to observe that both these entities are interspersed within RAAS. AHT acts through ACE axes while SARS-CoV-2 infection uses ACE2 to interact with RAAS. Both these axes function opposite to each other and ACE2 downregulation induced by SARS-CoV-2 infection can work parallel with AHT to promote proinflammatory, pro-oxidative and organ damage inducing activities. The immune deregulatory activities and proinflammatory activities also bridge these entities. Due to the complex nature of interaction of SARS-CoV-2 infection with the

RAAS, patients who recover from the infection should be encouraged to do mandatory follow-ups as *de novo* onset of AHT or worsening of existing AHT has been found in many patients. This can also be considered as a marker of damage induced on the vessel structures by SARS-CoV-2 infection by ACE2 downregulation. The potential of ACE2 as a decoy receptor also signifies the importance of their common pathophysiological roots. However, this also signifies the need to study host-factors and role of other proteases so that the combined knowledge can be laid down as a foundation into practical treatment options.

Conclusions

In simple terms it can be said that SARS-COV-2 infection and AHT are 2 entities acting on two regulatory axes of RAAS. Broadly speaking these two axes act opposite to each other, with areas of cross interactions and intricacies with their end effects culminating in the RAAS.

Regarding treatment RAAS inhibitors were not proved to have a detrimental effect on disease protraction or severity as of now. Additionally, by raising ACE2, inflammation and organ injury can be reduced as well. By realizing the effects of ACE2 in organ protection and inflammation and owing to it serving as a Covid entry receptor, engineered ACE2 is studied as a potential therapeutic agent.

However, other host factors and their interactions need to be further broken down. The pathogenic linkage between SARS-CoV-2 infection and AHT is complex. In a susceptible hypertensive patient SARS-CoV-2 infection can contribute to various immune dysregulations and can give rise to vicious cycles which once started can be difficult to break.

The advantage of therapy focusing on pathogenic entry is that if properly intervened, added benefit of preventing appearances of such vicious cycles and immune-inflammatory cycles can be prevented. The *de novo* onset of AHT in patients post SARS-CoV-2 infection may reveal the existence of a bidirectional relationship as opposed to a simple cause- effect relationship between the two entities.

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SG conceptualized and designed the idea; AS, SJ conducted literature review, and wrote the first version of the manuscript; SG and DS revised critically the manuscript and completed the final text. All the authors read and approved the final version of the manuscript.

Funding
This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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

Conflict of interests
The authors have no conflict of interests to declare.

https://doi.org/10.52418/moldovan-med-j.66-2.23.13
UDC: 611.149:616.149-008.341.1

Morphofunctional traits and reactivity of the portal vein

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Manuscript received May 10, 2023; revised manuscript July 17, 2023; published online December 27, 2023

Abstract

Background: Portal vein is the most enigmatic vessel of our body because it regulates own contractile performances using a special pace-maker mechanism represented by cells of Cajal. The contribution of various metabolic mediators and natural vasotropic agents in the control of the portal blood circuit is much less studied compared to the arterial system in general and the hepatic system in particular. The studies designed on the structure, function, and reactivity of the portal vein in different preconditioning have brought some common but also distinct evidence of the arterial system. Nitric oxide production is higher partly due to reduced arginase expression, but muscular media is thinner. Periodic spontaneous contractions directed towards the liver gate are characteristic for portal vein (PV), and the longitudinal muscle fibers are considered to be responsible for this phenomenon. Spontaneous rhythmic oscillations of the cells of Cajal are triggered by increasing calcium ion concentration leading to their depolarization. PV constrictor effect of phenylephrine is dependent on the activity of receptors to ET-1. For PV is characterized the acetylcholine induced contraction either *in vivo* or *in vitro*, and this effect is thought to be dependent on ET-1.

Conclusions: The establishment of main particularities of portal vein reactivity of action of different paracrine, endocrine, and hemodynamical stimuli represents an important tool for prediction of contractile disorders leading plausible to portal hypertension. Likewise, a well proven interplay between cholinergic and adrenergic stimulations and on the other hand between Ang II and ET-1 actions must be a support for pharmacological modulating of portal vein reactivity disorders.

Key words: portal vein, morphofunctional traits, vein reactivity.

Cite this article
Ojog V. Morphofunctional traits and reactivity of the portal vein. *Mold Med J.* 2023;66(2):83-90. https://doi.org/10.52418/moldovan-med-j.66-2.23.13.

Introduction

Portal vein is the most enigmatic vessel among vascular bed at least due to a unique capacity to realize a rhythmic spontaneous contraction so needed for a normal blood influx into the liver. Likewise, portal vein consists of a lot of receptors placed on both sensorial layers of the wall, endothelium, and muscular media, being high receptive to action of cholinergic and adrenergic natural stimuli, of Ang II, ET-1 and other vasoactive peptides and oligopeptides. The complex response of portal vein on neuroendocrine and hemodynamical signals mediated basically by calcium ions, nitric oxide, cAMP, and cGMP should complete and synchronize the spontaneous pace-maker-induced contraction in order to ensure an adequate blood pressure of about 10 mm Hg, capable to reach a suitable gradient of pressure between vena cava inferior and vena portae.

Morphofunctional traits of the portal vein

The portal vein (PV) provides 75% of blood flow to the liver anatomically positioned in the gastrohepatoduodenal ligament adjacent to the hepatic artery, which brings the last quarter of blood to the liver parenchyma by confluence of 3 big veins: gastric, splenic, and superior mesenteric. In the adult human body, the PV has an average length of 7-8 cm and a diameter of about 10 mm, and before entering the liver it ramifies into 2 branches: left and right. The role of PV in the body's homeostasis is notable, given the fact that it ensures the liver's access to the nutrients and metabolites of gastrointestinal digestion necessary for normal liver function, and on the other hand, it ensures the influx of metabolic toxins for the body's purification consistent with detoxification. The share of blood used by the liver from the general circulating minute volume is about 25%, so PV compliance in this context is announced as an important arrangement in view of the fact that hepatocytes are the cells with the highest level of perfusion [1]. At the same time, the blood stored in the liver can be an important element engaged in the cardiovascular mechanisms to compensate for fluid losses and dehydration of the body. Even a moderate elevation of PV pressure above the normal value (7-10 mm Hg) leads to increased PV/inferior vena cava pressure gradient (maximum value = 5.0 mm Hg) or an increase >6.0 mm Hg vs. of the right atrium and, respectively, at the risk of peripheral hemodynamics: from the risk of congestion and transudation in the abdominal cavity to the constriction of the renal arteries [2, 3]. The latter phenomenon is detected in the hepato-renal syndrome, which is triggered in the liver, when the portal blood flow

is compromised and associated with an increase in adenosine concentration, reflexively causing the activation of the renal sympathetic system, the release of renal vasoconstrictor agents and, respectively, constriction of the renal arteries [4]. Therefore, the feasibility of PV is orchestrated by a multifactorial system that is based on the conclusive cooperation between vegetative influences and humoral factors with paracrine and endocrine action.

The portal vein has sympathetic and parasympathetic innervations, the proximal mediators being involved in the regulation of basal vascular tone. Vegetative innervations are also common for the hepatic artery and bile ducts. Postganglionic sympathetic nerve fibers originate from the superior mesenteric and celiac ganglia, which in turn receive preganglionic fibers from the T7-T12 spinal ganglion. Parasympathetic fibers have a vagal origin and, similar to sympathetic ones, are involved in controlling the activity of paravascular liver neurons under the action of osmotic and metabolic stimuli, which send efferents to the main centers of the brain [5]. Recent reports indicate the role of the imbalance between sympathetic and parasympathetic influences with the predominance of adrenergic stimuli not only in affecting basal vascular tone, but also in hepatic metabolic disturbances, primarily carbohydrate and lipid [6]. Remarkably, the value of the ratio of sympathetic and parasympathetic nerve endings in the PV is dependent on the phenotype of the mammal, but it is normally accepted that the density of parasympathetic fibers predominates, their effects being, however, closely related to the functionality of the vascular endothelium. The increase in sympathetic activity in the regulation of PV tone or the impairment of parasympathetic control are estimated as trigger factors of the elevation of portal venous pressure, as well as intrahepatic vascular resistance in the arterial system inherent in various chronic liver diseases. J. Van Limmen et al. (2022) demonstrated that the sympathetic mediator, norepinephrine, is the cause of the reduction in portal blood circulation and hepatic arterial flow, an effect mediated by α1-adrenergic receptors expressed on vascular smooth myocytes [7].

The contribution of various metabolic mediators and natural vasotropic agents in the control of the portal blood circuit is much less studied compared to the arterial system in general and the hepatic system in particular. Under this aspect, the importance of highlighting the particularities of PV reactivity to the action of adenosine, acetylcholine, bradykinin, endothelin-1 (ET-1), angiotensin II (Ang II), adenosine triphosphate (ATP), lactate, pyruvate, is addressed. The regulation of the basal tone of the PV occurs under conditions different from those of the vascular zones, since the toxic substances of the portal system, after being metabolized in the liver, have an independent vasoconstrictor or relaxant action, a fact that interferes detrimentally with the intrinsic control system of compliance and portal pressure. In addition, the physiological entity of the "hepatic vascular buffer" system imposes in conditions of reduced blood flow to the liver via the hepatic artery (e.g., in atherosclerosis, stenosis, thrombosis) the increase of the portal circuit due to the dilatation of the PV, in order to maintain the adequate perfusion of the parenchyma. Accordingly, the presence of a liver ischemia signaling system intended to induce the dilation of the portal vein commensurate with the constriction of the hepatic artery, primarily through paracrine actions, is appropriate in this regard. The inverse relationship is also plausible, i.e., relaxation of the hepatic artery under conditions of increased portal pressure caused by exaggerated contraction of PV smooth myocytes. At present, the regulatory mechanisms of the "hepatic vascular buffer" system are apocryphal and require further elucidation. The research designed on the structure, functionality, and reactivity of the portal vein in different preconditioning have brought some common but also distinct evidence of the arterial system.

First of all, the vascular endothelium represents, as in the arteries, a single layer of cells that, beyond the mechanical barrier, performs important homeostatic functions. The capacity of the venous endotheliocyte to synthesize nitric oxide is higher compared to that of the arteries. This is due to the higher expression of endothelial nitric oxide synthase (eNOS) and in part to the reduced expression of arginase that engages the NO substrate (L-arginine) in the ornithic cycle. Regarding the contribution of the venous endothelium in the control of hemostasis, it should be noted that the expression of thrombomodulin, antithrombin III, as well as the receptors of the annexin-5 family compared to the anticoagulant protein C is estimated at similar levels to the arterial segment. At the same time, the expression of endothelial receptors for von Willebrand factor is considered to be lower, since the pro-coagulant pentamer in the smooth venous circuit does not discover all 5 ligand sites similar to arteries.

Second, the muscular media of the venous wall is thinner, and the elastic laminae that separate the intima (i.e., internal elastic lamina) and adventitia (i.e., external elastic lamina) media are missing. Smooth myocytes are numerically depleted and arranged centrally and longitudinally circularly, which are placed in the square of the circular ones or outside them. The muscular media of the arteries does not contain longitudinal muscle fibers. In the veins of the lower limbs, the structural arrangement of the muscular media excels through the formation of the valvular apparatus. The ratio of type I fibrillar collagen to type III in the extracellular matrix (ECM) inherent in the muscle medium is higher versus the index characteristic of arteries, which is in an agreement with the much higher compliance of veins. The expression of type IV reticular collagen is considerably reduced with the lack of elastic membranes. The adventitia has a composition similar to arteries and contains fibrillar collagen fibers, elastic fibers and all the cells of the MEC: fibroblasts, mast cells, macrophages.

The structure of the rat portal vein is better studied. The results obtained brought to highlight some significant features [8, 9]:

The endothelial layer of the extrahepatic segment of the PV has folds, which anatomically correspond to the areas of the venous wall containing well-developed longitudinal muscle fibers. Thus, it is suggested that the latter participate in the formation of endothelial folds. Endothelial cells are arranged between the folds of the intima in a circumflex fashion. This finding indicates that circumferential blood flow occurs locally on the luminal surface between the intimal folds. Endothelial cell alignment is determined by periodic mechanical loading, which in turn is contiguous with circular and longitudinal myocyte contraction. In the conditions when the endotheliocytes in the cell culture are exposed to periodic stretching and relaxation, then their long axis is reoriented perpendicular to the extension force. Such an orientation of the endothelial cells ensures *in vivo* a greater resistance against periodic mechanical stress and, therefore, a minimal deformation of the cell unit. Intimal folds in rats are circumflex and arranged parallel to each other.

Periodic spontaneous contractions directed towards the liver gate are characteristic for PV, and the longitudinal muscle fibers are considered to be responsible for this phenomenon, attested in several types of mammals (rodents, cats, rabbits, guinea pigs, etc.). The contraction of the longitudinal muscle fibers is stronger in the distal segment and decreases towards the liver gate, and the intima with circularly arranged endothelial cells is less exposed to tension and stretching stress.

Longitudinally arranged smooth myocytes are exposed in the outer zone of the PV wall, and circumflex myocytes – in the inner zone. These 2 types of myocytes have distinct contributions to the overall mechanical activity of the portal vein. The longitudinal myocytes are meant to counteract the gravitational force of the blood, and the contraction of the circumflex myocytes relieves the hydrostatic pressure of the blood. Remarkably, the elevation of the hydrostatic pressure of the blood during the postnatal period of body development leads to the more pronounced development of the circumflex smooth myocytes compared to the longitudinal fibers. It is also worth paying attention to the fact that in the proximal segment the ratio of circumflex myocytes to longitudinal cells is higher compared to the distal segment of the portal vein. In the proximal segment of the PV the hydrostatic pressure of the blood is obviously higher versus the distal segment.

The periodic spontaneous contractions of the portal vein are corroborated to be triggered and controlled by cells of Cajal, the presence of which was initially proven in the rabbit portal vein in the area of the muscular media and reported in 2004 by M. Harhun et al. [10]. By applying confocal microscopy and electron myography, the connection of these cells with smooth myocytes of the PV was established. Spontaneous rhythmic oscillations of cells of Cajal were triggered by increasing calcium ion concentration leading to their depolarization. Conceptually, it is important to mention that the depolarization of the smooth myocyte occurred at a distance of about 4 sec after the depolarization of the Cajal's cell. At the same time, the depolarization of the adjacent Cajal's cell was followed, only much faster: after about 200 msec. Thus, it was rightly concluded that the Cajal's cell can serve as a pacemaker of the muscle medium of the PV, and the depolarization of the smooth myocyte can be not only the repercussion of the electrical contact between them, but also the result of the action of some mediators released by the Cajal's cell able the induce depolarization of the adjacent myocyte.

Although discovered precisely in 1889 by the Spanish specialist in neuroanatomy, Santiago Ramon Cajal, these cells that bear his name (i.e., cells of Cajal) have remained an enigma to this day in terms of their functional role in various vital organs. Being abundantly detected in the gastrointestinal tract, their removal led to the disappearance of the slow depolarization wave of intestinal smooth myocytes, including under conditions of electrical stimulation. For a long time, experimental research could not highlight the mechanisms of the pacemaker activity of Cajal's cells, and the accumulated evidence points to the role of calcium unloading from the endoplasmic reticulum of the smooth myocyte, the activation of L-type channels in the myocyte membrane (the second source of calcium during of depolarization) and/or activation of Na-K membrane pumps.

The morphological remodeling of the portal vein, a ubiquitous phenomenon in portal hypertension, manifests itself through notable structural and geometric changes especially in the intima and the muscular media, which result in endangering the reactivity of the vein to the action of various natural stimuli. C. Ho et al. (2019) demonstrated that hypercholesterolemia and elevation of the circulating level of oxidized low-density lipoproteins are imposed by endothelial damage of the PV and its inflammation in association with the formation of imminent atherosclerosis plaques against the background of increased serum content of pro-inflammatory interleukins in patients with fatty infiltration of the liver [11]. Endothelial injury resulted in portal hypertension with detrimental effect on liver perfusion. At the same time, it is admitted that PV remodeling is accompanied by a decrease in the population of Cajal's cells due to the activation of their apoptosis induced by inflammatory mediators (extrinsic pathway of apoptosis induced by TNF-α), oxygen free radicals, energy deficit (intrinsic or mitochondrial pathway induced by cytochrome C) or their differentiation under the action of the same factors [12]. Portal vein endothelium controls circumflex and longitudinal smooth myocyte functionality via endothelial derivatives, NO and prostacyclin or prostaglandin I2 (PGI2). M. Trindade et al. (2017) demonstrated in cell culture experiments that the basal level of NO in PV endotheliocytes is similar to the characteristic index of inferior vena cava endotheliocytes, but incubation of the culture with ET-1 did not increase NO expression in PV, unlike the incremental response [13]. On the other hand, the basal level of PGI2 in PV endotheliocytes was significantly higher compared

to that in the inferior vena cava, but analogously to the change in NO, incubation of the culture with ET-1 was not imposed by increasing prostacyclin production, contrary to caval endotheliocytes. So, the endothelium of the portal vein is functionally marked by notable particularities, which can be important landmarks regarding its reactivity in different hemodynamic, paracrine, and neuroendocrine preconditioning. The PV endothelium expresses different types of receptors, the activation of which promotes the contraction and relaxation of the muscular media, consequently determining the reduction or increase of blood flow to the liver. Currently, the presence of the following types of receptors is proven, the expression of which is dependent on the phenotype of the mammal:

- Muscarinic type 1 and 3 (M1 and M3) to acetylcholine. The presence of M5 receptors is also indicated in rabbits.
- H1 to histamine (their expression in rat VP is low).
- ETB to endothelin 1.
- Alpha 2 adrenergic to norepinephrine (NE).
- AT2 to Ang II.
- Receptor mass to angiotensin 1-7.
- VIP2 to vasoactive intestinal peptide (VIP)
- B2 to bradykinin.

The effect of stimulating these receptors is in direct correlation with the ability of the PV endotheliocyte to release NO or PGI2, and in liver dysfunction their share and, respectively, the final effect can be notably influenced. Thus, I. Bockh et al. (2011) demonstrated in situ in rats with portal hypertension that stimulation of the hepatic branch of the vagus nerve with the frequency of 5 Hz decreased the value of hypertension against the background of increasing the concentration of acetylcholine in the portal circuit [14]. Pretreatment of animals with L-NAME (eNOS inhibitor) abolished the vagal effect on portal hypertension, a fact indicating the role of NO in promoting the cholinergic effect. Vagal stimulation with a double frequency (i.e., 10 Hz) led to an increase in the concentration in the portal circuit of vasoactive intestinal peptide also associated with a reduction in portal hypertension, an effect that was not abolished by the administration of L-NAME, but impaired in pretreatment with the blocker VIP2. Of note, both mediators of vagus nerve stimulation decreased intrahepatic vascular resistance, an important mechanism of portal hypertension. Therefore, vagus nerve stimulation may be an opportunity for pathogenetic treatment of portal hypertension and its impending consequences.

The portal vein smooth myocytes express the following receptors:

- M2 to acetylcholine. Their stimulation by acetylcholine produces contraction of smooth myocytes.
- ETA and ETB in distinct proportion in different mammals, but with notable superiority of ETA receptors: from 5:1 to 8:1. Remarkably, ET-1 is an important factor in the evolution of liver fibrosis, leading to increased hepatic vascular resistance and portal hypertension, respectively. ETA receptor antagonists improve both liver parenchymal remodeling and portal vein reactivity.
- AT1 to Ang II.
- Alpha 1 adrenergic to NE.
- Beta 2 adrenergic to epinephrine.
- H2 to histamine.

Activation of the majority of receptors expressed by smooth myocytes of the PV triggers and supports the remodeling process of the wall of the portal vein, which accelerates and facilitates the evolution of portal hypertension. In this context, the role of the TMEM16A protein in promoting the proliferative and growth effects of ET-1 and Ang II, the main vasotropic factors involved in the control of portal vein functionality, is important [15, 16].

Integrins, syndecans and alpha-dystroglycans are receptors of smooth myocytes of the PV, whose role in the remodeling of the portal vein is targeted through the prism of controlling the contractile phenotype of the muscle cell. Their activity is considered to be in tune with the functionality of aldosterone receptors, the stimulation of which stimulates the expression of MEC fibroblasts and the exaggerated synthesis of type I and type III fibrillar collagen. The impact of aldosterone on vein remodeling is estimated to be below the hormone's impact on arteries and myocardium at least because of the lower expression of the receptor in veins.

In vitro reactivity features of the portal vein

Adequate control of blood flow to the liver through the portal vein requires a feasible system to regulate reactivity to various paracrine and neuroendocrine actions, accomplished in contiguity with the property of the portal vein to spontaneously contract due to the presence of Cajal's pacemaker stromal cells. The response of the portal vein significantly influences the evolution of portal hypertension in view of the fact that a blood congestion caused by venous dilatation is a factor facilitating the formation of esophago-portal and gastro-portal anastomoses and the risk of their consequences. Fundamental research carried out *in vitro* on isolated rings or strips of portal vein taken from different laboratory animals is the main lever for studying the peculiarities of the response of the vein exposed to the action of a wide range of natural agents with physiological and pathological action, pharmacological substances, variations in concentration of ions, etc. The basic components of the experimental protocols involve estimations of the response of the portal vein to cholinergic, adrenergic, Ang II, ET-1, vasopressin actions, extra- and intracellular changes in the concentration of calcium, magnesium, sodium, potassium, pH value, etc.

Any experiment begins with the attestation of the spontaneous contraction of the portal vein caused by the cells of Cajal and which is imposed by the amplitude and frequency of the contraction depending on the phenotype of the laboratory animal. The presence of these spontaneous contractions indicates a sampling of bands or isolated rings without traumatizing elements, as well as the feasibility of

Krebs perfusion solution. In a recent publication W. Al-Aghawani (2022) demonstrated in this context the spontaneous phasic rhythmic contractions of the portal vein benzo taken from Sprague Dawly rats with the respective amplitude and frequency values [17]. The action of phenylephrine added to the infusion solution up to a concentration of 10-6 M obviously led to an increase in both contraction amplitude and frequency. The constrictor plateau induced by phenylephrine and which is mediated by the activation of α1-adrenergic receptors expressed on smooth myocytes is used as a benchmark to estimate the effect of agents with vasorelaxant action.

A. Chies and P. Rossignoli demonstrated that the constrictor effect of phenylephrine is dependent on the activity of receptors to ET-1, thus, premedication of the isolated portal vein with antagonists of ETA and ETB receptors (e.g., BQ-123 and BQ-788) decreased amplitude of adrenergic contraction [18]. A similar contractile impairment was detected in the denuded portal vein model, given that the endothelium is the main source of ET-1 synthesis, and inhibition of eNOS by L-NAME did not alter the contraction of the portal vein with intact endothelium consistent with the action of phenylephrine.

The portal vein, like the arteries, is influenced by hemodynamic stress, which triggers the process of vascular remodeling, which in the context of the venous wall is conclusively based on the proliferation of smooth myocytes. The evolution of portal vein remodeling against the background of portal hypertension is associated with increased ET-1 production and the expression of specific receptors ETA and ETB in tune with the activation of the respective endotheliocyte genes, including the genes responsible for NO production. Nitric oxide released into the wall of the portal vein is intended to counteract the effect of ET-1 in stimulating the proliferation of smooth myocytes and endotheliocytes. In the arterial wall, NO released in excess in hemodynamic stress also serves to counteract the vasoconstrictor effect of ET-1.

In the arteries, NO production is much more pronounced under hemodynamic stress, compared to the portal vein. With this connotation, the hypothesis based on the data obtained in the research of the portal hypertension model reproduced in pigs and rats is important, that the evolution of the remodeling of the portal vein is imposed by the more significant increase in the production of NO, a phenomenon defined as "venous arterialization" [19]. Its presence in the context of portal hypertension and remodeling of the portal vein has notable repercussions, first of all the dilation of the mesenteric veins and the increase of venous inflow in the portal vein, which will accentuate the portal hypertension and the remodeling of the portal vein, and on the other hand it will increase the venous congestion important in the formation esophago-gastro-portal anastomosis. What are the molecular mechanisms supporting the activation of genes that may be involved in the genesis of the phenomenon of "venous arterialization" remain complicated even now. Increased insulin-like growth factor receptor expression is suggested to be inherent in the process of venous remodeling associated with the progression of portal hypertension.

The potentiation of the vasoconstrictor and vasorelaxant response detected in portal hypertension understandably addresses the particularities of the adrenergic, angiotensin, endothelin and vasopressin reactivity of the portal vein in connection with the cholinergic response. Activation or inhibition of alpha1-adrenergic receptors results in changes in the expression and affinity of muscarinic (M3) receptors, which mediate the action of acetylcholine. Moreover, it is characteristic of the portal vein to change the activity of beta-adrenergic receptors in the context of blocking alpha-adrenergic receptors and vice versa.

Regarding the effect of cholinergic stimulation on the reactivity of the hepatic veins and the extrahepatic portae system, the data obtained *in vivo* demonstrated that the administration of acetylcholine leads to venous constriction, an effect dispensable by the action of atropine, but annihilated by phentolamine. This effect was also confirmed *in vitro*, being suggested that acetylcholine stimulates the synthesis and release of the constricting endothelial factor, ET-1, an effect plausibly mediated through nicotinic receptors, as their blockade by tubocurarine diminishes the constricting effect of acetylcholine on isolated rings of the portal vein.

There are solitary reports of the action of other members of the endothelin family (e.g., ET-2 and ET-3) on the response of the portal vein. Thus, both ET-2 and ET-3 have been shown to induce contraction of isolated rings of the portal vein and potentiate spontaneous contractility in a manner dependent on the concentration of oligopeptides in the perfusate, but in proportion underlying the effect of ET-1. The constrictor effect of ET-1 was stronger compared to that of bradykinin, Ang II, phenylephrine, thromboxane A2 (TxA2), and substance P, but underlying the constrictor plateau induced by depolarization with 80 mM KCl solution. The gradual reduction of the calcium ion concentration to zero caused the decline of the constrictor plateau, but not to the isoline, which was recovered with the addition of the cation in the Krebs infusion solution, a fact that confirms the role of extracellular calcium in the endothelin contraction of the portal vein and, in part, of intracellular Ca. L-type Ca channels have a significant role in promoting ET-1-induced contraction, but not the ultimate one, as their blockade with Nicardipine did not completely abolish the constrictor response. Blockade of T-type calcium channels by NiCl2 reduced the constrictor plateau more considerably compared to Nicorandine, but also the annihilation of the response was relative. Therefore, these results highlight the role of both types of calcium channels (L- and T-type) in achieving the contraction of the portal vein under the action of ET-1, which is released from the endothelium, but also record the input of other calcium channels, such as would be receiver-controlled channels and/

or non-selective channels. Regarding the release of ET-1 in the stimulation of the vessel with acetylcholine, it is important to mention that hypoxia and acidosis are factors that increase the endothelial production and release rate of ET-1, a fact that can significantly influence the reactivity of the cholinergic portal vein. Regarding the role of intracellular calcium in promoting the constricting effect of ET-1, the ability of the oligopeptide to stimulate phospholipase C and trigger the phosphoribosyl cascade mediated by prokinase-C, and IP3 and the resulting diacylglycerol mobilize calcium from the sarcoplasmic reticulum.

PV muscular media depolarization by ET-1 followed by extracellular calcium influx is evidenced by the fact that activation of K-ATP-dependent channels by Cromakalim, resulting in hyperpolarization of the muscle medium, prevented contraction of the rat portal vein. At the same time, the hyperpolarization of the smooth muscles did not abolish the constrictive effect of ET-1 on the portal vein in the absence of calcium in the perfusate, which justifies the feasibility of the constrictive mechanism of ET-1 linked to the IP-3/diacylglycerol system. It should be noted that a similar effect of Cromakalim was also obtained on isolated human portal vein [20]. The contribution of K_{ATP} channels in the control of smooth muscle reactivity proven in research on the rat portal vein is also confirmed on the pig detrusor muscle, a fact that substantiated the development of pharmacological remedies with a relaxing effect on the smooth muscles through the hyperpolarization mechanism.

The connection between K_{ATP} channels and the action of ET-1 is also claimed by the fact that blockade of these channels by Glimenclamide not only annihilates the vasorelaxant effect of Cromakalim but potentiates ET-1-induced contraction of the portal vein. Similar effect of K_{ATP} channel activation on portal vein reactivity to ET-1 action was also found in adrenergic stimulation with phenylephrine. Furthermore, removal of calcium from the Krebs perfusate abolished the impending contraction of the action of the alpha-1 adrenergic receptor agonist, phenylephrine, on isolated rings of the portal vein (fig. 1).

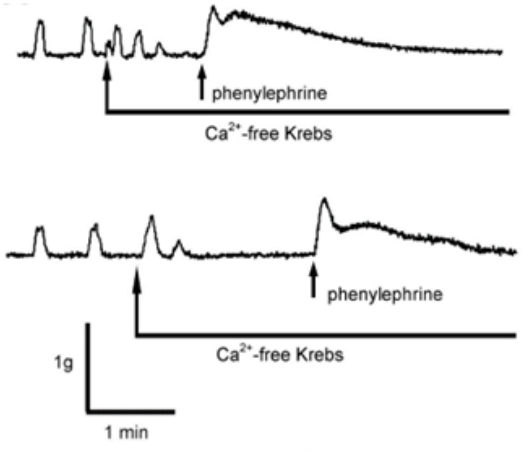

Fig. 1. Effect of phenylephrine on contraction of portal vein perfused without calcium [21]

Adrenergic and endothelinic contraction of the portal vein is naturally counteracted by nitric oxide, the level of which in reproduced portal hypertension in rats has been shown to be significantly elevated compared to intact animals. The contractile effect of phenylephrine (0.1 mM) and KCl solution (10-80 mM) was diminished relative to the reference pattern. The concentration of KCl solution required to induce ½ of the maximum contractile plateau of isolated rings by the depolarizing mechanism was significantly higher.

Thus, under the prism of the exegesis of the role of NO in promoting the reactivity of the portal vein, 2 important cardinal aspects are announced:

(1)- NO released by eNOS, as well as exogenous NO released in nitrate metabolism (e.g., sodium nitroprusside) diminishes the constrictor plateau induced by phenylephrine and ET-1.

(2)- Acetylcholine, although it stimulates eNOS to produce NO, induces contraction of the portal vein, an effect partly dependent on the stimulation of the endothelium to release ET-1. Denudation of the portal vein reduces the contractile activity of acetylcholine, which demonstrates the superiority of the ET-1 mechanism in promoting cholinergic reactivity over the NO mechanism.

These inherent subtleties are important in explaining the evolution of portal hypertension related not only to the increase in hepatic vascular resistance (e.g., inflammation, fibrosis, cirrhosis, etc.), but also to the peculiarities of the response of the portal vein, as well as in the development of the prevention of the clinical manifestations which are characteristic for portal hypertension. The main causes of increased circulating NO levels in portal hypertension are not fully established. The role of neuroendocrine activation in liver diseases is assumed, which increases the amplitude of hemodynamic stress, which results in increased eNOS expression, as well as increased inducible NOS (iNOS) expression against the background of increased inflammatory response and oxidative stress.

L. Caracuel et al. (2019) demonstrated an increased capacity of NO production and arterial endothelium, in the context of phenylephrine stimulation of the isolated rat mesenteric artery with acute or chronic liver disease [22].

There are reports calling for the ability of the vascular endothelium to synthesize and release ad-luminal and ab-luminal manner not only ET-1, but also catecholamines, norepinephrine, and epinephrine. Their autocrine and paracrine action on the cells of the vascular wall triggers the exaggerated production of oxygen free radicals, which results in the premature metabolism of NO and the increase in the expression of pro-inflammatory cytokines [23, 24]. Cumulatively, these factors alter the vascular reactivity of both arteries and veins.

Under the hood of the importance of the reactivity of the portal vein in the evolution of portal hypertension and its clinical manifestations, the effect of different morphofunctional improvement remedies of the liver or other consumed substances on the motor activity of the vein

must also be addressed. Thus, the caffeine found in coffee is interesting in this context, given its beneficial action on liver fibrosis and cirrhosis by stimulating angiogenesis, mitigating oxidative stress and inflammatory mediators. Basic research has demonstrated that caffeine induces contraction of isolated rings of the portal vein, engaging in exercise both the intracellular calcium stored in the sarcoplasmic reticulum (SR) and the extracellular cation [25]. Blocking the ryanodine receptors expressed on the SR leads to the annihilation of 35-40% of the phasic contractions induced by caffeine, which proves that by activating these receptors, caffeine produces the release of calcium from the SR deposits. Evidence of the role of extracellular calcium is the progressive reduction of the plateau constrictor of the isolated rings of the portal vein induced by caffeine in their perfusion with the reduced concentration of calcium or in conditions of premedication with calcium antagonists (fig. 2).

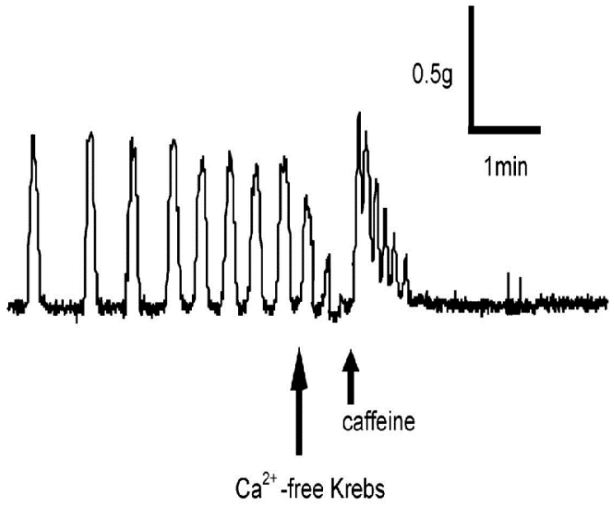

Fig. 2. Effect of caffeine on contraction of portal vein perfused without calcium [21]

Under conditions of perfusion of isolated rings of the portal vein with increased concentrations of potassium, caffeine produces only a contraction that quickly extinguishes. In high concentrations, the potassium in the perfusate (20-100 mM) produces the phasic contraction of the portal vein due to the depolarization of the vascular smooth myocyte. It is important to note that this depolarization consistent with excess potassium produces a short-term phasic contraction of the portal vein, even in conditions of lack of calcium in the perfusate, a fact that indicates the direct action of depolarization on the release of calcium from the SR. Activation of potassium channels attenuates portal vein contractility induced by increased extracellular potassium concentrations [26].

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Author's contributions
VO reviewed and analyzed the scientific literature, exposed the main postulates of the material entity, and approved the final version of the manuscript.

Funding
This study was supported by *Nicolae Testemitanu* State University of Medicine and Pharmacy. The trial was the author's initiative. The author is independent and takes responsibility for the integrity of the data and accuracy of the data analysis.

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

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.14
UDC: 616.8(091)

History of the creation and activity of the Functional Neurology Research Unit over 30 years

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Manuscript received November 3, 2023; revised manuscript December 5, 2023; published online December 27, 2023

Abstract

Background: The Functional Neurology Research Unit (FNRU) was created in 1992 at the State University of Medicine and Pharmacy of the Republic of Moldova. The activity of the scientific unit was essentially based on the concept of Functional Neurology, which was published in an article in the prestigious Italian journal Functional Neurology (1998) and continues to be further developed by Professors I. Moldovanu and V. Vovc.
Conclusions: Physiological aspects in the researches carried out by collaborators of the FNRU concerned research of breathing pattern, autonomic parameters of suprasegmental and segmental level (research of heart rate variability, segmental vegetative samples, etc.), reactivity of cerebral vessels to modeled hypocapnia, trigeminal, somesthetic, visual, auditory evoked potentials in patients with chronic headache, etc. To conclude, the scientific objectives of the research carried out by the collaborators of the Functional Neurology Research Unit during 30 years of activity – the development of the problem of identifying and using the huge, so far still insufficiently researched possibilities of the "reserves" of the human brain for therapeutic purposes (treatment of chronic pain, movement disorders as a result of organic brain lesions, autonomic disorders, etc.) in the context of a new, modern and advanced understanding of the relationship and interaction of the human brain, psyche and body within a social background which is the essence of the *functional neurology*.
Key words: functional neurology, research, autonomic disorders, chronic pain

Cite this article
Odobescu S, Grosu O, Rotaru L, Vovc V, Moldovanu I. History of the creation and activity of the Functional Neurology Research Unit over 30 years. *Mold Med J*. 2023;66(2):91-100. https://doi.org/10.52418/moldovan-med-j.66-2.23.14.

Introduction

The Functional Neurology Research Unit (FNRU) was created in 1992 at the State University of Medicine and Pharmacy of the Republic of Moldova by the university professor, PhD in medical sciences Ion Moldovanu, who had previously worked for 11 years in Moscow in the team led by Professor Alexandr Vein. This laboratory was the only one of its kind in the former USSR, and its research was largely focused on functional neurological pathologies. After a two-year internship in France, Ion Moldovanu returned to Moldova.

The activity of the scientific unit was essentially based on the concept of Functional Neurology, a concept further developed by professors Ion Moldovanu as a neurologist, and Victor Vovc as a physiologist, based on both the clinical-physiological and the conceptual-theoretical approach, largely on the brilliant acquisitions of the Russian school of physiology (Anohin P. K., Bernștein N. A., etc.), which strongly influenced the development of neurology in general.

This concept, which was published in an article in the prestigious Italian journal Functional Neurology (1998) [1], continues to be further developed by Professors I. Moldovanu and V. Vovc.

It should be noted that in the last 20 years neuroscientists have made an enormous leap forward in the study of the human brain. The scientific language relating to the nervous system has been enriched not only with new terms, but also with new concepts, thanks in large part to the appearance of remarkable investigative tools: computer tomography, magnetic resonance and, in particular, functional magnetic resonance, which has opened up unsuspected ways of penetrating the mysteries of the brain and the human psyche.

In 1997 Professor Ion Moldovanu also presented a comprehensive report on "Functional Neurology and its prospects for development" as a visiting professor at the Institute of Neurosciences in Paris (University of Paris VII). Of the 3 scientific monographs and 4 textbooks, in which Professor Ion Moldovanu is co-author, a reference manual for neurologists is worth mentioning: "Disorders of the autonomic nervous system", a fundamental textbook, which has gone through 4 editions (last in 2010, edited in Moscow) [2].

Physiological aspects in the researches carried out by collaborators of the FNRU concerned research of breathing pattern, autonomic parameters of suprasegmental and segmental level (research of heart rate variability, segmental vegetative samples, etc.), reactivity of cerebral vessels to modeled hypocapnia, trigeminal, somesthetic, visual, auditory evoked potentials in patients with chronic headache, etc.

It is important to mention that Professor Ion Moldovanu has succeeded in creating a consolidated team of researchers, who carry out a polyvalent study of autonomic (vegetative) disorders in different forms of neurological pathologies. He is rightly considered the founder of the local school of autonomic disorders.

The scientific directions of the research unit were established on the basis of the urgent needs of both clinical and scientific knowledge of Functional Neurology – a branch of modern neurology, which had not previously developed in the Republic of Moldova and which presents a comprehensive analysis of various functions in physiological or pathological conditions addressed to the functional system, to the relationship between voluntary and involuntary, as well as to the mechanisms of semantic regulation of concrete function. The "Functional Neurology" department included the research of headaches, different pain syndromes and pathologies from a pathogenetic, clinical and epidemiological point of view; and the areas of scientific activity of the Functional Neurology Research unit concerned the following compartments: primary and secondary headaches, chronic pain, segmental and suprasegmental autonomic disorders, sleep disorders, movement disorders (Parkinson's disease, Tourette's syndrom, tics and others).

Functional neurological disorders (FND) are a common cause of disability and distress in neurological practice, being the second most common reason for consulting a neurologist after headache. Karina Bennett et al. performing an extensive review of publications on the prevalence and incidence of FND, found that they account for 5%-10% of neurological consultations internationally. Estimates of incidence are 12 per 100000 population per year. FND disproportionately affects women (approximately 3:1) [3].

Basically, the scientific research guided by university professors Ion Moldovanu and Victor Vovc focused on the study of the mind-body relationship [4]. This led to the specialization of the scientific research in the problems of the autonomic disorders (of the vegetative nervous system – involuntary and unconscious), but which are largely dependent on psychological processes (affective or emotional phenomena and motivational processes). After all a person, this 'psycho-somatic' being par excellence, being in the socio-cultural environment and being shaped by this environment, is in reality a bio-psycho-social being (George Engel, 1977) [5].

On the basis of the laboratory's study directions, two Centres of Excellence were created: the Headache Centre (2010), the Somnology Centre (2013), three scientific societies were founded: the Headache and Pain Society (president – Prof. Ion Moldovanu), the Psychoanalysis and Psychosomatics Association (president – Prof. Ion Moldovanu), the Sleep Medicine Society (president – Prof. Victor Vovc). Key collaborators of the research unit were and are: Ion Moldovanu, Victor Vovc, Stela Odobescu, Lilia Rotaru, Oxana Grosu, Adrian Lupusor et al.

The scientific directions developed by the collaborators are:

1. The problem of episodic, frequent and chronic pain in various forms of neurological, somatic and functional pathologies, in particular headaches (migraine, tension-type headache, trigeminal-autonomic headaches, etc.), extracephalic algic syndromes (fibromyalgia, dorsolumbalgia, visceral pain). Research has also been carried out on pain in Parkinson's disease, multiple sclerosis, arterial hypertension, etc.

2. Pathology of the vegetative (autonomic) nervous system at both segmental and suprasegmental (central) level. Functional neurological disorders (hyperventilation syndrome, panic attacks, syncopal (non-epileptic) states, sleep disorders, neurogenic tetany phenomena, neurological manifestations of hysterical conversion, etc.) were used as a model for study. Contributions have been made to the systematization of autonomic disorders conditioned by psychic factors and hyperventilation phenomena, revealing their role in various clinical structures.

Argumentation of the scientific directions of the Finctional Neurology Research Unit in fundamental aspect

The clinical-morphological method of classical neurology, the method which laid the foundation for the great discoveries in neurology and which has retained its theoretical-practical value into the 21st century, did not always make it possible to explain clinical phenomena. For example, in Parkinson's disease, a disease with established organic dysfunction, the patient can episodically completely get rid of the motor deficit existing in certain particular states (sleep, stress, emotions). Explanation of this phenomenon requires a broadening of the frontiers of the neurological discipline and can be approached partly in accordance with the concept of functional neurology.

Functional neurology (FN) attempts to overcome the limits of the clinic-morphological approach which are partly determined by the, organism-centrism' of traditional concepts with the exclusion of the subject from the analysis of complex brain and mental mechanisms. Modern research has shown that there are „purely human" mechanisms, absent in the animal behavioural model, which need to be taken into account.

The notion of *functional* itself can have several different meanings. For classical neurology the term "functional" was conceived as an antithesis of the notion of organic, i.e. organic damage to the nervous system. For example, J.-M. Charcot considered that in patients with hysteria manifested by pseudo-epileptic seizures, functional hemiplegia,

balance disorders without neurological signs, etc., the organic substrate was not detected and was conceptualised by the author as a "dynamic lesion" [6].

Another aspect of the term *functional* comes from the notion of "functional state of the brain", a notion inspired directly from the framework of "activation theory" [7]. The functional state of the brain depends, according to the authors of this notion, on the level of activation of the brain. This approach, elaborated and further developed in the Moscow School of Neurology, for patients with Parkinson's disease, contains the following emblematic statement: "Clinical and electrophysiological particularities ... are determined, on the one hand, by the lesional focus and, on the other, by the functional state of the brain".

Another interpretation of the term *functional* in neurology can be related to the analysis of a specific function.

Thus, functional neurology presents a diverse spectrum of processes, functions in physiological or pathological conditions addressed to the functional system, the relationship between voluntary and involuntary, as well as the mechanisms of semantic regulation of the concrete function, investigated from a pathogenetic, clinical and epidemiological point of view [7].

Autonomic (vegetative) disorders

It is known that the autonomic nervous system together with the endocrine system are the main systems connecting to the psyche, the human brain and the body. Since Descartes the psyche (the soul – in Cartesian terms) and the human body have been conceived (and continue to be perceived today) as separate entities. This Cartesian dualism, being deeply implanted in Western concepts, persists, to the detriment of the integral, holistic approach of Eastern thought.

While in Moscow, then later in France, and continuing their scientific research in Moldova, Prof. I. Moldovanu together with Prof. V.Vovc studied functional disorders of the respiratory system in the context of affective and neuro-vegetative disorders, disorders of the cerebral control of the breathing pattern, the voluntary-involuntary relationship, the use of breathing as a method of treatment (respiratory biofeedback) and the induction of altered states of consciousness for therapeutic purposes [7]. As a major object of study, the respiratory dysfunctional syndrome – a distressing functional disorder, insufficiently known by physicians in their clinical work – was deeply investigated and analyzed.

In 1996 Prof. I. Moldovanu travelled to France together with Prof. V. Vovc as invited professors to carry out for 2 months a joint research with Professor Gila Benchetrit (head of the respiratory physiology laboratory of the Faculty of Medicine of the University "Joseph Fourier" in Grenoble, France), where breathing patterns in patients with Parkinson's disease were recorded and analysed.

The problem of chronic pain

If acute, nociceptive pain really is a warning sign, then chronic pain is considered a neurological disease. The high rate of chronic pain in the population leads scientists to consider chronic pain – a veritable pandemic. Chronic pain has become a public health problem because of its high prevalence, associated comorbidities and the disability it generates [8]. Analysis of pain prevalence varies from country to country, ranging from 2-40% [8, 9]. It is considered that globally 10% of the population is affected by chronic pain, i.e. 60 million people suffer from pain, and national and regional studies indicate prevalence of 20-25% [10]. In Europe 20% of people suffer from chronic pain, prevalence higher than 40% reported by Italy, France and Ukraine, i.e. every year 1 in 5 Europeans is affected by chronic pain [11]. Despite multiple researches conducted all over the world, chronic pain being usually very resistant does not give way, striking with great violence in the quality of human life [12].

In the Headache Centre of the *Diomid Gherman* Institute of Neurology and Neurosurgery about 65-70% of the patients who are referred suffer from chronic headache: migraine or tension-type headache (chronic headache is considered to be when the presence of headache is 15 or more days per month in the last 3 months). In the vast majority of these patients certain comorbidities are associated, which amplify and maintain the pain: medication abuse with analgesics, anxiety, depression, sleep disorders and multiple vegetative suprasegmental and, less frequently, segmental disorders. A new, relatively recently recognised factor in the chronicity of pain is post-traumatic stress disorder, particularly the poor family atmosphere in childhood [13]. Consideration of comorbid illnesses, stresses, psychological states, etc. is necessary for an effective treatment.

A mixed study (quantitative and qualitative) was carried out in the framework of the master thesis in public health management of Mrs Oxana Grosu (scientific collaborator of Functional Neurology Reserch Unit), which included 355 patients, who completed a special questionnaire, the aim of the work being to analyze the management of the patient with chronic non-cancer pain in the health system of the Republic of Moldova. The results of the study showed the following: patients with chronic non-cancer pain are middle-aged, predominantly female, suffer from pain on average 10 years, with abuse medication, have failed drug treatment, having functional disability and are not satisfied with the management of chronic pain they receive in the health system. It is worth noting, that the chronic pain patient overburdens the health care system through visits to the family doctor and specialists, use of urgent care and frequent hospitalizations in the desire to receive the services they require [12, 14, 15].

The Functional Neurology Research unit's team works together with colleagues from the Department of Neurology and the Department of Physiology of *Nicolae Testemitanu* State University of Medicine and Pharmacy (SUMPh) in the current project under the State Programme (2020-2023) which is specifically addressed to the study

of chronic pain with the theme: "Using 4P principles in the analysis of risk factors for the onset, perpetuation and progression of chronic pain" (20.80009.8007.01).

Pain, autonomic nervous system dysfunction and somatic disturbances of a psychogenic nature

Psychogenic pain or the psychogenic component of chronic pain is one of the problems still unresolved by algologists. In one third of patients with various pain disorders who come for neurological consultation, especially in patients with persistent pain associated with various comorbidities, the presence of a precarious, violent atmosphere is found both in the childhood period of the future adult and recent unhealthy relationships in the family or at work [13]. As a rule, patients do not realize the importance of this factor, and doctors unfortunately do not actively question patients on this topic. Working with patients suffering from chronic pain (headaches and pain in other locations) as well as psycho-vegetative disorders requires a special approach. Three principles need to be observed:

1) to identify the presence or absence of organic pathology;
2) to clarify the full range of the pain spectrum, the type of pain;
3) to elucidate the presence or absence of functional disorders.

Only a thorough investigation with an exhaustive anamnesis and a thorough analysis of psychological disorders, the use of a set of psychological performance tests can reveal the extent of psychological mechanisms, which generate pain. The use of the bio-psycho-social continuum paradigm is imperative in these cases.

Consciousness and altered states of consciousness

The brain has huge and largely unexplored reserves. Identifying and then using these reserves is one of the most important and promising ways for the development of further therapeutic strategies, not only in the field of neurology, but also for most existing pathologies. In recent years Prof. I. Moldovanu has succeeded in organizing an interdisciplinary research team (scientific researchers, residents, psychologists, students, etc.), which jointly conducted both the debate of various concepts and the research of certain brain functions, studied various altered states of consciousness - all this for a deeper understanding and application of the results obtained in the therapy of patients. A great success for the research was the collaboration with specialists in quantum physics of the Academy of Sciences of Moldova, namely on the same topic: the study of brain, consciousness and creativity from the point of view of modern quantum physics approaches and concepts [16].

The development of non-pharmacologic therapeutic strategies

Modern medicine is dominated by predominantly drug treatments, especially for chronic pain. The main idea of Prof. I. Moldovanu lies in the conviction that medical science does not make sufficient use of all the possibilities offered by human nature. The treatment of chronic pain and autonomic disorders by non-pharmacologic methods is currently a fundamental and highly perspective goal in the world of therapies [17]. Recently, an important fact has been realized: the human brain can be compared to a mega-computer, which simultaneously processes a huge number of programs, manages dozens of biochemical „laboratories" of the body, influences complex neuro-physiological processes and makes various connections at both intracerebral and psycho-physiological (psychosomatic) levels. In the Functional Neurology Laboratory extensive research has been conducted on the use of transcranial electrical stimulation with direct current in non-pharmacologic therapy of chronic migraine [18], also - in the group of patients with chronic migraine and ventricular asymmetry, as well as beneficial results have been noted in the treatment of algic syndrome in patients with Parkinson's disease [19].

In the years of scientific activity of the Functional Neurology Research Unit several research projects have been carried out:

1. Multivariate study of cephalalgic, vegetative and extrapyramidal disorders. Epidemiological, pathogenetic and therapeutic aspects. 06.420.050F.

2. Preventive treatment of chronic migraine by transcranial direct current stimulation method. 10.820.05.10 GF.

3. Multivariate study of chronic pain in nervous system disorders. Epidemiological, pathogenetic, clinical, therapeutic and preventive aspects. 11.817.09.25A.

4. Research on chronic cephalic pain carried out in the Mayo Clinic, Department of Neurology, Arizona (USA) with Professor David Dodick in the framework of the Fulbright Program sponsored by the US Department of State (2002-2003, Ion Moldovanu, Stela Odobescu).

5. Epidemiological research on primary headaches in the Republic of Moldova funded by the International Headache Society (2005-2006) (Ion Moldovanu, Stela Odobescu, Lilia Rotaru, Oxana Grosu, Gabriela Pavlic).

Professor Ion Moldovanu has always been intrigued by the pathology of the autonomic nervous system, a complex, understudied field that was still developing. The leading specialist in the former USSR in this field, as well as in sleep medicine, was, at that time, Professor Alexander Vein from Moscow. Between 1980 and 1991 Mr Ion Moldovanu had the great opportunity to be a doctoral student and then a scientific collaborator in the Neurology Clinic of the Moscow Institute of Medicine „I.M. Secenov" (nowadays „I. M. Secenov" Moscow Medical Academy). A world-renowned scientist, well-known in the field of neuro-vegetative pathology, Prof. A. Vein succeeded in creating a highly scientific atmosphere in the research of the brain and the vegetative nervous system, as well as the human psyche, within a highly efficient team (10 professors and 20 medical doctors). The performance of this group was determined by the intersection of research from several disciplines: neurology, physiology, psychology, endocrinology. It was an innovative approach

at the time, it was new and fascinating. This allowed him to study not only clinical aspects of neuroscience, but also the fundamental science: neuro-physiology and especially psycho-physiology. In Moscow Mr Moldovanu defended two theses: "Neurogenic tetany syndrome" (1983) and "Neurogenic hyperventilation and vegetative disorders" (1991). Both theses were of great interest to neurologists because they were absolutely new, unstudied and unknown topics in the context of the pathology of the autonomic nervous system [20, 21].

These approaches, which Mr Moldovanu had learned in the team of Professor Alexandru Vein, were later transposed and developed in the research of the collaborators of the Functional Neurology research Unit, thus creating the first and the only laboratory of vegetology in the RM. The aforementioned aspects were also the target of the scientific research carried out by the collaborators of the laboratory - Victor Vovc, Stela Odobescu, Larisa Bobeico, Octavian Razlog, Ludmila Ciobanu, Severin Sohotchii, Gabriela Pavlic, Lilia Rotaru, Dorina Tiple, Oxana Grosu, Galina Corcea, Adrian Lupusor, Crsitina Chicu-Hadarca, etc.

In the Functional Neurology Research Unit, which later became part of the Institute of Neurology and Neurosurgery, researches in various aspects of functional neurology have been carried out over the years, most of which were scientific theses:

1. **Odobescu Stela.** Breathing pattern dysfunction in patients with vegetative suprasegmental disorders (clinical, psychological, neurophysiological and therapeutic study) (PhD).
2. **Ciobanu Ludmila**. Vegetative patterns in children of the first year of life (PhD).
3. **Siric Ala.** Neurogenic syncope in children (PhD).
4. **Tiple Dorina**. Specificity of vegetative disorders in patients with Parkinson's disease (PhD).
5. **Rotaru Lilia.** Chronic migraine in patients with cerebral ventricular asymmetry - clinical-electrophysiological and neuroimaging study (PhD).
6. **Corcea Galina.** Syncopy in migraine patients – clinical and neurophysiological study (PhD).
7. **Maticiuc Violeta.** Migraine as a risk factor in cerebral and coronary ischemia (PhD).
8. **Grosu Oxana**. Chronic migraine associated with arterial hypertension (PhD).
9. **Pavlic Gabriela**. Algic syndromes in patients with Parkinson's disease (PhD).
10. **Razlog Octavian**. Breathing pattern disorders in patients with panic attacks (PhD).
11. **Sidorenco Irina**. Peculiarities of neurophysiological indices changes under stress conditions (PhD).
12. **Besleaga Tudor.** Ventilatory and cardiac effects of voluntary hyperventilation - study in healthy volunteers and patients with panic disorder (PhD thesis by cotutelle).
13. **Profire Liliana.** Correlations of hormonal decline and psycho-vegetative disorders in menopausal patients (PhD).
14. **Plesca Viorica.** Autonomic disorders in children with irritable bowel syndrome (PhD).
15. **Curca Cristina**. Ophthalmological manifestations in patients with chronic migraine (PhD).
16. **Jubarca-Tulum Svetlana**. Clinical, psychological and neuro-vegetative peculiarities of pregnancy and birth in teenagers (PhD).
17. **Raileanu Gheorghe**. The role of perinatal factors in the clinical manifestations and pathogenesis of neuro-vegetative disorders in children (PhD2 - doctor habilitate thesis).
18. **Odobescu Stela.** Chronic migraine and associated autonomic disorders (epidemiological, clinico-neurophysiological and therapeutic study) (PhD2 – doctor habilitate thesis).
19. **Lozan Tatiana**. Epidemiological and medico-social aspects of primary headaches in adolescents (PhD).

Currently, the following theses are in progress, some even at the completion stage:

1. **Concescu Diana**. Analysis of the clinical profile of patients with post lumbar puncture headache and the therapeutic efficacy of blood-patch (PhD thesis carried out in cotutelle with the Headache Emergency Centre, Lariboisiere Hospital, Paris).
2. **Sajin Valeria**. Neuroanatomical correlation of tics and sensory phenomena in Gilles de la Tourette syndrome (PhD thesis carried out in cotutelle with the Department of Movement Disorders and Neuropsychiatry in Children and Adults, Institute of Neurogenetics, Luebeck, Germany).
3. **Lupușor Adrian**. Morning headache in patients with sleep-disordered breathing.
4. **Gavriliuc Olga**. Effect of deep brain stimulation on gait and posture disorders in patients with Parkinson's disease.
5. **Ganenco Andrei.** Relationship between heart and respiratory rhythms in healthy and panic attack patients.
6. **Lozovan Svetlana**. Psycho-physiology of respiratory paternity.

The research work has been carried out in international collaboration with numerous international institutions and scientific centres:

1. International Headache Society and the Russian language subcommittee (Prof. Zaza Katsarava) – methodology, research, academic exchange.
2. European Headache Federation – methodological support, research, academic exchange.
3. Russian Headache Society (Moscow) – methodology, research, academic exchange.
4. Italian Headache Society, Mondino Center for Head pain and adaptive disorders Pavia, Italy – research, academic exchange.
5. Headache Centre of the Neurology Department of the Roger Salengro University Clinic in Lille – Professor Christian Lucas – research, academic exchange.

6. The French Society for the Study of Migraine and Headache and the Headache Emergency Centre (Centre Urgences Cephalees), Lariboisiere Municipal Hospital, Paris, France – research, academic exchange.

7. Department of Movement Disorders and Neuropsychiatry in Children and Adults, Institute of Neurogenetics, Luebeck, Germany – research, academic exchange.

8. Palliative Care Service of Malestroit (France) – promotion of the concept of palliative care in the public health care system in the Republic of Moldova by completing the university curriculum with this new discipline for the medicine of the Republic of Moldova.

9. Rehabilitation Clinic, Iasi, Romania – research, academic exchange.

10. Swiss National Scientific Foundation Centre – Scientific cooperation between Eastern Europe and Switzerland (SCOPES 2013-2016) – funding, methodological and logistic support, academic exchange.

11. Institute of Applied Physics, Laboratory of Quantum Optics of the ASM – research.

12. School of Public Health Management of *Nicolae Testemitanu* SUMPh – research, methodological support.

13. Department of Neurology and Physiology of *Nicolae Testemitanu* – research, academic exchange.

14. Doctoral School of *Nicolae Testemitanu* (SUMPh) – methodological and academic support.

A great influence for the advancement in the problem of pain and headaches had the scientific training in the Mayo Clinic Scottsdale (Arizona, USA) of Prof. Ion Moldovanu for 6 months (2002-2003). One of the scientific results of this stay was the publication of the monograph „Headache, facial and cervical pain" (authors – Moldovanu I., Dodick D., Odobescu S.), which was the first practical guide for the diagnosis and treatment of this neurological problem in the Republic of Moldova [22].

On the initiative of the director of the Institute of Neurology and Neurosurgery (INN), Prof. I. Moldovanu, inspired by this training, new performance centres in certain neurological fields were created within the INN. So, in 2010 the National Centre for Headache and Vegetative Disorders was created (head of the centre Stela Odobescu), in 2011 – the National Centre for Spinal Pain (head of the centre Svetlana Pleșca), and in 2013 – the National Centre for Sleep Medicine (head of the centre Ion Moldovanu).

For many years the Headache and Vegetative Disorders Centre associated with the Functional Neurology Research Unit, together with the Headache and Paroxysmal Disorders Department have played an essential role in promoting the knowledge of headaches, vegetative disorders, paroxysmal states both in the medical environment of the Republic of Moldova and for the population of our country.

Main scientific results of the Functional Neurology Research Unit in the years of activity

The first epidemiological study of primary headaches in the Republic of Moldova was conducted (I. Moldovanu, S. Odobescu, L. Rotaru, O. Grosu), which revealed the prevalence of episodic and chronic primary headaches in the urban and rural adult population [23], as well as among adolescents in Moldova (PhD thesis of Mrs Tatiana Lozan) [24, 25]. Prevalence data have been published internationally, and the study has been appreciated and mentioned by the International Headache Society and the European Headache Federation [23]. The overall prevalence of migraine, resulting from the first epidemiological study of primary headaches in the Republic of Moldova [23], is estimated at 20%: 16.5% for episodic migraine and 3.5% – for chronic migraine, i.e. higher than reported in most previous studies in Europe and the USA. Possibly this finding can be attributed to major social stresses, related to the period of socio-economic transition, through which Moldova is passing, and migraine, as it is known, is a disease sensitive to psycho-emotional disorders. The prevalence of chronic migraine of 3.5%, highlighted in this study, is also higher compared to most epidemiological studies conducted in the world so far [23].

The classification of headache disorders has been translated, implemented and disseminated in the Moldovan medical environment, being the first translation of this comprehensive and exhaustive diagnostic compendium in the field of headache in the European space [26].

There has been developed and proposed for use in scientific autonomic research the questionnaire Patient Vegetative Profile – a remarkable clinical tool, which elucidates polysystemically from the qualitative-structural and quantitative point of view 12 vegetative scales in patients with various organic and functional pathologies of the nervous system [27].

The role of risk factors (biological, psychological) in the manifestation and progression of chronic pain has been elucidated. The identification of biological (heart rate variability parameters, cardiovascular segmental vegetative evidence parameters, trigeminal evoked potentials, motor and somatosensor evoked potentials, blink reflex, etc.) and psychological markers important in the pathogenesis of chronic headaches has allowed a more precise differential diagnosis and the development of a more adjusted therapeutic strategy in chronic migraine [28].

Chronic migraine has been studied multilaterally – epidemiologically, neuro-physiologically, clinically and therapeutically (PhD2 thesis by Stela Odobescu) [28]. Thus, the specificity of autonomic regulation in the cardio-vascular system in patients with chronic migraine vs. frequent migraine was revealed by means of measurement of heart rate variability indices, namely, the impairment of vegetative balance with increased activity of the sympathetic nervous system and decreased activity of the parasympathetic nervous system, with indicators of amplification of the degree of centralization of autonomic heart rate regulation [29]. Neuro-physiological research of patients with chronic migraine by means of trigeminal evoked potentials has demonstrated a more pronounced activation of the functional state of the trigeminal system at different levels (nuclear-thalamic, thalamo-cortical) in the interictal period and

a tendency towards intensification of trigeminal system activity at the nuclear-thalamic level during migraine attack [30].

Various coping strategies and pain acceptance have been studied in patients with different forms of chronic pain (chronic migraine vs. chronic low back pain). The therapeutic approach of modifying the coping strategy (coping with pain) was implemented in the complex non-pharmacologic treatment of chronic headache syndromes, which contributed to decrease the expenses for abortive and preventive drugs of the chronic pain patient [31, 32].

Chronic migraine in association with asymmetry of the lateral cerebral ventricles has been investigated from an imaging, clinical and electrophysiological point of view (PhD thesis of Mrs Lilia Rotaru); the lateral cerebral ventricles asymmetry being proven as a factor influencing the severity of clinical manifestations in patients with chronic migraine. The increased degree of asymmetry of the lateral ventricles in patients with chronic migraine was associated with an earlier age of onset of headache, a longer duration of migraine attacks, a higher risk for the development of very severe and bilateral migraine attacks, and susceptibility to a greater number of triggers. Severe degree of ventricular asymmetry in patients with chronic migraine was more frequently associated with genetic predisposition for migraine (90%) and cutaneous allodynia (90%) [33-35].

A distinct clinical entity – syncopal migraine – was studied multilaterally (PhD thesis of Mrs Galina Corcea), which allowed to highlight the fact that patients with syncopal migraine and positive response to the tilt-test, compared to those who do not have syncope during migraine attacks, showed increased frequency and more severe intensity of migraine attacks, predominantly constrictive character of headache, increased frequency of syncopal attacks, shorter duration of premonitory symptoms, more profound degree of loss of consciousness, higher values on the vegetative motor profile scales and increased personality anxiety score [17, 36].

Chronic headache associated with hypertension was investigated (PhD thesis of Mrs Oxana Grosu), which demonstrated that hypertension amplifies the effect of migraine on the vascular wall leading to increased endothelial dysfunction of cerebral vessels. Another conclusion of the study is that subjects with chronic migraine and arterial hypertension present in 50% of cases a "non-dipping" circadian pattern suggesting an increased cardiovascular risk as this abnormal circadian pattern is associated with an elevated risk of stroke, cerebral white matter lesions, etc. [37, 38].

The relationship between migraine and cardiovascular and cerebrovascular pathology has been studied (PhD thesis of Mrs Violeta Maticiuc), which allowed the establishment of a practical algorithm to identify the presence of migraine algic phenomenon in patients with acute vascular events (cerebral and cardiac). The severity of the ischemic stroke was evaluated depending on the presence or absence of migraine in these patients (according to the Rankin scale). The study allowed to assess the peculiarities of the cephalalgic syndrome in patients with ischemic events (stroke and acute coronary syndrome). The weight of risk factors in patients with migraine and ischemic events (cerebral and coronary) was highlighted. Serum markers of endothelial dysfunction (nitric oxide metabolites) and oxidative stress were determined in patients with ischemic stroke and acute coronary syndrome with and without migraine. This research demonstrated that migraine has a serious contribution to the clinical and evolutionary manifestations of stroke and acute coronary syndrome by involving several pathogenetic mechanisms (oxidative stress, endothelial dysfunction). Thus, it has been shown that migraine is a true vascular risk factor for ischemic vascular events (cerebral and coronary) [39, 40].

An important pathogenetic aspect has been highlighted, that the presence of morning headache increases the likelihood that the patient has undiagnosed sleep apnea. The combination of psychological and biological factors triggers morning headache, which is a „red flag" for suspecting the presence of sleep-disordered breathing, the most common being Obstructive Sleep Apnea Syndrome, a known risk factor for sudden sleep death, myocardial infarction, hypertensive disease, excessive daytime sleepiness with falling asleep at the wheel, etc [41]. A very important research and investigation method – polysomnography – has been implemented in the clinical experience of INN (professor Victor Vovc, scientific collaborator Adrian Lupusor).

Respiratory dysfunctional syndrome (RDS), which plays a fundamental role in amplifying affective disorders, has been studied. Of all the mental phenomena, the most sensitive to respiratory dysfunction syndrome are the body sensation disorder and anxiety scales. The most pronounced vegetative disorders associated with RDS are cardiovascular disorders, vertigo-syncope and gastrointestinal disorders.

The specificity of the algic syndrome in patients with Parkinson's disease has been studied (PhD thesis of Mrs Gabriela Pavlic) [42], the importance of non-motor disorders in parkinsonian patients has been highlighted (PhD thesis of Mrs Dorina Tiple) [43], the neuro-physiological exploration of sensory phenomena in Tourette's syndrome and in patients with tics has been carried out. Multilateral research of patients with Parkinson's disease in association with chronic pain has allowed to highlight clinical features correlating pain with the severity of motor and non-motor signs, with predominance in patients with akinetic-rigid phenotype with more advanced dopaminergic deficit. The study provided support for the dopaminergic hypothesis in the pathogenesis of pain; the concept of clinical heterogeneity of the disease was confirmed by the association of pain-related variables and more severely expressed motor parameters with the akinetic-rigid phenotype of the disease [44]. Another area of movement disorders, such as tics has also been investigated – namely, the very complex relationship between subjective phenomena (desire for move-

ment – urge) and the actual movement (tic), which involve various brain systems and interact in a unitary way with each other, has been studied. The results of this study have allowed a deeper understanding of the pathogenetic mechanisms of tics, opening up real prospects for more effective treatment, both drug and non-drug (psychotherapy, biofeedback, electrical stimulation, etc.) [45, 46].

Non-pharmacological treatment methods for chronic headaches using transcranial electrical stimulation with alternative and direct current have been implemented. Estimation of therapeutic efficacy of non-drug treatment method by transcranial electrical stimulation by means of serum beta-endorphin assessment (before and after treatment) contributed to the implementation of the method in clinical experience in pain control [47].

A set of powerful tests was developed, which allowed to identify vegetative (patient's vegetative-motor profile) and individual psychological parameters, in order to determine the patient's psycho-vegetative profile, with the aim of establishing an appropriate specific treatment.

The results of the study investigating the relationships between personality disorders and migraine or tension-type headache concluded that the patient's psycho-emotional background plays a very important role in the manifestation of primary headache and affective and vegetative disorders. As inflexible and maladaptive as personality traits are, so great is the subjective suffering and functional impairment. The patient's personality disorder negatively influences vegetative manifestations (accentuates respiratory dysfunction and somatoform disorders), affective manifestations (aggravates anxiety and depression), cephalalgic manifestations (increases duration of access, number of days per month with pain, use of analgesics).

Functional Neurology Research Unit collaborators have published more than 400 scientific papers in national and international scientific journals, registered 6 patents, participated in numerous European and world congresses in the field of pain, headache, extrapyramidal system pathology, as well as organized national scientific events with international participation. National and institutional clinical guidelines and protocols for the management of migraine, tension-type headache, Parkinson's disease, cognitive disorders, anxiety, depression, etc. have been elaborated and published.

Thus, a new field – *the vegetology of chronic pain* – has been developed, with elucidation of the role of the segmental and suprasegmental autonomic nervous system in the pathogenesis of frequent and chronic headaches.

If there wasa task to formulate in a single sentence the scientific objectives of the research carried out by the collaborators of the Functional Neurology Laboratory during 30 years of activity – they would be the development of the problem of identifying and using the huge, so far still insufficiently researched possibilities of the "reserves" of the human brain for therapeutic purposes (treatment of chronic pain, movement disorders as a result of organic brain lesions, autonomic disorders, etc.) in the context of a new, modern and advanced understanding of the relationship and interaction of the human brain, psyche and body within a social background which is the essence of the *functional neurology*.

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Funding
This study was supported by the grant from the National Agency for Research and Development of the Republic of Moldova, Project code 20.80009.8007.01. The authors take responsibility for the integrity of the data and accuracy of the data analysis.

Ethics approval and consent to participate
The research protocol was approved by the Research Ethic Board of the *Diomid Gherman* Institute of Neurology and Neurosurgery and the tests have been done according to the contemporary principles in biological standardization of experiences and Declaration of Helsinki with further amendments (Somerset West Amendment, 1996).

Conflict of interests
No competing interests were disclosed.

https://doi.org/10.52418/moldovan-med-j.66-2.23.15
UDC: 616.858:616.89-008.45

Current level of knowledge about Parkinson's disease cognitive impairment

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Manuscript received November 7, 2023; revised manuscript December 11, 2023; published online December 27, 2023

Abstract

Background: Parkinson's disease (PD) affects more than 1% of the population aged over 65 years and manifests with both motor symptoms – bradykinesia, rest tremor, rigidity, and non-motor symptoms. Cognitive impairment and dementia are recognized non-motor symptoms that can significantly affect the quality of life of both the patient and caregivers and are a risk factor for institutionalization in nursing homes and a risk factor for early mortality. Cognitive impairment is frequent in Parkinson's disease (PD) that can develop even before the diagnosis of Parkinson's disease based on its motor features.
Conclusions: There are several clinical, molecular, and imaging factors that constitute risk factors for the development of Parkinson's disease dementia, in which basal cholinergic and prefrontal dopaminergic systems are involved. Histological changes are Lewy-body, Alzheimer, but also vascular pathology. Clinically can be distinguished subjective cognitive decline, mild cognitive impairment and, subsequently, Parkinson's disease dementia. There are no remedies with a proven effect to prevent the occurrence of cognitive decline in PD. The only approved drug for already developed D-PD is the cholinesterase inhibitor – donepezil. Non-pharmacological interventions are thought to be beneficial. A multidisciplinary approach to cognitive impairment is recommended, with specific pharmaceutical treatment of the cognitive disorder and comorbidities, and appropriate rehabilitation.
Key words: Parkinson's disease, mild cognitive impairment, dementia.

Cite this article
Rotaru L, Grosu O, Groppa S. Current level of knowledge about Parkinson's disease cognitive impairment. *Mold Med J*. 2023;66(2):101-110. https://doi.org/10.52418/moldovan-med-j.66-2.23.15.

Introduction

Parkinson's disease (PD) affects more than 1% of the population aged over 65 years [1] and manifests with both motor symptoms – bradykinesia, rest tremor, rigidity, and non-motor symptoms [2]. Cognitive impairment and dementia are recognized non-motor symptoms that can significantly affect the quality of life of both the patient and caregivers and are a risk factor for institutionalization in nursing homes and a risk factor for early mortality [3]. Cognitive impairment can develop and manifest at any stage of the disease, even before the diagnosis of Parkinson's disease based on defining motor symptoms and most often begins insidiously [4]. There are several clinical, molecular, and imaging factors that constitute risk factors for the development of Parkinson's disease dementia (D-PD). Executive and visuospatial impairments, visual hallucinations, changes in cerebrospinal fluid and/or blood serum biomarkers, and structural and functional imaging changes are recognized as risk factors of D-PD [5]. In D-PD, the basal cholinergic and prefrontal dopaminergic systems are involved; and histological changes of Lewy-body type, Alzheimer type, but also vascular type are observed [6]. Subjective cognitive symptoms, which can appear even from the premotor and early stages of PD, progress to mild cognitive impairment and, subsequently, to Parkinson's disease dementia. There are no remedies with a proven effect to prevent the occurrence of cognitive disorders in PD or to treat the minor cognitive deficit associated with PD, while the only approved drug for already developed D-PD is the cholinesterase inhibitor – donepezil [7]. Physical training and cognitive training are thought to be beneficial. A multidisciplinary approach to cognitive impairment is recommended, through the administration of specific pharmaceutical treatment of the cognitive disorder, treatment of comorbidities, and appropriate rehabilitation.

Epidemiology of cognitive disorders in Parkinson's disease. Epidemiologic studies of PD do not always include minor cognitive impairment associated with Parkinson's disease (MCI-PD) or dementia associated with Parkinson's disease (D-PD). Therefore, data on the epidemiology of cognitive impairment in PD are incomplete. About 30% of newly diagnosed patients with PD presented subjective complaints related to memory; their risk was significant for developing MCI within the next 2 years compared to patients who had no memory-related complaints [8]. Up to 25.8 – 64% of patients with PD suffer from MCI-PD [9]. At the time of diagnosis of PD, MCI is found in approximately 20% of patients [10]. A multicenter prospective longitudinal study of PD patients found that at the time of PD diagnosis, 20.2% had MCI, and at 5 years of follow-up, its

incidence increased to 40–50% [11]. Two cross-sectional studies estimated the prevalence of MCI-PD to be 33% and 64%, respectively [12]. At the same time, the prevalence of MCI in the general elderly population (60 - 90 years) is much lower and varies between 16 and 20% [13]. The conversion rate to D-PD is significant in PD patients with MCI and is almost 60% at 5 years of follow-up [11]. More than 75% of patients, who develop cognitive decline in the early stages of PD, will later develop Parkinson's disease dementia, but a stabilization of cognitive function or even a reversal from MCI-PD to normal cognition has also been reported in approximately 25% of MCI-PD patients [11]. The results of longitudinal studies show that the risk of patients with PD to develop dementia is up to 6 times higher than that of the age-matched healthy population [14]. In the population older than 60 years, the prevalence of dementia is 5-7% [15]. 3 - 4% of dementia is thought to be caused by PD, and the estimated prevalence of D-PD in the general population aged over 65 is 0.2 to 0.5% [16]. Research indicates an association between the prevalence of D-PD and the duration of PD: the cumulative prevalence of D-PD 5 years after diagnosis is 17%, 10 years after diagnosis – 46%, and 20 years after diagnosis – 83% [17]. After 10 years of PD evolution, dementia is present in approximately half of the patients, and after 20 years – in most patients [5]. Several general and clinical characteristics are associated with an increased risk of developing cognitive decline. Some predictors, which were independently associated with the development of cognitive impairment and/or dementia, have been described: (1) general factors: older age, male gender, lower education level, older age at onset of PD, (2) non-motor symptoms: visual hallucinations, depression/mental state, hyposmia/anosmia, orthostatic hypotension, (3) motor signs: akinetic phenotype, postural instability, (4) disease severity: high motor impairment score, high bradykinesia score, advanced Hoehn and Yahr stage, (5) response to treatment: cognitive adverse effects of dopaminergic treatment, poor therapeutic response to dopamine agonists, (6) specific cognitive deficits: posterior cortical cognitive deficits, frontal executive dysfunction, (7) comorbidities: cerebrovascular disease, diabetes, obesity, heart disease, (8) lifestyle factors: alcohol consumption, smoking [2, 18, 19].

Pathophysiology of cognitive decline in Parkinson's disease. PD patients suffer an early cholinergic degeneration in the anterior basal part of the brain, but abnormalities of the prefrontal dopaminergic system, and other neurotransmitter systems (noradrenergic and serotonergic) of the neocortical, limbic and basal ganglia regions are also characteristic [20]. Along with the early loss of dopaminergic neurons in the substantia nigra, an abnormal deposition of α-synuclein in the Lewy bodies also occurs; first – in the cholinergic and monoaminergic neurons of the brainstem and olfactory system, which leads to significant synaptic deficiencies [21].

Compared to patients with PD and normal cognition, those with MCI-PD and D-PD, show an increased dopaminergic loss in the region of the frontal, parietal and temporal cortex [5]. Dopamine depletion in the nigrostriatal pathways has been involved in the impairment of working memory, planning/sequencing, task switching, response inhibition, recall, verbal fluency, and psychomotor speed [22]. On the other hand, impairments in amnestic memory, language, and visuospatial impairments involve neurotransmitter systems, such as acetylcholine, noradrenaline and serotonin [23].

Several studies have identified associations between the progressive loss of cerebral noradrenaline and cognitive decline in patients with PD [24]. Dopaminergic, noradrenergic, and serotonergic CSF markers were compared in healthy controls, PD patients and D-PD patients. Progressive changes were found in all markers, but only the noradrenergic markers were significantly reduced in all D-PD patients and in all brain regions [25].

Loss of cortical cholinergic innervation is independently associated with the cognitive decline of PD, and its association with a greater dopaminergic denervation in the caudate nucleus correlates with an even more pronounced cognitive decline [26]. The density reduction of cholinergic neurons in the anterior basal cortex and of their projections to the neocortex, amygdala and hippocampus is associated with cognitive decline in patients with de novo PD, and is predictive of cognitive decline in patients with PD and normal cognition [5, 27]. The loss of cholinergic projections from the forebrain to the hippocampus correlates with memory deficits and conversion to D-PD [27]. The loss of brainstem serotonin is associated with non-motor symptoms of PD (depression, anxiety) and correlates with β-amyloid deposition [28]. The loss of cholinergic fibers is more pronounced than the loss of cholinergic neurons in patients with D-PD [29]. In MCI-PD there is a loss of cholinergic fibers and a decrease of cholinergic activity in the hippocampus, while in D-PD a progressive deposition of α-synuclein occurs, with subsequent dysfunction, not only in the hippocampus, but also in the basal forebrain [30].

Morpho-pathological changes in PD-associated cognitive dysfunction include Lewy bodies and neurites, coexisting Alzheimer's tau and amyloid pathology, and ischemic microvascular changes [31]**.** The most common neuropathology in D-PD is limbic and/or neocortical Lewy pathology [32], but other types may coexist. Alzheimer-type and Lewy-type pathologies frequently coexist, and this coexistence is a better predictor for the development of D-PD than the severity of either pathology alone. Neuropathological studies argue for the dual hypothesis of cognitive impairment in PD. A morpho-pathological study showed that 38% of D-PD patients only had Lewy body accumulations, 59% had Lewy bodies in combination with beta-amyloid plaques, and 3% had Lewy bodies, beta-amyloid plaques, and neurofibrillary tangles [33].

An increased share of white matter hyperintensities has been reported in subjects with PD who later progressed

to dementia [34]. The total volume of white matter hyperintensities in patients with early PD and in patients with MCI-PD, was predictive for the occurrence of longitudinal cognitive decline [35]. A prospective study found that progression to dementia was more frequent in PD patients who had a moderate-severe ratio of parieto-occipital white matter hyperintensities associated with low CSF β-amyloid levels [36]. At the same time, other studies found no correlation between the severity of subcortical small vessels injury and dementia [37].

Certain genetic polymorphisms are associated with the onset and development of PD-related cognitive dysfunction. In patients with Parkinson's disease and GBA mutations, diffuse neocortical pathology with Lewy bodies is more common and is associated with hallucinations, cognitive decline, or early dementia [38]. Executive functions, visuospatial skills and working memory are affected. COMT Val/Val polymorphisms stimulate dopamine catabolism and lead to a decrease in postsynaptic dopaminergic stimulation, while Met/Met polymorphisms decrease COMT enzyme activity and lead to increased dopamine levels [39]. Therefore, there is not necessarily an association of the COMT genotype with later cognitive impairment or dementia. COMT genotype is associated with executive dysfunctions based on the frontostriatal system. The MAPT H1/H1 genotype, which influences tau transcription, is thought to be an independent predictor of D-PD and is associated with significant posterior-type cortical cognitive deficits [39].

There are a series of predictive biomarkers of cognitive decline in Parkinson's disease. The function of biomarkers of cognitive decline is to predict the developmental perspective of cognitive decline and dementia in PD [40]. Protein biomarkers are predictive of cognitive decline in Parkinson's disease. Cortical pathological synuclein accumulation in patients with idiopathic PD correlates with cognitive decline, while the lack of Lewy bodies in autosomal recessive PD, caused by Parkin gene mutations, is associated with normal cognitive function throughout the course of the disease [41]. Likewise, in autosomal dominant PD caused by mutations in the LRRK2 gene, in which there are subgroups without Lewy bodies, the risk of cognitive dysfunction is lower, and cognitive impairment and dementia correlate with the presence of Lewy bodies [42]. Autosomal dominant PD due to SNCA [43] and GBA mutations, patients with Gaucher disease and idiopathic PD [44], may frequently manifest cortical Lewy bodies and significant associated cognitive dysfunction. Comorbid morpho-pathological changes, such as cerebrovascular disease and Alzheimer-like changes (hippocampal sclerosis, accumulation of β-amyloid plaques and tau neurofibrillary tangles) may contribute to D-PD [45]. The severity of Alzheimer-type pathology is associated with a shorter time-frame between the onset of motor symptoms and the onset of dementia, as well as a lower survival rate [45] in D-PD and also correlates with the severity of cognitive impairment. Total α-synuclein CSF levels are lower in PD patients compared to controls, with no significant difference between patients with and without dementia [46]. The potentially pathogenic forms of α-synuclein: phosphorylated, ubiquitinated, nitrated, oligomeric, could be more sensitive indicators of underlying disease progression and of a more severe cognitive decline. Thus, oligomeric synuclein has significantly higher CSF levels in patients with D-PD and Lewy body dementia, compared to Alzheimer dementia patients and the control group and correlates with UPDRS-III, MMSE scores, semantic and visuo-perceptual fluency [47]. Plasma levels of total α-synuclein are higher in PD with a more severe cognitive dysfunction and they also correlate with lower Mini Mental State Examination (MMSE) scores [48]. In most studies, the level of CSF ß-amyloid was lower and could predict future cognitive decline in PD patients [49]. This reduced level was associated with worse verbal learning, semantic fluency, and reduced visuo-perceptual scores as well as cortical atrophy in the superior frontal/anterior and precentral cingulate regions— which is predictive for D-PD [50].

Cerebral atrophic changes are predictive structural neurodegenerative biomarkers of cognitive decline. In patients with PD and normal cognition, loss of gray matter volume in the temporal cortex, prefrontal cortex, insula, hippocampus, and caudate nucleus may predict the occurrence of MCI-PD. In MCI-PD, neuronal loss has a pattern of posterior, parietal and frontal cortical involvement, as well as hippocampal atrophy. The severity of this atrophy correlates with the memory decline, and its progression towards the parahippocampal and cingulate gyrus is associated with the progression of cognitive decline in PD. When applied to PD patients, the SPARE-AD Alzheimer's Dementia brain atrophy model predicted long-term cognitive decline in PD patients who were dementia-free at that time point [51].

Functional neurodegenerative biomarkers predictive of cognitive decline. Through PET and SPECT, the activity of acetylcholine and dopamine can be determined. Acetylcholinesterase (AChE; the enzyme which catalyzes acetylcholine breakdown) activity in the lower cortical regions is associated with reduced cognitive performance scores for: attention, memory, and executive function. In D-PD, reduced AChE activity becomes more severe and widespread; it involves the occipital, temporal, frontal, and medial cortex, as well as the thalamus.

Connectivity-biomarkers predictive of cognitive decline in Parkinson's disease. Altered neurotransmitter signaling associated with neurodegeneration leads to dysfunctions of regional brain activity and circuit connectivity in PD. Brain activity, measured by regional glucose metabolism (PET) and regional perfusion (SPECT), is lower in PD patients in the occipital and inferior parietal lobes. Decreased brain activity correlates with performance on neuropsychological tests [52]. PD patients without cognitive decline show a decrease in the functional connectiv-

ity of the right medial temporal lobe and bilateral inferior parietal cortex, in the brain connectivity network; this low connectivity correlates with cognitive parameters. Patients with D-PD reveal decreased connectivity in the inferior occipital gyrus bilaterally, compared to healthy subjects; and in the right frontal gyrus, compared to both non-D-PD patients and controls [53].

A sole biomarker that could represent the multiple pathological substrates of cognitive impairment in PD is not yet available. Therefore, to improve the accuracy of cognitive decline prediction in PD, the following combinations of biomarkers could be used:

(1) patient age + UPSIT score (University of Pennsylvania test) + REM sleep behavior disorder score (RBDSQ) + CSF Aβ42 level + caudate nucleus DAT uptake; this model allows the prediction of cognitive decline in 2 years.

(2) Alzheimer's disease biomarkers: total tau CSF level + phosphorylated tau CSF level + Aβ42 CSF level + APOE genotype + SPARE-AD imaging score; this model separates patients with PD and normal cognition from patients with D-PD with an accuracy of 80%.

(3) PD age at onset + gender + number of years of education + MMSE score at study initiation + presence of depression as a symptom at enrollment + MDS-UPDRS III score at enrollment + presence of GBA gene mutation; this model predicts cognitive impairment with an AUC score of 0.85, and dementia or disabling cognitive impairment with an AUC score of 0.88, within 10 years of disease onset [4, 54].

Clinical characteristics of cognitive decline in Parkinson's disease. Cognitive impairment in PD varies highly in severity, rate of progression, and cognitive domains affected [12]. The phenotypic severity of cognitive impairment ranges from bradyphrenia, subjective cognitive complaints without objective evidence of cognitive dysfunction to MCI-PD later, with a decline that can be highlighted by standardized neuropsychological tests which reflect a worsening of previous functionality, but do not significantly interfere with daily activities; and D-PD with more severe cognitive deficits affecting more than one cognitive domain and significantly interfering with daily activities.

Subjective cognitive decline. Subjective cognitive changes must be monitored; and although they are not always associated with objective changes, in a number of cases, they could herald incipient cognitive decline [8]. In a study of subjective memory complaints in patients with de novo PD (recently diagnosed and/or drug-naive) it was found that about 30% of patients who complained of memory issues had a higher risk to develop MCI-PD during the next 2 years of follow-up compared to patients with no such complaints [8]. Several factors, including affective symptoms, could contribute to progression to MCI-PD [9].

Minor cognitive impairment. The difference between MCI-PD and D-PD is the extent to which cognitive impairment interferes with daily activities. Although cognitive impairment is present in MCI-PD, it does not interfere with daily activities. One or more cognitive domains (attention, executive, language, memory, and visual-spatial functions) may suffer within MCI-PD. Depending on the number of affected cognitive domains, MCI-PD is classified into single-domain MCI-PD and multi-domain MCI-PD. First level assessment for the diagnosis of MCI-PD requires one neuropsychological test for each of the five cognitive domains, while second level assessment includes at least two tests for each cognitive domain which allows the sub-typing of MCI-PD in single-domain and multiple-domain MCI-PD [55]. It was established that multiple-domain MCI-PD occurs more frequently than single-domain MCI-PD, the most affected cognitive domains being executive, visuo-spatial, memory and attention [56]. The most common subtype of MCI-PD is the non-amnestic subtype, while speech disorders are less common [57]. MCI-PD is usually a precursor to D-PD, with 19-62% of patients with MCI-PD developing Parkinson's disease dementia within 2 to 5 years after receiving a diagnosis of MCI-PD [58]. The risk of developing D-PD in the next 5 years for patients with MCI-PD is 6.5 [59]. However, in some patients with MCI-PD cognition may be restored. According to a meta-analysis, 28% of MCI-PD patients followed-up for one year, returned to a normal cognitive state; but had a higher rate of progression to D-PD and a lower rate of return to normal cognition at a follow-up of over 3 years [60]. This fact can be explained by the "dual syndrome hypothesis" which mentions two types of MCI-PD: *MCI-PD* with predominant frontal striatal involvement and MCI-PD with predominant temporal and posterior cortical dysfunction [61]. The MCI-PD type with predominant frontal striatal involvement manifests with disfunctions related to planning, working memory and response inhibition, which are modulated by dopamine. It may be present even in the initial stages of PD and rarely progresses to D-PD. The MCI-PD type with predominant temporal and posterior cortical dysfunction presents with deficits in attention, semantic verbal fluency, and visuospatial difficulties and leads to a higher risk of developing D-PD [61].

Parkinson's disease dementia. Parkinson's disease dementia is a common late manifestation of Parkinson's disease and is characterized by a cognitive decline that is stereotyped, rapid, devastating, and has an impact on daily activities. The essence of cognitive changes in D-PD is executive dysfunction that is characterized by impaired planning, mental inflexibility, deficiencies in abstract thinking and verbal fluency, and apathy. Attention, visual-spatial functions, and memory may also be impaired, while speech is usually preserved [62]. Patients with D-PD suffer memory impairments, especially impacting rapid memory, that improve when given clues [63]. A diagnosis of D-PD is based on the presence of deficits that are severe enough to affect activities of daily living, which occur in at least two of the four basic cognitive domains (attention, memory, executive and visuospatial) [55]. D-PD is accompanied by neuropsychiatric symptoms, such as: mood disorders, psy-

chosis, and hallucinations. Visual hallucinations are usually complex, with preserved discrimination.

Assessment of cognitive impairment in Parkinson's Disease includes questioning both the patient and the caregiver [64]. It is important to understand if the person presents with a new symptom or has had problems of this kind in the past. In the case of an acute onset of cognitive decline, potential causes are evaluated, such as: infections; metabolic disorders, like the decompensation of chronic somatic diseases with renal or hepatic insufficiency; craniocerebral trauma with acute subdural hematoma; other somatic diseases with acute onset; the patient's medication (high doses of dopaminergic drugs, the use of amantadine and dopamine agonists, the use of drugs with a pronounced anticholinergic effect, both antiparkinsonian and non-antiparkinsonian). In the case of an insidious onset of cognitive decline, potential causes are evaluated, such as: coexisting cerebrovascular disease, vitamin B12 deficiency, thyroid dysfunction, craniocerebral trauma, chronic subdural hematoma, autoimmune diseases, visual or auditory sensory disorders, depression and anxiety, sleep disorders, overwork, psychosis, orthostatic hypotension associated with PD [65].

There are a series of screening tests for cognitive decline in Parkinson's disease – rating scales.

Validated scales with good inter-rater reliability are recommended for the screening of cognitive decline in PD [66]: Montreal Cognitive Assessment Scale (MoCA), Dementia Rating Scale 2 (DRS-2) and Parkinson's Disease-Cognitive Rating Scale (PD CRS) [67].

The Montreal Cognitive Assessment (MoCA) was developed as a screening test for mild cognitive impairment. It covers visual, executive, attention, memory, language, and orientation functions and can be completed in 10-30 minutes. A total score below 26 points suggests MCI-PD, and a total score below 21 points indicates D-PD [68]. The Dementia Rating Scale 2 (DRS-2) is a global test of cognitive function that assesses cognitive domains such as: attention, initiation/perseveration, construction, conceptualization, and memory. It can be performed in approximately 20-30 minutes and has cut-off scores of 139 out of 144 for MCI-PD and 132 out of 144 for D-PD [69]. The Parkinson's Disease-Cognitive Rating Scale (PD CRS) is designed for the entire spectrum of cognitive disfunctions of Parkinson's disease. It includes tasks for frontal and subcortical functions (sustained attention, working memory, alternating and action verbal fluency, clock drawing, immediate verbal recall and long-term memory) but also tasks for posterior cortical functions (clock copying) and can be completed in approximately 20 minutes. By keeping copies of previous tests – clocks, copied numbers, etc. – it's possible to monitor the evolution of cognitive performance over time. The Mini-Mental State Examination (MMSE) (traditionally used as a standard clinical test for assessing cognitive dysfunction) evaluates cortical cognitive aspects that are usually preserved in D-PD, so it is not recommended as a first-choice neuropsychological test. Screening tests of executive function are not specific, they are sensitive to the deterioration of other cognitive domains, but by using several screening tests, the pattern of cognitive deficit can be obtained.

Neuropsychological testing aims to identify the affected cognitive domains. Specific neuropsychological tests have been proposed to evaluate the different cognitive domains affected in PD. Executive function implies planning, cognitive flexibility, motor inhibition, cognitive inhibition, working memory, motor sequencing, timing, concentration/attention. Planning deficit manifests through organizational problems. It can be quickly tested by drawing the clock (circle, placing basic figures, etc.) or describing the stages of planning a trip. Cognitive inflexibility manifests as perseveration and difficulties in changing tasks. It can be quickly assessed by testing phonetic fluency (number of words beginning with "C" in 60 seconds). Motor inhibition deficits or motor inhibition errors can be highlighted by "Start! / Stop!" commands, Luria loops or Luria parapets [70]. Cognitive inhibition deficit is manifested by impulsivity, sexual disinhibition, obsessions; it can be inferred from swearing during testing of the phonemic fluency [71]. Patients with impaired working memory mention situations like: "I forgot the reason why I entered the room". This deficit can be objectified by counting forward and backward, simple calculations (addition / subtraction) [72]. Impairments in motor sequencing can manifest as difficulties in using new tools and can be objectified by the "fist-lip-palm" motor sequence repetition test. The synchronization deficit is indicated by the wrong estimation of time and can be objectified by the test of touching a surface with a finger to a certain stimulus. Patients with attention/concentration deficits often ignore road signs. This deficit can be tested by counting backwards, auditory target detection, surface touching for each 'A' in a letter sequence [72]. Visuospatial function refers to: perceptual discrimination, face recognition / discrimination, emotion recognition, spatial orientation, visual construction, visual memory. Patients with impaired perceptual discrimination may not recognize the items in a refrigerator. This dysfunction can be revealed by the "overlapping digit recognition test". Patients with face recognition/discrimination disorders are overall confused in the social environment. This disfunction can be objectified by testing the recognition of celebrities. Emotion recognition deficits can result in interpersonal difficulties and can be assessed by the ability to recognize the examiner's mimicked emotion. Spatial orientation deficit may manifest as wandering. These patients will not be able to correctly describe the route to their own home. Impairments of visual construction cause difficulties in making minor repairs or cooking. It can be objectified by the test of copying a figure. Deficits in *visual memory* can be indicated by keys or wallet misplacement and can be tested by contemplating a figure and then drawing it from memory. Episodic memory deficits in D-PD are usually mild and present

late in the course of the disease [73]. Patients with episodic memory impairment forget conversations and events. Memorizing a list of heard words with their subsequent recall, using category or multiple-choice cues for guidance, is a test that can be used to identify deficits in episodic memory. Speech is usually preserved in PD and occasional language deficits may be indicators of a superimposed dementia of neurodegenerative or vascular origin. Although the diagnostic criteria of D-PD state that basic language functions should be preserved [62], more than half of patients with D-PD also have comorbid amyloid pathology [73], and screening for these speech deficits is important for prognosis and treatment. During the general neurological examination attention is drawn to the use of nouns, repetitions, and the fulfillment of commands. Patients may show word-finding difficulties or paraphasia. Fluency tests for nouns or categories of nouns (60 seconds time allowed) or tests for completing simple commands are useful. Speech disorders, as well as episodic memory impairment in a patient with D-PD, may indicate a superimposed dementia within an associated proteinopathy (tau or ß-amyloid). The model of cognitive decline is best identified by applying standardized, validated neuropsychological tests with appropriate population norms, adjusted according to age, level of education, area [74]. Certain cognitive domains are targeted through certain neuropsychological tests: (1) Attention and working memory – the counting-back test; route making test; word-color Stroop test; WAIS-IV-number/letter sequencing; WAIS-IV-coding; (2) Executive – drawing the clock; verbal fluency test (letters, categories of objects/beings), Wisconsin / Nelson card sorting test; (3) *Speech* – WAIS-IV-similarities, Naming-matching test, Boston naming test; (4) Memory – word list learning test with delayed recall and subsequent recognition (Rey Auditory Verbal Learning Test, California Verbal Learning Test, Hopkins Verbal Learning Test, Selective Recall Test); the delayed prose-text recall test (the Wechsler Scale-IV logical memory subtest or the Rivermead paragraph recall subtest), the short visual memory test; (5) Visuo-spatial – copying the clock; the Benton test for determining the orientation of lines; the Hooper test of visual organization.

Because poor cognitive performance can be due to deficits in multiple domains, it is necessary to use multiple tests to identify the most significant deficit suggestive for a particular etiology of the cognitive impairment.

Diagnostic criteria of different degrees of cognitive decline in Parkinson's disease. To establish a diagnosis of cognitive decline associated with PD, the DSM-V criteria for diagnosing major or minor cognitive disorders associated with Parkinson's disease [75] or the criteria of the International Parkinson and Movement Disorder Society can be used.

According to the DSM-V criteria: Major or minor cognitive impairment *can be attributed* to Parkinson's disease if: it occurs within established PD; has an insidious onset and slow progression. The cognitive impairment is considered probably attributable to Parkinson's disease if: (1) there is no evidence of another disorder that could contribute to the cognitive decline, and (2) Parkinson's disease clearly precedes the onset of the cognitive impairment. Cognitive impairment is *possibly attributable* to Parkinson's disease if only one of the two criteria is met. Associated features that support the diagnosis are – apathy, depression, anxiety, hallucinations, personality changes, REM sleep behavior disorder, and excessive daytime sleepiness [75]**.** The International Society of Movement Disorders has developed diagnostic criteria for the diagnosis of Parkinson's disease-associated minor cognitive impairment MCI-PD [55] and for Parkinson's disease dementia [75]**.**

According to the International Society of Movement Disorders Criteria, the diagnosis of minor cognitive impairment associated with Parkinson's disease (MCI-PD) is established at two levels. Level I is a shortened assessment and consists of denoting the existence of cognitive deficit, according to a scale suitable for cognitive testing in Parkinson's disease (Montreal Cognitive Assessment Scale (MoCA), Dementia Rating Scale 2 (DRS-2) or Parkinson's Disease-Cognitive Rating Scale (PD CRS)) and impairment on at least two neuropsychological tests on an abbreviated assessment (one test per domain; fewer than five cognitive domains assessed). Level II is an extensive assessment, using at least 2 tests for each of the five cognitive domains (attention and working memory, executive functions, language, memory, visuospatial skills). MCI-PD can be diagnosed if there is impairment in two tests within one domain or impairment within one test in two different domains. Cognitive impairment is manifested by scores of 1–2 standard deviations below the norm, significant decline in serial tests, significant decrease in functioning compared to the premorbid level. Level II allows the identification of MCI-PD subtype: single-domain MCI-PD (impairment on two or more tests in one domain) and multi-domain MCI-PD (impairment on at least one test in two or more domains) [67]**.**

According to the International Society of Movement Disorders, the diagnosis of Parkinson's disease dementia also occurs at two levels. The first level consists of establishing a diagnosis of PD according to the UK Brain Bank criteria for PD, before the onset of dementia; a Mini-Mental State Examination (MMSE) score of less than 26; an impact of cognitive impairment on daily life that is independent of motor symptoms. Cognitive impairment must be present in more than one cognitive domain. Major depression, delirium, or other disorders that would obscure the diagnosis must be absent. *Level II* consists of an extensive assessment of four compartments: global cognitive efficiency, subcortical-frontal characteristics of D-PD, cortical characteristics of D-PD (language, visuo-constructive, visuo-spatial, visuo-perceptual) and neuropsychiatric characteristics of D-PD (apathy, depression, visual hallucinations, psychosis) [76]**.**

The differential diagnosis of Parkinson's disease de-

mentia is mainly done with Lewy body dementia and is based on an arbitrary distinction between the time of onset of motor and cognitive symptoms [77]. In Lewy body dementia, dementia precedes the development of parkinsonian motor symptoms. Lewy body dementia is diagnosed when dementia has developed within one year of the onset of motor symptoms, while D-PD is defined as dementia occurring in already established PD, with motor symptoms having lasted more than one year [78]. Both Lewy body dementia and D-PD present with neuropathological changes related to Lewy bodies [79] and have similar clinical profiles including visual hallucinations, cognitive fluctuations, and parkinsonian motor symptoms [79]. In favor of the single spectrum "Parkinson's disease dementia – Lewy body" is the fact that neuropsychological testing shows a severe deficit in executive functioning, visuospatial processing and verbal learning in both D-PD and Lewy body dementia. Lewy body dementia and D-PD are considered to represent entities within the same spectrum, with a similar pattern of impairments [31].

What is the impact of dopaminergic therapy on cognition in Parkinson's disease patients? Dopaminergic drugs can improve the performance on tasks modulated by the dorsal caudate, but they may worsen the performance on tasks modulated by the ventral striatum, due to dopaminergic deficits that are different in these regions [80]. The effect of dopaminergic drugs on executive function differs depending on the stage and severity of the disease, the dose of the drug, and the specific cognitive task assessed [81]. There is evidence of improvement of working memory, planning and behavioral flexibility in patients taking levodopa [82]. Low doses of levodopa and dopaminergic agonists cause drowsiness, while high doses of levodopa promote wakefulness and alertness [83]. Dopaminergic drugs, especially dopamine agonists, are associated with the onset or worsening of visual hallucinations in some patients with PD. However, the latency of the hallucination's onset is influenced by the mechanisms of the disease itself rather than by the dopaminergic regimen administered. Dopaminergic drugs improve cognitive flexibility, planning, working memory, attention, timing, motor inhibition, perceptual initiation and discrimination; and can worsen motor sequencing, cognitive inhibition, visual memory and emotion recognition [84].

The choice of initial medication at the onset of PD – levodopa, or a dopamine agonist, or a monoamine oxidase-B inhibitor, was found to make no difference to the cumulative rates of dementia [85]. However, drugs with strong anticholinergic properties (for PD (benztropine, trihexyphenidyl) or for issues other than PD) are associated with worse long-term cognition, both in the general population and in PD patients, especially in the cases of a long-term exposure to several anticholinergic drugs or to a drug with more pronounced anticholinergic properties [86]. In patients with PD and comorbid psychosis, it is necessary to simplify antiparkinsonian treatment by gradually stopping non-levodopa antiparkinsonian drugs, in the following order: anticholinergic drugs, amantadine, selegiline, dopaminergic agonists, COMT inhibitors [87].

Several studies have found that deep brain stimulation can worsen cognitive function [88]. Continuous dopaminergic stimulation, such as continuous subcutaneous infusion of apomorphine and intrajejunal infusion of levodopa (IJL), was previously avoided in patients with PD-related cognitive impairment. Currently, continuous subcutaneous infusion of apomorphine is considered for patients with MCI, while intrajejunal infusion of levodopa is considered for patients with MCI and mild-moderate D-PD [89]. Continuous dopaminergic stimulation could especially benefit patients with cognitive complaints as a manifestation of non-motor fluctuations [90].

Management of cognitive impairment in Parkinson's disease. The optimal management of cognitive impairment in PD is a multidisciplinary approach, with pharmacological, non-pharmacological and psychosocial strategies. It begins with assessing the presence, severity, and impact of cognitive impairment, as well as investigating factors that contribute to cognitive decline: comorbidities, medications, modifiable risk factors. Counseling patients and families and developing a management plan is essential. Adequate management is required for orthostatic hypotension associated with advanced PD, cerebrovascular disease and vascular risk factors (diabetes mellitus, obesity, hypertension, arrhythmia), alcohol consumption, depression and associated sleep disorders [19]. Neuroimaging is useful for identifying structural etiologies (stroke, chronic subdural hematoma, intracerebral neoplasm) [31], and laboratory examinations – for diagnosing systemic infections or metabolic abnormalities (hypothyroidism, vitamin B12 or vitamin D deficiency) [31].

Pharmacological treatment of cognitive decline associated with Parkinson's disease. If the patient presents with psychosis and hallucinations, the first step is to rule out secondary causes – infections or toxic-metabolic etiologies. The next step is to stop administering non-essential non-parkinsonian drugs – anticholinergics, benzodiazepines, opioids. Subsequently, a reduced and simplified parkinsonian treatment is considered [91]. If optimal improvement of psychotic symptoms still does not occur, an atypical antipsychotic may be added. However, acetylcholinesterase inhibitors have been proposed by some authors for the management of psychosis and hallucinations as a step preceding antipsychotic drugs. To date, only rivastigmine has been approved for the treatment of D-PD [92]. There is insufficient evidence for the use of acetylcholinesterase inhibitors in MCI-PD [93].

Non-pharmacological management of cognitive impairment in Parkinson's disease. Non-pharmacological interventions for D-PD and MCI-PD include psychological rehabilitation, cognitive training, exercise, music, art therapy and non-invasive brain stimulation techniques [94].

Neuropsychological rehabilitation comprises the use of

cognitive and behavioral psychological interventions and includes educational, psychotherapeutic and motivational components, as well as "exercises" to activate specific cognitive functions [84]. Neuropsychological rehabilitation protocols include common goals (applicable to most patients, like the management of stress, sleep disturbances, limited social stimulation [84]; and specific goals (applicable to a particular patient), which are identified as a result of neuropsychological assessment and discussions with the patient and family which refer to interventions, such as not to lose certain objects around the house or organizing tips in order not to miss meetings, etc. Cognitive-behavioral therapy (CBT) aims to modify cognition and behavioral routines [95]. Optimizing cognitive function is achieved indirectly through physical exercise, proper sleep hygiene, adaptive stress management, social engagement, and continuous cognitive stimulation. Problems are identified jointly by the CBT provider, patient, and family. The final goal is the awareness of the negative influence of some harmful routines on the motor and cognitive symptoms of the disease and their optimization. Cognitive training improves global cognition, working memory, executive function, processing speed and attention. This is achieved through different types of tasks: computer programs, arts, crafts, reading, puzzle games, card games or board games [84]. Several randomized clinical trials stipulate significant positive effects of aerobic and resistance exercise on cognitive function in patients with D-PD [96]. Tango, cognitive training combined with motor training and treadmill training have positive effects on global cognitive function, processing speed, sustained attention and mental flexibility [97]. Compensatory strategies and devices – the most direct and practical method of addressing problems arising from cognitive deficiencies – refer to: drawing up lists of activities to be performed or objects to be purchased, using diaries for noting activities in advance, implementing organizers for drugs, alarms for finding objects.

An important aspect of neuropsychological rehabilitation, as part of the multimodal management of cognitive impairment in PD, is the patient's involvement in his own care, giving him a sense of self-control in dealing with his own illness. In this case, all these interventions will have the potential to have a significant impact on the patient's functionality and quality of life.

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Authors' contribution

LR, OG conceptualized the idea, conducted literature review, wrote the manuscript; SG revised and finalized the text. All the authors revised and approved the final version of the manuscript.

Funding. This study was supported by the grant from the National Agency for Research and Development of the Republic of Moldova (NARD), Project code 20.80009.8007.01.

Ethics approval and consent to participate. The research project was approved by the Research Ethics Committee of *Diomid Gherman* Institute of Neurology and Neurosurgery (protocol No 4, 29.09.2023).

Conflict of interests. No competing interests were disclosed.

Moldovan Medical Journal. December 2023;66(2):111

The monograph

"Chronic pain – classification, pathophysiology, personalized management"

Printed by Bons Offices, Chisinau, 2023, 358 Pages

The authors: **Oxana Grosu**, MD, PhD, Senior Researcher; **Natalia Belii**, MD, PhD, Assistant Professor; **Lilia Rotaru**, MD, PhD, Associate Professor; **Daniela Cepoi**, MD, Assistant Professor; **Stela Odobescu**, MD, PhD, Associate Professor
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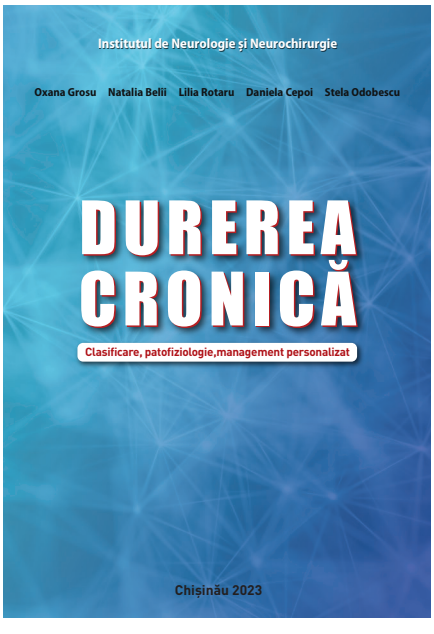

Pain is the most common cause of patient's referral to health services; the correct diagnosis and treatment of pain is a challenge for doctors of various specialties. Recognizing chronic pain as an independent entity (ICD11) and classifying of chronic pain syndromes allows a faster and more accurate diagnosis of pain, so that the patient can benefit from treatment as early as possible, in order to prevent chronicity. The patient can thus be placed at the center of a multidisciplinary team of specialists to offer him a personalized treatment.

The book "Chronic pain – classification, pathophysiology, personalized management" was developed under the editorship of Professor Ion Moldovanu, MD, PhD, by the prestigious group of authors from the *Diomid Gherman* Institute of Neurology and Neurosurgery of the Republic of Moldova.

The work addresses an extremely current topic, much studied in the literature. The monograph is impressive due to the clear and synthetic presentation of the types of chronic pain, with the description of the clinical aspects, the modern concepts regarding the pathophysiological mechanisms involved in the occurrence of chronic pain and the proposed current treatments.

The plan of the work follows the international classification of chronic pain according to the principles of the International Classification of Diseases (ICD11) and the Classification of the International Association for the Study of Pain, presenting the chapters of primary chronic pain, chronic pain associated with cancer, chronic post-surgical and post-traumatic pain, chronic pain neuropathy, secondary chronic musculoskeletal pain, secondary chronic visceral pain, headache or secondary chronic orofacial pain.

Developed in a modern way, the work has a real scientific value, through the latest literature data that is presented, based on an up-to-date and exhaustive bibliography. The rich iconography facilitates the understanding of the text.

The work has a special practical importance. This monograph is intended for a wide audience, addressing neurologists, neurosurgeons, intensive care specialists, internal medicine, surgery, rheumatology, oncology, family doctors, as well as residents and students. I recommend the book "Chronic pain – classification, pathophysiology, personalized management" for reading.

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The monograph

"Major cognitive disorders (dementia) in patients with neurodegenerative and vascular pathology. Pathophysiology, diagnosis, treatment"

Printed by Capatina Print, Chisinau, 2023, 483 Pages

The authors: **Lilia Rotaru**, MD, PhD, Associate Professor; **Ana-Maria Bubuioc**, MD, PhD Student;
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The undersigned **Cristinel Stefanescu**, qualified university professor, doctor of medical sciences, discipline of Psychiatry, *G. T. Popa* University of Medicine and Pharmacy, Iasi, Romania, primary psychiatrist, head of the V-th Acute Section, Socola Institute of Psychiatry in Iasi, I carefully read the monograph *"Major cognitive disorders (dementia) in patients with neurodegenerative and vascular pathology. Pathophysiology, diagnosis, treatment"*. In the following lines, I will briefly express my considerations.

Major neurocognitive disorders have as their common denominator the progressive decline of cognitive and socio-occupational functions, which leads to a significant and progressive decrease in the overall functioning of the person. These disorders are a major cause of morbidity and mortality in old age, with a great impact on the quality of life, both for patients and those around them. The global prevalence of dementia was 24 million in 2011, safely under-reported, and it is estimated to double every 20 years. The global incidence of dementia continues to increase annually in both low- and middle-income and high-income countries.

Major cognitive disorders are a frequent cause of referral of patients and their relatives to health services. Recognizing these complex disorders as early as possible allows the patient to benefit from therapy within a multidisciplinary team of specialists.

Monograph *"Major cognitive disorders (dementia) in patients with neurodegenerative and vascular pathology. Pathophysiology, diagnosis, treatment"* was developed by a team of renowned specialists in neurology and psychiatry. The work presents a real scientific value through data from the latest literature, based on a generous bibliography.

Neurodegenerative processes have a multifactorial etiology, being the subject of extensive research. The work attracts and impresses by presenting different types of dementia, with the description of modern concepts regarding biological, genetic, epigenetic, environmental risk factors. The authors of these materials elucidate the pathogenetic and physiopathological mechanisms in major cognitive disorders, the involvement of different neurotransmitter systems, the presence of neuropathological and morphopathological changes in the brain.

Extensive material is devoted to contemporary clinical approaches in major neurocognitive disorder secondary to Alzheimer's disease, major frontotemporal neurocognitive disorder, dementia with Lewy bodies, major vascular neurocognitive disorder, substance-induced brain injury, autoimmune, demyelinating and inflammatory diseases of the nervous system, also secondary to prior diseases, Parkinson's, Huntington's, structural brain disorder (brain tumors), and metabolic disorder.

For all these types of dementia, diagnostic markers, key elements, examples of neuropsychological tests, essential clinical features, examples of clinical manifestations are presented.

In the framework of the research, the peculiarities of onset and evolution, the functional consequences of patients with major cognitive disorders, the prognosis of the patients were studied. The paper presents modern ancilary investigations and their principles of interpretation. Essential comorbidities considered for differential diagnosis are also described.

The last chapter is dedicated to the assessment of cognitive deficit, tools for measuring cognitive decline and principles of treatment of patients with major neurocognitive disorders. In the opinion of the authors, the management of patients with dementia must focus not only on pharmacological therapy, but also on biopsychosocial aspects. Pharmacological approaches and non-pharmacological interventions for these patients are detailed.

In conclusion, major cognitive disorders are a challenge for the healthcare system. The monograph, with certainty, will contribute to the creation of necessary skills for doctors of various specialties to delay the onset of major cognitive disorders at different levels of healthcare.

Based on the above, I believe that the monograph "Major cognitive disorders (dementia) in patients with neurodegenerative and vascular pathology. Diagnosis, treatment" can be recommended for studying.

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The monograph

"**Newborn survival: progress and priorities for action**"

Print by Bons Offices, Chisinau, 2023, 214 pages

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The monograph "Newborn survival: progress and priorities for actions" is an up-to-date paper, focusing on the health and well-being of newborn children, anchored since 2015 in the Sustainable Development Goals (SDG Target 3.2.2), a fact whereby this category of children is given increased attention on the global agenda. The paper is based on the global research dedicated to the newborn in *The Lancet* journal included in two series: "Newborn survival" in 2005 and "Every Newborn" in 2014, which provided evidence to set the direction for this research.

Although newborns were invisible in the Millennium Development Goals, sustained efforts were made in the Republic of Moldova to increase survival, especially of premature newborns, with the transition to the WHO viability criteria (2008), as well as the reduction of impairment among premature babies and sick term newborns.

At the global level, the SDG 3.2.2 target for neonatal mortality is 12 or fewer deaths per 1000 live births, while in Moldova the indicator is 6.4 deaths per 1000 live births (2015), remaining at the same level in 2020, which imposes the need for in-depth study of the factors that contributed to success.

The monograph includes four chapters. In Chapter 1 – *"Progress at the global, regional level and in the Republic of Moldova regarding the newborn survival increase"* – the author carries out a deep analysis of the evolution of neonatal survival in the world and in the Republic of Moldova. Progress in improving child survival was accelerated between 2000 and 2015 compared to the 1990s. Globally, the annual reduction in under-five mortality rate (U5MR) increased from 1.8% in 1990-2000 to 3.9% in 2000-2015. Neonatal mortality is studied as a distinct age segment in the U5MR. It is found that in the period of 1990-2020 there was a decline in the U5MR by 58.33%, which occurred mainly due to the age category of 1-12 months (75%), being followed by the age period of 1-5 years (72.58%) and in the first month of life (31.9%). The 31.9% progress in reducing neonatal mortality must be properly appreciated, because reducing mortality in the first month of life is not easy to achieve.

Chapter 2 describes global and national initiatives on maternal, newborn and child health (MNCH). Among them, it is necessary to mention the *Global Strategy for Women's and Children's Health* on MDG 4 and 5 and the *Action Plan for Every Newborn* (2014) launched by WHO. The programs and strategies for improving the health of the mother, newborn and child in the Republic of Moldova were described by the authors to present the progress achieved.

The chapter "Continuity of health care for mothers and children" reflects the continuum of health care and care throughout the life cycle to improve the MNCH and survival. Continuity is approached through two key dimensions: time and place. The time dimension highlights the continuity of care over time, according to the life cycle, including: preconception, pregnancy, birth, postnatal, infant and up to 5 years. The place dimension depends on the level of service provision (family/community, outpatient, and home visits and in hospitals). 7 integrated packages for maternal, newborn and child health with evidence-based interventions across the continuum of care are presented.

Chapter "Survival of the newborn, infant and child aged 1-5 years. The action of biomedical interventions and other determinants of health" addresses the action of MNCH determinants on survival: *proximate* – biomedical, *intermediate* – such as effective coverage with interventions across the continuum of medical services, and *distal* – socioeconomic context, policy implementation of health and the direct or indirect contributions of the health system. For this purpose, the author applied a conceptual framework of results to examine the survival of newborns and children under the age of 5 between 1997 and 2015, in terms of coverage with preventive and curative biomedical interventions (total 47 interventions contained in 6 intervention packages – preconception, antenatal, labor, birth and postpartum, neonatal, infancy and the age of 5) and health system indicators, as well as macroeconomic factors, the national policy framework relevant to the field, financial flows, governance. Through modeling, the factors that influenced the reduction of the mortality of newborns and children 0-5 years were determined.

I consider the monograph "Newborn survival: progress and priorities for actions", authored by Curteanu Ala, represents a coherent work, it addresses an important topic from the point of view of medical research and practice. The content of the monograph is based on a wide current bibliographic material, presented concisely and logically. The work has scientific value and is of interest both to scientific researchers in the field and medical practitioners. Representing a scientific work, the monograph is recommended for studying, being an objective requirement for increasing the qualitative level of evaluation of scientific activity in the country.

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