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CONTENTS

CUPRINS

REAL-TIME IMAGINING OF NK-92 CELLS INTERACTION WITH TUMORAL CELL LINES

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ABSTRACT

One priority in developping better immunotherapies in cancer is the use of natural killer cells to treat different types of cancer. The citotoxicity of natural killer cells to tumoral cells depends on the type of immunological synapse formed. By establishing a litic immunological synapse, the effector cells is activated and release enzymes to destroy the target cells. The termination of the interaction between two cells is as important as the engagement in immunological synapse, because the detachment of the target cell can influence the serial killing of natural killer cells. Here, we followed the behavior of NK-92 cells in interaction with 3 different types of tumoral cell lines. The diferences observed in the interaction of natural killer cells with tumoral cells gives insight into the factors invovled in citotoxicity.

Keywords: immunological synapse, cytotoxicity, natural killer cells, tumor cells, time-lapse

INTRODUCTION

Natural killer cells are the first line of defense of the immune system against viral infected cells and tumoral cells [1-3]. The cytotoxic activity of natural killer cells depends on the close contact with the target cell. Detection of target cell is followed by adhesion to the cell to form a stronger contact. Signals inside the cell determine rearrangements of actin filaments causing changes in the morphology of the cell [4]. Depending on the signals received from the activating and inhibitory receptors the NK cells form an inhibitory or a lytic immunological synapse with the target cell [1]. The activated NK cells induce apoptosis of target cells through release of lytic enzymes in the immunological synapse interface [5].

The initiation of immunological synapse starts with the binding of integrin and selectin receptors to their ligands on the surface of target cell. Adhesion molecules like LFA-1 (CD11a/CD18) and MAC1 (CD11b/CD18) provide signals for further recruitment of receptors to form a stronger cellular contact and determines the clustering of activating and inhibitory receptors [6] Activating receptors NKG2D, Nkp30, Nkp44, NKp46, DNAM-1, 2B4 and NTB-A activate the NK cells through phosphorylation of the tyrosine kinase family Src and Syc, phospholipase C and PI3K. The activation signal induces the actin reorganization which allows the lytic molecules to be transported at the immunological synapse cleft. Degranulation of lytic vesicles with peforin and granyzime results in death of connected target cell [7].

For the lytic immunological synapse to be established is essential for the NK cell to identify the target cell and to receive activating signals. The cytolytic activity of NK cells was highly studied in order to understand the process which determine the activation stage and the evasion of tumor cells [8]. The death of target cell determine the detachment of an activated NK cell which can establish faster a new immunological synapse [9]. The serial killing of NK cells depends on the activation status and the type of target cells [10]. The former interaction with the target cell dictates the ongoing activated signal and so the efficiency of consequently killing. Studies have shown the formation of a new immunological synapse only after the detachment from the old target cells [11].

Challenges in obtaining sufficient NK cells from the donors and to genetically manipulate NK cells lead to the use of NK-92 cell line for immunotherapy. The NK-92 cell line is interleukin-2 dependent and tend to form clumps in culture [12]. The cell line has a higher degree of cytotoxicity

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than NK primary cells due to higher content of peforin and granyzime B and the lack of almost all the KIR receptors expressed on normal NK cells [13]. Also, NK-92 cells lack the CD16 receptor involved in ADCC [12].

In this study we investigated the behavior of NK-92 and establishment of immunological synapse with three different types of target cells. Most cytotoxic assay analyzes the individual contact between the cells and forget that the cell-to-cell communication is relevant in activation and proliferation response. The tendency of clumping for NK-92 indicates the health and activation status of the cells [12]. We decided to follow the morphology and contact dynamics of NK-92 cells in contact with target cells with focus on the clump formation and kinetics.

MATERIAL AND METHODS

Cell lines

NK-92 cell line was maintained in X-vivo 10 [Lonza] supplemented with 5% Human Plasma, 500 U IL-2 [Peptrotech]. The target cells NALM-6 and MCF-7 were cultivated in RPMI 1640 [Lonza] with 10% FBS [Gibco] and 1% Penicillin/Streptomycin [Sigma], SKBR-3 cell line was cultivated in DMEM [Sigma] + 10% FBS [Gibco] and 1% Penicillin/Streptomycin [Sigma].

Cell labeling

NALM-6 were washed with serum free media and labeled with 5uM lipophilic tracer spDII [Invitrogen] for 10 minutes at 37°C. The cells were resuspended in media with serum and seeded into the 4 wells. The adherent cells MCF-7 and SK-BR-3 were trypsinized at confluence, washed with serum free media and labeled in the same way like NALM-6 cells. The cells were left to adhere for 24 hours.

Fibronectin Coating

The imaging dish used µ-Dish 35 mm Quad [iBIDI] was coated with 100 uL of 15 ug/mL Fibronectin [Invitrogen] in each of the four wells and left for 1 hour in the hood. After 1 hour the solution was removed and the dish was left to dry for another 30 minutes before adding the NALM-6 cells.

Cytotoxicity assay

The NK-92 cells were centrifugated, resuspended in X-Vivo media supplemented with 5% HP, 500 U/mL IL-2, counted and added at the E:T ratio of 1:1 in each well. Live cell imaging of cells was performed using Nikon Biostation IM-Q at 37°C in a 5% CO_2 atmosphere with image acquisition every 5 minutes for 8 hours.

Data analysis

The size of the NK-92 clumps was measured using NIS-Element Software [Nikon] and the data is represented as mean+-SD.

RESULTS

NK-92 cells have activated morphology on contact with target cells (Fig.1).

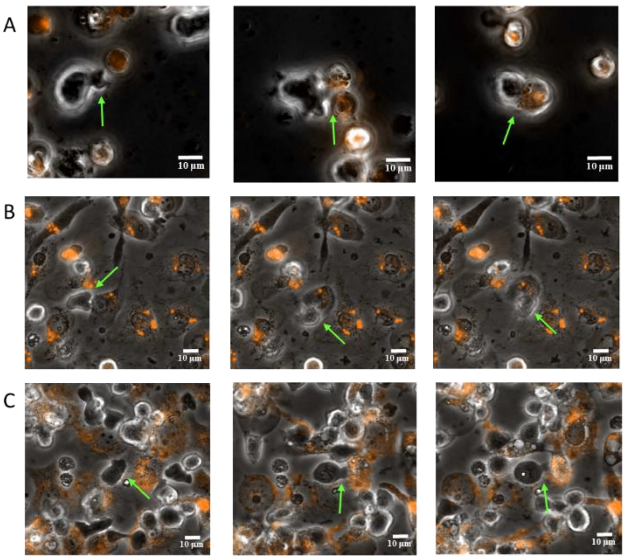

Fig. 1. Morphology of NK-92 cells during contact with target cells labeled with spDII. Green arrows indicate the conjugate formation. A. NK-92 cell is conjugated with NALM-6 cell (orange) B. NK-92 cell is conjugated with SK-BR-3 cell (orange) C. NK-92 cell is conjugated with MCF-7 cell (orange)

Morphology of NK-92 cells in contact with target cells was followed during the 8 hour time-lapse. Initially NK-92 cells spread lamellipodia in search for the target cell. Also, the lamellipodia are present for a period of time after the contact was established. The activated NK-92 cells have a symmetrical spreading across target cells surface. The large immunological synapse is followed by a contraction phase of NK-92 cell which determines shrinkage of adherent cells. Visible changes occur in morphology of adherent cells, the target cells became more granulated and rounder, indicating the apoptosis of cells.

Size of NK-92 clumps is influenced by the interaction with target cells (Fig. 2).

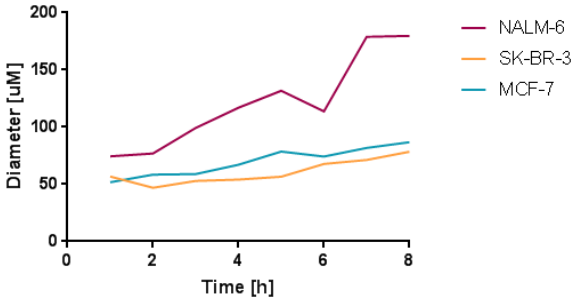

Fig. 2. Measurement of NK-92 cell clumps during the 8-hour time lapse imaging

To determine if the NK-92 cells have a different clumping behavior depending on the type of target cells we measured the diameter of formed clumps every hour during the 8 h time-lapse. After 2-hour incubation with target cells, the NK-92 cells started to form bigger clumps when in contact with NALM-6 cells. A drop in the size of clumps is registered after 5 hours in case of NK-92 incubated with NALM-6 cells. The increase in size of clumps continue after 6 hours and reach a plateau size after 7 hours. The aggregation size of NK-92 cells in contact with adherent cells was comparably smaller with NALM-6 cells, but followed an increasing path during the incubation time.

Differences in NK-92 target cell attachment to target cells (Fig. 3.)

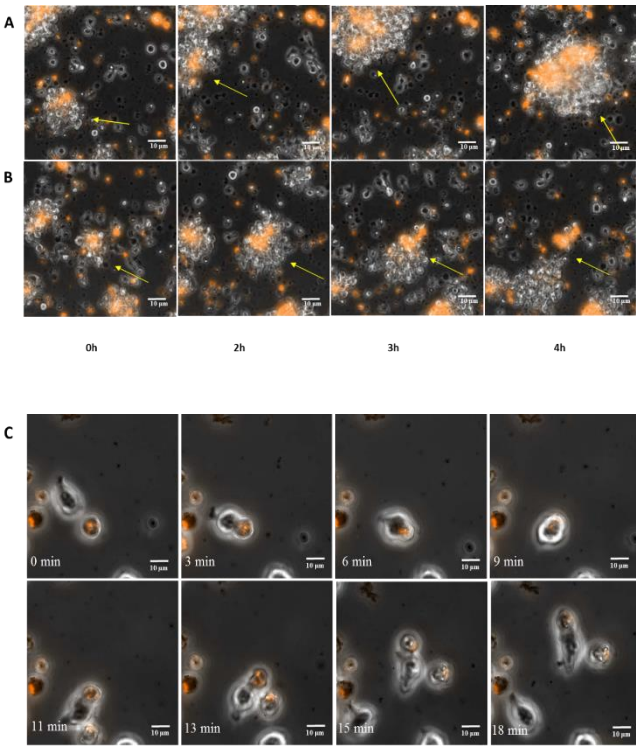

Fig. 3. NK-92 cell contact dynamics with NALM-6 target cells labeled with spDII. **A**. Smaller NK-92 clumps fuse with other NK-92 clumps to form a bigger cluster. The NK-92 cells are strongly attached to NALM-6 cells (orange) and transport them inside the NK-92 clumps; **B.** After the binding and close interaction of NK-92 cells to NALM-6 cells the NK-92 clumps shed away the NALM-6 aggregates (orange) from the clump formation; **C.** Motility of a NK-92 cells which binds to NALM-6 cell (orange) and drag the cell.

During the time-lapse imagining we observed a different interaction of NK-92 cells with NALM-6 cells adherent on fibronectin compared to NK-92 cells co-incubated with adherent target cells MCF-7 and SK-BR-3. The NK-92 cells in close contact with a NALM-6 target cells spread across the surface of the cell and drag the target cell to a close multicellular cluster (Fig.3 C). Moreover, we observed NK-92 cells that form conjugates with the target cells drag them into the NK-92 cell clumps, which results in an accumulation of NALM-6 cells inside the NK-92 clumps.

Only at the encounter with NALM-6 cells, the NK-92 cells accumulate target cells at the site of NK-92 cell clusters. The tendency of smaller clumps to fuse with other smaller clumps determine the aggregation into much bigger clusters formed of NK-92 cells and NALM-6 cells (Fig.3 A). Some of the NALM-6 cells brought together inside an NK-92 clumps were shaded away (Fig.3 B).

The engagement of NK-92 clumps towards MCF-7 and SK-BR-3 cells was different, without the integration of target cells in the NK-92 clumps. Some of the times the killed target cells remain attached to the NK-92 cells for a short period, but the NK-92 cells release them before engaging in another kill.

DISCUSSION

Using time-lapse imaging we have compared the behavior of NK-92 cells co-incubated with different types of target cells. Leukemia tumor NALM-6 cells are single small, round cells cultivated in suspension and MCF-7, SK-BR-3 cells are adherent tumor breast cancer cells. NK-92 cells were added at the same E:T concentration of 1:1 for all type of cells.

The cytotoxic activity of NK-92 cells is established during the immunological synapse when the NK-92 cell forms a firmer contact with the target cell. The duration of the contact is important, because shorter contacts indicate an inhibitory synapse where the NK-92 cells does not kill the target cell. Prolonged contact lead to stronger conjugate effector-target cells with efficient cytotoxicity [14]. NK-92 cells activation is known to be determined by the engagement of activating receptors like NKG2D, NK 1.1, 2B4, DNAM-1 and NCRs [15]. The activation status enables the rearrangement of actin filaments inside the cells to transport the lytic enzymes at the interface of the immunological synapse [7,16]. The release of the enzymes such as perforin determine the pore formation on the target cells in which granzyme B can enter and induce apoptosis of the cell [17].

Each of the immunological synapse stages determine modification in the morphology of the effector cell. Changes in membrane morphology are characterized by spreading over the target cells which increases the contact of ligand-receptors at the level of immunological synapse [14,18]. We have noticed that individual NK-92 cells form lamellipodia during their surveillance for target cells and also at the first contact with the target cell. After the contact was established the NK-92 cells become bigger and spread over the target cell. Olofsson et al. [14] showed that IL-2 activated cells are larger and spread across target cells during conjugation. Results from our imaging method are in line with other

studies where during synapse formation activated NK cells display elongated and irregular shape compared to resting NK cells which are smaller and rounder [14,17,19].

We identified a different behavior of NK-92 cells towards NALM-6 cells. The NK-92 cell form conjugates with target cells and remain in contact with them, migrating towards the closest cluster of cells. Thus, the formation of clumps has integrated NALM-6 target cells with-in, NK-92 cells scanning over the target cells. The motile scanning of NK cells was also described by [14], but only for isolated conjugates. Netter P et al. [20] demonstrated that initiation of new contact with a target cells is followed by a rapid detachment of NK cells from the last target. In our study, the NK-92 cell clusters were observed to contain smaller aggregates of NALM-6 cells, which were shed together after a period of 4 hours. The process through which NK cells could dissemble the immunological synapse and engage in sequential killing has not been identified. The termination of the immunological synapse was studied more using NK cells, which have different array of receptors compared to NK-92 cell line [21]. Is possible that the integration of NALM-6 target cells in the NK-92 clumps is necessary for the cells to maintain the activation status for a longer period of time. LFA-1 integrin plays a role in NK cell binding to target cell and is regulated by the balance of activating signals from 2B4 and NKG2D receptors [6].

CONCLUSION

Imaging techniques allows for a better understanding of the biological processes through real-time cell tracking. Moreover, the experiments will enable us to define the interactions between cells that can have further consequences in the function of the cell.

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IMAGISTICĂ TEMPORIZATĂ PENTRU EVIDENȚIEREA INTERACȚIUNILOR DINTRE CELULELE NK-92 ȘI LINII CELULARE TUMORALE

REZUMAT

O prioritate în dezvoltarea imunoterapiei mai eficiente este folosirea celulelor natural killer pentru tratarea difertelor tipuri de cancer. Citotoxicitatea celulelor natural killer față de celulele tumorale depinde de tipul de sinapsă imunologică formată. Prin stabilirea unei sinapse imunologice litice, celula efectoare este activitată și eliberează enzime ce distrug celula țintă. Terminarea interacțiunii dintre cele două celule țintă este la fel de importantă ca și crearea unei sinapse imunologice deoarece detașarea celulei țintă poate influența citotoxicitatea continuă a celulelor natural killer. În acest studiu am urmărit comportamentul celulelor natural killer în prezența a 3 tipuri diferite de linii celulare tumorale. Diferențele observate în interacțiunea celulelor NK-92 cu celulele tumorale dă o perspectivă asupra factorilor implicați în citotoxicitate.

Cuvinte cheie: sinapsă imunologică, citotoxicitate, celule natural killer, celule tumorale, time-lapse

MECHANISMS OF BONE LOSS IN GLUCOCORTICOID INDUCED OSTEOPOROSIS

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ABSTRACT

Osteoporosis (OP), defined by decreased bone mass and altered bone microarchitecture, is a generalized skeletal disease associated with a potentially high fracture risk and consequent severe complications; it has become a major health problem, with over 200 million people diagnosed and over 9 million new fractures annually. Glucocorticoid (GC) therapy is used with high efficiency in a wide variety of inflammatory and autoimmune diseases and in long term administration constitutes the most common cause of osteoporosis. Glucocorticoid induced osteoporosis (GIO) is recognized to be associated with a secondary increased prevalence of vertebral fractures due to a main effect on trabecular bone. The fracture risk is directly related to dose, appears even at small doses, and it has been published to be increased, after the first 6 months of treatment, both for vertebral and non vertebral fractures. It is reported that over 10% of the patients that have chronic GC treatment are diagnosed with a fracture.

GC effects on bone mass are visible as two types: first, a rapid phase, with a percentage between 6 and 12 reduction in the first year, due to an enhanced regulation of osteoclastogenesis and increased bone resorption, and a second phase, with a maximum 3% loss per year, explained by an altered bone formation. The mechanisms concurring to GIO pathogenesis are complex and imply both systemic effects, as well as local consequences on every bone tissue cell type.

Keywords: glucocorticoids, osteoporosis, fracture risk

INTRODUCTION

Osteoporosis (OP), defined by decreased bone mass and altered bone microarchitecture, is a generalized skeletal disease associated with a potentially high fracture risk and consequent severe complications [1]; it has become a major health problem, with over 200 million people diagnosed and over 9 million new fractures annually [2]. Usually fractures occur in spine, hip, distal forearm and proximal humerus; vertebral fractures are known to be associated with further vertebral fractures, with a reported incidence of 19.2% in the first year; more than 60% of them aren't diagnosed and represent one of the major causes of death among elderly [3, 4].

Glucocorticoid (GC) therapy is used with high efficiency in a wide variety of inflammatory and autoimmune diseases and in long term administration constitutes the most common cause of secondary osteoporosis. GC remain major anti-inflammatory drugs in many rheumatic diseases, both at the time of diagnosis, as well during flares, observed both by clinical examination, as well as using imagistic techniques, like high resolution ultrasonography, which has

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become an important diagnostic and evaluation tool; it is published that about 0.5-2% of the general population receives GC therapy, with a percentage up to 90 among patients with rheumatoid arthritis [5-9].

Glucocorticoid induced osteoporosis (GIO) is recognized to be associated with an increased prevalence of vertebral fractures due to a main effect on trabecular bone. The fracture risk is directly related to dose, appears even at small doses, and it has been published to be increased, after the first 6 months of treatment, both for vertebral and non vertebral fractures. It is reported that over 10% of the patients that have chronic GC treatment are diagnosed with a fracture [10]. GC determine a more evident bone loss mostly in the first 6 months of treatment, due to an increased bone resorption but also associated to the concurrent inflammation of the underlying disease, with a slower decrease during long term administration [11, 12].

GIO has been intensively studied, both regarding pathogenesis and improving therapeutic measures, adapted to each category of patients and it should receive greater awareness, with early intervention, in order to prevent future complications.

We aimed to make a brief review of the most recent aspects regarding GIO pathogenesis, assessment and treatment.

MECHANISMS OF BONE LOSS

GC effects on bone mass are visible as two types: first, a rapid phase, with a percentage between 6 and 12 reduction in the first year, due to an enhanced regulation of osteoclastogenesis and increased bone resorption, and a second phase, with a maximum 3% loss per year, explained by an altered bone formation [13]. The mechanisms concurring to GIO pathogenesis are complex and imply both systemic effects, as well as local consequences on every bone tissue cell type [14].

Osteoblasts. In patients with GC therapy appear intricated actions on osteoblast progenitors, osteoblasts and osteocytes, which contribute to bone loss and GC induced osteoporosis [10]. Several experimental studies demonstrated that GIO is characterized by an important decrease of osteoblast cells [15].

MSCs (mesenchymal stem cells), a subpopulation of bone marrow cells capable of bone formation, that can differentiate into multiple cellular lines, are altered by GC therapy, by stimulating the expression of different adipocyte-specific transcription factors, like upregulation of PPARγ2 (peroxisome proliferation-activated receptor gamma receptor 2) and CCAAT (cytosine-cytosine-adenosine-adenosine-thymidine)-enhancer-binding proteins (C/EBPs) α, β and γ; consequently, the balance is shifted towards adipogenesis and limited capacity of osteogenesis and osteoblast production [16-21].

Osteoblasts proliferation is inhibited by GC antianabolic effects, as suppression on the synthesis of different growth factors, such as insulin growth factors (IGF), hepatocyte growth factor (HGF), or inhibiting the activity of kinases/phosphatases [22, 23]. Another mechanism responsible for osteoblasts' impaired proliferation is represented by attenuation of cell cycle progression; several studies reported that cell cycle regulators CDK2, 4,6 cyclin D, c-Myc and E2F are downregulated and cyclin-dependent inhibitors, such as p21 and p27, and the tumor suppressor gene, p53, is stimulated [24-26].

High doses of GC inhibit osteoblasts differentiation by interfering with pathways essential for this process: downregulation of Wnt/β-catetin and BMP (bone morphogenetic protein) signaling pathway or upregulation of Notch inhibitory pathway. Physiologically, Wnt signaling prevents osteoblasts apoptosis and stimulate their proliferation. Wnt/ β-catetin signaling pathway is activated by Wnt binding to frizzled (Fz) receptors and co-receptors for low density lipoproteins receptor related protein (LRP) 5 and 6, which determines a reduced osteoclasts generation and osteoblasts differentiation and maturation [15, 27, 28]. High GC doses reduce Wnt expression and increase sclerostin and Dkk1 expression, thus inhibiting osteoblasts differentiation; by inducing Notch1 and Notch2 mRNA in osteoblasts, their differentiation is also inhibited [2, 29, 30].

Osteoblasts apoptosis. It is extensively reported that an important contributor to bone loss is represented by GC-induced apoptosis in osteoblasts and osteocytes [15, 28, 31]. There are several mechanisms that potentially contribute to apoptosis: excessive GC determine an increase of pro-apoptotic factors, Bim and Bak, and decrease the expression of BclXL, a pro-survival factor; also, high GC doses upregulate p53 protein levels, activate proline-rich tyrosine kinase 2 (Pyk2), by Ca entrance from extracellular space, and consequently JNK (c-Jun N-terminal protein kinase) activation, cell detachment and apoptosis [32]. Experimental studies showed that in human osteoblasts dexamethasone reduces β1 integrin expression and prevents cell-matrix adhesion, with cell death [33]. Therefore, osteogenic cellular line is prone to apoptosis due to GC administration through inhibiting anti-apoptotic factors and enhancing different pro-apoptotic pathways.

Osteocytes. Osteocytes, the most abundant bone cell type, with a tight connection that allows them to communicate, sense mechanical alterations, repair and maintain bone integrity, are differentiated osteoblasts with characteristic dendrites. GC dramatically influence osteocytes function and number, as they upregulate the hormonal factor FGF23, event that decreases serum 1,25 (OH)2-vitamin D3 levels and phosphorus [34, 35]; also, osteocytes are a major source of RANKL, and GC stimulate the expression of RANKL and of inhibitors of Wnt signaling, as Dkk1 (dickkopf signaling pathway inhibitor 1) and Sost (sclerostin) [36, 37]. Several studies have proposed to establish the dose and time of GC exposure that determines a decrease of osteocyte number; it was reported that long

term high GC doses (2.8-5.6mg/kgc/day) induce their apoptosis and low doses, under 2.8mg/kgc/day, determine autophagy [38, 39]. *Weinstein el al* reported that bone aging is directly related to an increased activity 11-beta-HSD1, a GC-activating enzyme, sustaining an increased sensitivity of osteocyte response to GC in older subjects [40].

Autophagy. Autophagy, a fundamental process for cell survival, can become insufficient or extensive and produce apoptosis. Bone homeostasis is maintained by autophagy in all cellular lines (osteoblasts, osteocytes and osteoclasts), and is relevant to several pathologies, including GIO [41]. GC interfere with osteoblast autophagy, the results of several experimental studies indicating that they may inhibit the process in a dose and time dependent manner [42, 43]. In a similar way, apparently autophagy is a protective mechanism for osteocytes in response to GC administration [32].

Osteoclasts. GC induced bone lost begins with a fast process of bone resorption due to an enhanced osteoclasts' number and activity [44]. GC increase the production of receptor activator of nuclear factor-κB ligand (RANKL) and decrease osteoprotegerin (OPG) production by osteoblastic cells and osteocytes, with a consequent enhancing of osteoclasts' proliferation and differentiation [45, 46]. RANKL, receptor activator of nuclear factor-kappa B (RANK) and OPG, members of TNF family, have a major role in bone remodeling and disorders of mineral metabolism. The differentiation and activation of osteoclasts is promoted by RANKL binding to its receptor, RANK [47]. OPG functions as a decoy receptor for RANKL, which prevents RANKL binding to RANK and constitutes a protective factor against bone loss [48]. The reports of several studies that investigated the RANKL/RANK/OPG system, concluded that is represents a major regulator of bone resorption [49]. Experimental studies have reported that GC administration induces the production of RANKL in bone marrow adipocytes and consequently supports osteoclasts differentiation in vitro [37, 50-52]. Pro-inflammatory cytokines, as TNF-α, IL-1, IL-6 and IL-17 can activate osteoclasts directly; production of RANKL and M-CSF is stimulated by TNF α and IL-17, produced by Th17 cells, that can release directly RANKL; these proves suggest that Th17 cells can be considered a major factor involved in osteoclastogenesis [53-58].

CONCLUSIONS

GC therapy is used with high efficiency in a wide variety of inflammatory and autoimmune diseases and in long term administration constitutes the most common cause of osteoporosis. GIO is an important pathology with potentially severe complications and identifying risk categories and initiating proper therapeutic measures is essential for osteoporosis and fracture prevention in chronic GC users.

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MECANISMELE PIERDERII OSOASE ÎN OSTEOPOROZA GLUCOCORTICOID INDUSĂ

REZUMAT

Osteoporoza, reprezentată de scăderea densității și masei osoase, alături de alterarea microarhitecturii osoase, este o afecțiune generalizată a scheletului osos, asociată cu un risc fracturar înalt și complicații potențial severe; este recunoscută ca o problemă majoră de sănătate publică, cu peste 200 de milioane de cazuri diagnosticate și peste 9 milioane de fracturi anuale. Terapia glucocorticoidă este utilizată cu eficiență înaltă în afecțiuni inflamatorii și autoimmune, iar administrarea pe termen lung constituie cea mai importantă cauză de osteoporoză secundară. Osteoporoza glucocorticoid indusă este recunoscută a fi asociată cu o prevalență crescută a fracturilor vertebrale, în special datorită efectelor asupra osului trabecular. Riscul fracturar este direct asociat cu doza, este prezent chiar și la doze mici, în special în primele 6 luni de tratament, atât pentru fracturile vertebrale, cât și pentru cele non-vertebrale; peste 10% dintre pacienții cu terapie cronică cu glucocorticoizi sunt diagnosticați cu o fractură.

Efectele glucocorticoizilor asupra masei osoase apar în două faze: prima, rapidă, determină reducerea masei osoase cu 6-12% în primul an, consecutive osteoclasogenezei și intensificării resorbției osoase, și a doua, datorată alterării formării oasoase, ce determină o pierdere a masei osoase de aproximativ 3% pe an. Mecanismele ce determină apariția osteoporozei glucocorticoid induse sunt complexe și asociază efecte sistemice și locale, asupra fiecărui tip de celulă osoasă.

Cuvinte cheie: glucocorticoizi, osteoporoză, risc fracturar

SIMULTANEOUS CANCERS OF THE HEAD AND NECK – CASE PRESENTATION

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ABSTRACT

We present a 76 years old patient with a Laryngeal tumor stage III, T3N0Mx with a s simultaneous neoplasm localized at the oropharyngeal level stage IIA (left palatine tonsil T2aN1Mx). The patient underwent a complex oncologic surgical procedure: total laryngectomy, left palatine tonsillectomy with bilateral lymph node dissection, level I-IV, followed by radiotherapy. Histopathologic exam revealed a squamous cell carcinoma in both tumors. Patient was followed-up for 5 years, being free of disease. Multiple head and neck cancers are not rare entities and according to studies in the literature, their incidence is increasing. A patient who suffered a head and neck cancer is exposed to a lifetime risk of developing a second malignancy.

Keywords: simultaneous cancers, synchronous cancers, laryngeal cancer, oropharyngeal cancer, head and neck cancers

INTRODUCTION

The head and neck represents the most complex parts of the human body, and cancers with this localization presents a tremendous importance. Head and neck cancer includes mucosal and tissues malignant tumors. Head and neck tumors implies tumors occurring in oral cavity, oropharynx, rhinopharynx, hypopharynx, larynx, nasal cavity, paranasal sinuses and salivary glands, etc. [1,2]. Simultaneous, synchronous and methachronous cancers are explained by the phenomenon of "field cancerization", not being rare entities; according to studies in the literature, patients with a head and neck cancer are at risk of a concurrent, synchronous and methachronous cancers appearance.

A patient who suffered a head and neck cancer is exposed to a lifetime risk of developing a second malignancy [3]. The real incidence of simultaneous, synchronous and metachronous cancers is not known, but according to some studies, it appears to be increasing [4]. Head and neck cancers presents a incidence of approximately 4% in the United States, and up to 35-45% in less developed countries [1,5]. Annually are diagnosed about 650,000 new cases of head and neck cancers all over the world, with a ratio of 3:1 between men and women [3], the majority being in advanced stages (stage III or IV) [6]. From histopathological point of view, more than 90% of head and neck cancers are squamous cell tumors, and the oral cavity and larynx interests approximately 60-70% of patients, followed by the pharynx, nasal cavity, salivary glands, thyroid gland, middle ear, etc. [1,5,7]. Besides squamous cell carcinomas, there are adenocarcinomas in approximately the rest 10% of the cases, followed by melanoma, soft tissue tumors and lymphomas [1,8].

Despite the medicine evolution regarding head and neck cancer patients' survival rate, we did not encountered an improvement, explained probably by the high risk of recurrence and the development of a second primary cancer.

CASE PRESENTATION

We present a 76 years old patient which presented in our ENT Department with dysphonia for 6 months, mild odinophagya, 10 kg weight loss and intermittent headache. ENT clinic and endoscopic exam, performing 70º Rigid Hypopharyngolaryngoscopy revealed a tumor with supraglottic localization with glottic extension (the right hemilarynx being fixed) (Figure 1) and a simultaneous tumor localized on the left palatine tonsil (oropharynx).

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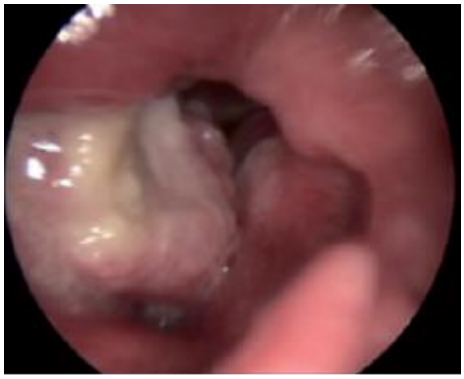

Fig.1. Supraglottic tumor with glottic extension (right hemilarynx)

Being incriminated several factors and affections such as lifestyle, genetic susceptibility, immune deficiencies, radiotherapy, chemoradiotherapy etc. a complete anamnesis was performed, revealing that the patient smokes 30 cigarettes / day, drinks approximately 100 ml pure alcohol/day and his father died with a pharyngeal tumor (without following any treatment modality).

A double biopsy and panendoscopy followed clinic examination. The biopsies revealed carcinomas of the larynx and oropharynx (squamous cell carcinoma).

The following diagnostic was established: Laryngeal cancer staged III, T3N0Mx (T3 supraglottic carcinoma with glottis extension (right hemilarynx) with a simultaneous neoplasm localized at the oropharyngeal level stage IIA (left palatine tonsil T2aN1Mx). The patient underwent a complex oncologic surgical procedure: total laryngectomy, left palatine tonsillectomy with bilateral lymph node dissection, level I-IV. At 14 days after the surgical procedure the nasogastric tube was removed and the patient was addressed to radiotherapy. The follow-up continued for 5 years as follows: in the first year at 2 months, in the second year at 3-4 months, in the 3rd year at 6 months and in the 4th and 5th year annually. Patient was free of disease.

Simultaneous, synchronous and methachronous cancers are not a rare entities. In our ENT Department, evaluating a period of 10 years, we encountered a number of 23 patients with head and neck simultaneous, synchronous and methachronous cancers (5 patients presented simultaneous cancers, 2 patients presented synchronous tumors and 16 patients presented metachronous tumors). The most frequently involved organ was the larynx, followed by the hypopharynx, oropharynx and oral cavity.

DISCUSSIONS

Patients who presented during their life a head and neck cancer and who were treated for it, have a high risk to develop multiple cancers. Simultaneous, synchronous and metachronous cancers appears in 3-5% of patients, presenting three cancers involves approximately 0.5% of patients and the concomitance of four cancers occur in less than 0.3% of patients [9].

From historical point of view Billroth reported for the first time in 1889 a simultaneous-synchronous cancers a stomach cancer and an external ear cancer (the second primary cancer) [10,11]. The term “field cancerization” might explain the phenomenon that occurs in the upper aero-digestive tract when mucosa at this level is exposed to the same types of carcinogens, if they act consistently and for a long time [5,12,13]. “Field cancerization” mainly affects the oral cavity, oropharynx and larynx, lung, esophagus, vulva, cervix, colon, breast, bladder and skin [14].

From clinical and oncological point of view it is important to differentiate between recurrence and second primary metachronous cancer.

The cancers might be simultaneous tumor appears in the same time with the primary (initial) tumor; synchronous tumors, which develop within 6 months of initial cancer diagnosis and metachronous tumors that occur at least 6 months after initial primary tumor development [15,16]. From statistically point of view a second primary tumor may appear with an incidence of 15% for synchronous tumours and 4% of the metachronous ones [17].

In our ENT Department simultaneous, synchronous and methachronous cancers are not a rare entity. In a period of 10 years we encountered a number of 23 patients with head and neck simultaneous, synchronous and methachronous cancers (5 patients presented simultaneous cancers, 2 patients presented synchronous tumors and 16 patients presented metachronous tumors). More than 20,000 patients were evaluated, out of which 8,000 there were oncologic patients. The most frequently involved organ was the larynx, followed by the hypopharynx, oropharynx and oral cavity.

Mortality risk is higher in patients with second primary head and neck cancer, having the primary tumor in an early stage, compared with mortality risk from the primary tumor [7,18,19].

The prognosis for simultaneous and synchronous cancers is worsten than the prognosis of and methachronous cancers [16].

There are incriminated several factors and affections such as lifestyle, genetic susceptibility, immune deficiencies, radiotherapy, chemoradiotherapy etc. Follow-up of patients who have had a head and neck cancer and periodic control are important for early detection of multiple cancers.

CONCLUSION

Multiple cancers of the head and neck are not as rare as we might think. The number of patients with synchronous or methachronous cancers in head and neck area has increased. Due to increased life expectancy it is very important to monitor and investigate all oncologic patients regarding tumor evolution and the entire upper aerodigestive tract for a possible early detection of second primary cancers.

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CANCERE SIMULATANE DE CAP ȘI GÂT – PREZENTARE DE CAZ

REZUMAT

În această lucrare prezentăm un pacient de 76 de ani, cu tumoră laringiană stadiul III, T3N0Mx cu un neoplasm simulatan localizat la nivel orofaringian stadiul IIA (amigdala palatină stângă T2aN1Mx). Pacientul a suferit o procedură chirugicală oncologică complexă: laringectomie totală, tonsilectomie palatină stângă cu disecție bilaterală a ganglionilor limfatici, nivelul I-IV, urmată de radioterapie. Examenul histopatologic a arătat carcinom cu celule scuamoase la nivelul ambelor tumori. Pacientul a fost urmărit timp de 5 ani, neavând remisiuni. Cancerele multiple de cap și gât nu sunt entități rare și, conform datelor din literatură, incidența acestora este în creștere. Un pacient care a dezvoltat un cancer la nivelul capului sau gâtului prezintă un risc pentru tot restul vieții de a dezvolta o afecțiune malignă.

Cuvinte cheie: cancer simultan, cancer sincron, cancer laringian, cancer orofaringian, cancere de cap și gât

ALERGIC RHINNITIS – METHODS OF PREVENTION

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ABSTRACT
Allergic rhinitis is a frequent pathology affecting about 20% of the population. The risk is higher if the patient associates asthma, different types of eczema or has a history of allergic diseases. You don't have an age limit for allergic rhinitis – it can develop also in newborns or in elder people. Symptoms have remission periods – meaning they can disappear completely for a long period of time in which the patient can feel he has been completely cured. Allergic rhinitis is caused by the reaction of the nasal mucosa to small particles called allergens. Most commonly we classify the type of allergic rhinitis by its duration. We can have seasonal allergic rhinitis (rhinitis that occurs in allergy-specific seasons) and perennial allergic rhinitis (which is present anytime during the year.)
Key words: allergic rhinitis, nasal obstruction, allergens

INTRODUCTION

Allergic rhinitis is a frequent pathology affecting about 20% of the population. Allergic rhinitis is defined as a symptomatic affection of the nose induced by allergen exposure, its inflammation being mediated by IgE.

The risk is higher if the patient associates asthma, different types of eczema or has a history of allergic diseases. It is important to remember you don't have an age limit for allergic rhinitis – it can develop also in newborns or in elder people. Usually the symptoms have remission periods – meaning they can disappear completely for a long period of time in which the patient can feel he has been completely cured [1].

Main causes for the development of alergic rhinitis

Allergic rhinitis is caused by the reaction of the nasal mucosa to small particles called allergens.

This allergic reaction is characterized by activation of two types of inflammatory cells: mast cells and basophils. These cells produce pro-inflammatory substances such as histamine and these substances cause congestion and stimulate fluids to gather in the nasal mucosa leading to itching and sneezing. After this cascade is activated, other mechanisms continue to activate, and the symptoms become persistent and increasingly unpleasant [2].

Most commonly we classify the type of allergic rhinitis by its duration. We can have seasonal allergic rhinitis (rhinitis that occurs in allergy-specific seasons) and perennial allergic rhinitis (which is present anytime during the year.)

Seasonal allergic rhinitis is most often caused by allergens coming from tree pollen, grass, various plants (the most common being ambrosia) or molds. Seasonal allergies generally do not occur in children under 2 years of age [3].

Allergens that produce perennial rhinitis are the ones that surround us throughout the year and are usually found right inside our house. The most commonly encountered are dust, mites, hair or puff of certain animals, kitchen beetles. This type of allergy is the most difficult to treat because it is very difficult to remove the allergen from the patient's environment [4].

ARIA (*Allergic Rhinitis* and its Impact on *Asthma)* classifies allergic rhinitis in intermittent : less than 4 days a week or less than 4 weeks or persistent: more than 4 days a week or more than 4 weeks.

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Main symptoms of allergic rhinitis

The most common symptoms are:

- Nose related: the nose will usually flow in the form of aqueous secretions, it will be clogged, the patient breathes hard, sneezes, and has the feeling of secretions in the back of the nose . Sometimes they can associate a lack of taste or a sense of pressure or pain in the sinus area.
- Eyes related: itching may occur and the eyes may be red. Sometimes the patient can describe a feeling of sand in their eyes.
- Throat related - there is a feeling of numb, dry throat, the voice can be hoarse and a chronic cough that does not respond to classic cough treatment can appear.
- In addition, the patient can relate that he breathes more than the mouth, sleeps poorly and wakes up often, during the day is tired and focuses hard on work.

Allergic rhinitis is diagnosed by detailed history, including questions about possible asthma, and nasal examination, together with inspection of throat, ears and chest where possible, backed up by specific allergy tests, either skin prick or blood tests, for specific IgE to allergens suggested by the history.

Clinical history: symptom type, duration and frequency and exacerbating factors are important for diagnosing and classifying rhinitis which is characterised by two or more nasal symptoms: itching, sneezing, obstruction and rhinorrhoea. The timing of these in relation to exposure to allergen (i.e., specific season or animal) is highly relevant. Upon such exposure,symptoms occur in minutes and last for hours. Late-phase symptoms can include nasal obstruction, hyposmia, postnasal mucous discharge and nasal hyper-reactivity [5].

What can we do to prevent allergic rhinitis?

The best advice for the patient is to eliminate the allergen from the environment. For that the first step is to identify the allergen. There are four major categories of trigger:

- Pollen - spring and summer - trees, grass, autumn - hay
- Insects - kitchen beetles, ladybugs
- Allergens of animal origin - skin, fur, fluff, saliva.
- Molds

The most common indoor allergens are dust and dog or cat hair. It is good to inform your patient with regard to indoor allergens, that a period of 3 to 6 months is required until the symptoms are resolved after the allergen is removed [6].

Mites - these are a very small type of insect microscopic living in mattresses, sofas or other types of material. They do not bite or produce any harm other than allergies. They absorb moisture from the atmosphere and feed on organic matter (human or animal skin). They can be removed by using special shells against the mites. The patient should be advised to cover the bed with special anti-mucus covers that are very tight on the bed. Improvise plastic bedding glued with adhesive tape as described by various patients should not be improvised. They are inefficient and put the patient's life in danger by choking. Lingerie should be washed weekly in warm water and dry in the dryer if this is possible [7].

Exposure may be reduced if the patient uses a vacuum with a HEPA filter, wipes the dust frequently, and does not sleep on upholstered furniture (for sofas).

Air humidity must be maintained at a level between 30 and 50%. Humidifiers are not recommended because there are some studies that claim their use amplifies the problem.

When possible unnecessary items should be removed, excess furniture, curtains, drapery rugs, especially in rooms where the patient spends a lot of time (bedroom, office) because they are sources of dust. Plush toys are to be avoided [8].

Puff / animal fur -any animal can cause allergies. In cats, allergies are produced by a protein found in its saliva, the skin, the urinary and reproductive tracts, and not the hair as it is popular believe. So a cat with short fur is as allergenic for a patient with allergic terrain as a long-haired cat. If the patient really wants a pet, he can try a reptile, turtle or fish - although fish are not recommended because they live under the pool they live in is a perfect environment for allergy development.

If the patient is allergic to an animal then it must be removed from the house. Limiting it to an area of the house is not effective because allergens are frequently carried by the air and the clothes of the people who come in contact with the animal. After the animal has been removed from the home, the carpets, sofas, curtains, drapes and linen should be thoroughly cleaned. You should recommend a thorough cleaning of the home especially after cats because the allergens produced by it are very adherent and their elimination from the environment lasts months after the cat has been removed [9].

Other advices for allergic patients [10]:

- Discard garbage every night to prevent the bugs from appearing
- Wash dishes and cutlery immediately after use
- Do not leave water overnight in the sink or bathtub.
- Molds live in wet environments, such as the shower wall, sink pipe area, basements, drain trays. It is very important to remove the mold every time you view it. Mold grows well on the soap film that covers the sink, the shower or the faience.
- Change the air filters frequently
- If the patient is allergic to a factor outside the house, try to avoid exits in the allergic season. If he should expose to a known allergen, it is advisable to use a specially designed mask.
- The shower before bedtime is essential

- Nasal lavage twice a day is very important to eliminate allergens.

There are a few medications that can be helpful:

- Sea water sprays - helps treat sneezing, dry nose and throat.
- Spray with glucocorticoids (steroids) - these are the first line in the treatment of allergies. They have few side effects and are very effective in solving aergic rhinitis. Studies show that in some cases they are even more effective than antihistamine pills. These are usually applied as one or two puffs twice a day. Although the effect can be noticed from the first day, treatment should be used for at least a few weeks, and in some patients it may be necessary to use it continually even when the patient is well [11].
- People with severe allergic rhinitis may need to use a decongestant nose spray in the first few days toghether with the steroid .
- Antihistamines - are antiallergic drugs that reduce itching, sneezing but do not reduce the feeling of a stuffy nose. They must therefore be taken in combination with the nasal spray. Some drugs of this type can cause drowsiness, although those of the new generation no longer produce this symptom.
- In some selected cases, vaccins can be made to reduce the body's sensitivity to a specific allergen. They can only be given for certain allergens such as pollen, dog or cat hair, dust, mites or mold. The most important role of immunization is the possible prevention of asthma. Studies show that this treatment method is more effective if administered over a period of 3-5 years [12].

CONCLUSION

If we know the allergen we will avoid exposing the patient to this factor as much as possible. For example, if the patient is allergic to pollen, he should not go out in the high-grade pollen days, keep the doors and windows of the house and car closed, wash himself every evening before bed and use a HEPA filter vacuum cleaner to keep the air in the room as clean as possible.

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INVESTIGATION OF NEUTRAL POLYSACCHARIDES FROM *FOMES FOMENTARIUS*: A PRELIMINARY STUDY

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ABSTRACT
The combined chemical and spectral analysis of the polysaccharide content from the *Fomes fomentarius* mushroom, harvested from the spontaneous flora, highlights the presence of a neutral polysaccharide. This glycan is a linear or branched galactomannan which does not contain any fucose residues, in contrast to what was found in previous research regarding neutral polysaccharides isolated from this species.
Key words: *Fomes fomentarius*, MALDI-TOF-MS, Glycan

INTRODUCTION

The total number of mushroom species on the planet is estimated to be around 140 000, of which only 10% (~ 14 000 named species) are known. They comprise a vast and yet largely unexplored resource of promising new pharmaceutical products and have been valued as food and medical resource for thousands of years [1]. Several bioactive molecules, which include antitumor compounds, have been identified in mushrooms. The fruit bodies, cultured mycelium and culture broth of many Basidiomycetes mushrooms contain biologically active polysaccharides [1]. Most polysaccharides have unique structures in different species, and even different strains of one species can produce polysaccharides with different properties. They are the best known and most potent mushroom derived substances which possess antitumor and immunomodulating properties [2-10]. Such polysaccharides do not attack cancer cells directly, but instead they activate various immune responses in the host, thus producing their antitumor effects. This requires an intact T-cell component; their activity is mediated through a thymus-dependent immune mechanism. However, besides the biological properties, the biotechnological availability is also important for practical applications [1].

Fomes fomentarius, which is a member of Basidiomycetes, has been used in traditional oriental medicine for treating various diseases, i.e. gastroenteric disorder, hepatocirrhosis, oral ulcer, inflammation, and various cancers. Previous research revealed various appealing biological activities for this fungus, including antidiabetic, anti-inflammatory, antioxidant and anticancer properties [11-14]. The polysaccharides from this species were investigated in the 1970s by Lindberg and Kubala. They determined the composition and structure for two types of polysaccharides in the aqueous extract, i.e. neutral and acidic [15-17].

Lindberg examined the fruit bodies of *Fomes fomentarius* for polysaccharide content. The materials, which were extracted with hot water, were further investigated. Neutral and acidic polysaccharides were separated by treatment with DEAE cellulose. Neutral

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polysaccharides were subjected to stepwise acid hydrolysis, and the fragments of low molecular weight were isolated. The monosaccharides were separated and identified as D-mannose, D-galactose, and L-fucose. From the structural studies, it was found that the mannofucogalactans from *Fomes fomentarius* and *Fomes igniarius* have similar structures. They consist of a backbone of α(1→6) linked α-D-galactopyranose residues, of which about 30% *(F. fomentarius)* and 40% *(F. igniarius)* are substituted in the 2-position. Most of the substituents are either 3-*O*-α-D-mannopyranosyl-L-fucopyranosyl or fucopyranosyl residues. [15,16]

At the same time, Kubala determined the structure of neutral polysaccharides, extracted with water from *Fomes fomentarius*. It was found that the backbone of the polysaccharide consists of D-galactose units, linked together with (1→6)-glycosidic linkages. Some of the galactose units are found as branch points substituted in the 3-position by side chains. It can be concluded that the predominant amount of L-fucose is found in the side chains. On the other hand, some of the side chains are formed by single L-fucose units, while others consist of mannosyl-fucose [17].

Bellow we present the results obtained during the extraction, isolation, purification and deciphering of the structure of neutral polysaccharides from the fruiting body of *Fomes fomentarius*, harvested from the spontaneous flora of the Vâlcea County in Romania.

EXPERIMENTAL

Equipment

Rotary Evaporator RII (Buchi); Alpha 1-2 Freeze Dryer (Christ); 200 Benchtop Centrifuge (Labofuge); pH meter inoLab 7110; Glass Oven B-580 (BUCHI); CAMAG Model-3 TLC scanner equipped with CAMAG CATS 4 software; CAMAG Horizontal Developing Chambers (for plate 10x10); Bruker Vertex 70 (Bruker Daltonik GmbH, Germany) ultrafleXtreme MALDI-TOF workstation (Bruker Daltonik).

Reagents

TRIS hydrochloride (ROTH), sodium hydroxide, phenol (>99%), sulfuric acid 95-97%, 2,5-DHB (2,5-dihydroxybenzoic acid) (all from Merck); sodium chloride 99.8%, ethyl alcohol (abs) (from Chimopar S.A., Bucharest); 2,5-dihydroxybenzoic acid (DHB) >99%, α-cyano-4-hydroxycinnamic acid (HCCA) 99%, trans-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene]malononitrile (DCTB) ≥99.0%, 2',4',6'-trihidroxyacetophenone monohydrate (THAP) ≥ 99.5% (all from Sigma-Aldrich, Spruce Street, St. Louis, USA); Sepharose Fast Flow (GE Healthcare Sweden); dialysis membranes, molecular weight cutoff (MW/CO) 1000 were purchased from Spectrum Europe B.V. (Breda, The Netherlands).

Sample

The main steps of extraction, isolation, purification and fractionation of the polysaccharide blend obtained from the fruiting body of *Fomes fomentarius* are presented below:

a) **Isolations and control:** The fungus was harvested from Vâlcea County (Romania) in October 2015 and stored until used at -78 ºC. The analysis and taxonomic classification of the collected material was carried out by experts in systematic botany from the Faculty of Chemistry, Biology, Geography within the West University of Timisoara. The fungus was treated with liquid nitrogen and grounded into a powder.

b) **Extraction:** An aliquot of 300 g from the powder was extracted by refluxing with:

- hexane (3 x 0.5 L);
- acetone (3 x 0.5 L);
- water (3 x 0.5 L).

The aqueous volumes were combined and brought to minimum volume by evaporating the solvent (in a rotary evaporator) at low pressure.

c) **Purification:** The minimum aqueous extract volume (50 mL) was diluted with EtOH (abs) – ten times the minimum volume (500 mL) – precipitating the polysaccharide fraction, followed by cold (0–4 ºC) filtration. The obtained precipitate was washed repeatedly with methanol and acetone (0–4 ºC) and dialyzed (MW/CO = 1000) against water for 48 hours. The dialysate was then lyophilized, yielding 118 mg of yellow-brownish powder.

d) **Column chromatography:** The column (20 x 2.5 cm), was filled with DEAE Sepharose Fast Flow and eluted with a 0.01 M Tris solution adjusted to pH 7 with 0.1 M NaOH. A sample of 30 mg of raw mixture of polysaccharide in 3 mL eluent was loaded into the column and washed with 750 mL of 0.01 M Tris solution adjusted to pH 7, collecting a series of 3 mL fractions. The presence of polysaccharide in the fractions was checked by using a spectrophotometric method [18]. The column was then eluted with 0.1 M, 0.4 M and, respectively, 1 M NaCl in 0.01 M Tris solution (3 x 750 mL). The representation of the absorbance as a function of the number of tubes gives us the elution profiles (Figure 1).

Mass Spectrometry

MALDI-TOF mass spectra of the samples were recorded by using a Bruker ultrafleXtreme MALDI-TOF workstation controlled by FlexControl (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam II laser (Bruker Daltonics) of 355 nm and operating in positive mode. The following settings were used: laser frequency, 2,000 Hz; smartbeam, '4_large'; sample rate and digitizer settings, 1.25 GS/sec; accelerator voltage, 20.07 kW; extraction voltage, 18.87 kW; lens voltage, 5.58 kW; and delayed extraction, 250 nsec. A 1,000 laser shots were used for each individual spectrum and a minimum of ten individual spectra were cumulated and saved. External calibration was

performed using maltodextrin (Paselli MD-6, AVEBE, Veendam, The Netherlands). FlexAnalysis version 3.3 (Bruker Daltonics) was used for data processing. The analyzed sample was spotted on MTP 384 polished steel BC targets (Bruker Daltonik). Four types of matrices, with three different concentrations, known to be used in the MALDI-TOF determination of oligosaccharides, glycans and glycoconjugates, were used in combination with three different sample concentrations to find the optimal ionization conditions. The trials as well as the evaluation of the quality of the spectra by using various settings are given in Table I.

Preparation of the matrix solution:

DHB matrix solution: 50 mg DHB in 0.5 mL H_2O + 0.5 mL ACN (C1); for C2 and C3 (dilution 1:3 and, respectively, 1:5)
DCTB matrix solution: 50 mg DHB in 1 mL ACN (or THF) with 5µL TFA (C1); for C2 and C3 (dilution 1:3 and, respectively, 1:5)
HCCA matrix solution: 50 mg HCCA in 0.5 mL ACN + 0.5 mL MeOH (C1); for C2 and C3 (dilution 1:3 and, respectively, 1:5)
THAP matrix solution: 50 mg THAP in 0.5 mL H2O + 0.5 mL ACN (C1); for C2 and C3 (dilution 1:3 and, respectively, 1:5)

Infrared spectroscopy (IR)

The FT-IR spectra were obtained in attenuated total reflectance (ATR) mode by using a Bruker Vertex 70 instrument (Bruker Daltonik GmbH, Germany) equipped with Platinium ATR, Bruker Diamond Tip A225/ Q.I. 128 of co-added scans were collected in the range of 4000-400 cm^{-1}, with a resolution of 4 cm^{-1}.

UV spectrophotometry

Carbohydrate determinations using the $PhOH/H_2SO_4$ method [18] were performed on a Biochrom Libra S12 UV/VIS Spectrophotometer in 1 cm cuvettes at λ=490 nm.

TL-Chromatography

The linear ascending development was carried out in a CAMAG glass twin through chamber (10 cm × 10 cm), which was previously saturated with 10 mL of mobile phase, i.e. ethyl acetate:1-propanol:anhydrous acetic acid:water = 4:2:2:1 (v/v). Plates were developed to a distance of 80 mm, after which the TLC plates were dried in an air current with a hair dryer. The dried plate was visualized by spraying with $Ce(SO_4)_2$:$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$:$H_2SO_4$:$H_2O$ = 1:5:10:90 (w) and then dried 5 min to 120 °C.

HPTL–chromatography

Samples (20 µL) were deposited at 10 mm from the bottom of the plate, after which the plate was developed in the planar HPTLC chamber (CAMAG). After elution (9 cm from bottom) the plate was dried and visualized using $Ce(SO_4)_2$:$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$:$H_2SO_4$ and 5 min heating at 120 °C. The color is stable for 75 minute.

Densitometry

The CAMAG densitometry (CAMAG Model-3 TLC scanner equipped with CAMAG CATS 3 software) used a reflectance spectrometer with 190–700 nm monitoring domain. The slit was set to 8x0.4 mm and data acquisition and processing were performed using the WinCATS software. The measurements were performed at λ=580 nm wavelength.

Total hydrolysis of NP2

3 mg of NP2 was pre-hydrolyzed with 72% (w/w) sulfuric acid for 1 h at 25 °C, followed by hydrolysis with 1 M sulfuric acid for 3 h at 100 °C. The reaction mixture was neutralized with $BaCO_3$, filtered and then lyophilized [19]. The obtained product, dissolved in the minimum quantity of water, was analyzed by TLC.

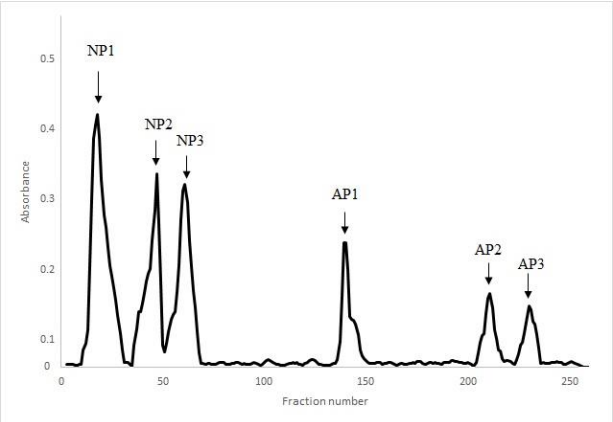

Fig. 1. Elution profiles of raw polysaccharides on DEAE-Sepharose FF column (20 × 2.5 cm). The column was equilibrated with 10 mM Tris-HCl buffer (pH 7.0), then eluted as follows: a) 1-90 tubes 10 mM Tris-HCl buffer; b) 91-170 tubes with 0.1 M NaCl in 10 mM Tris-HCl c) 171-220 tubes with 0.4 M NaCl in 10 mM Tris-HCl d) 221-250 tubes with 1.0 M NaCl in 10 mM Tris-HCl; elution speed: 1mL/min; volume =3 mL/tube; $PhOH/H_2SO_4$ colorimetric method ($A_{\lambda=490\ nm}$); NP = neutral polysaccharides, AP = acidic polysaccharides

Table I. The quality of the spectra strongly depends on the sample application protocol.

M/P first 1 µL matrix solution, then 1 µL sample solution; P/M first 1 µL sample solution then 1 µL matrix solution; MP-1 µL matrix solution + 1 µL matrix solution, vortex and application of 1 µL to the target. Quality of spectra: m - missing; p - poor; g - good, e - excellent; P-sample; P1-5 mg sample in 1 mL H_2O; P2-1.7 mg sample in 1 mL H_2O; P3-1.7 mg sample in 1 mL H_2O; M1=2,5-DHB; M2=4-HCCA; M3=THAP; M4=DCTB; C1=50 mg matrix; C2=17 mg matrix; C3=10 mg matrix; # add 0.5 µL TFA; * add 0.5 µL HCOOH

SPOTTED	M1			M2			M3			M4		
	C1	C2	C3	C1	C2	C3	C1	C2	C3	C1	C2	C3
M/P1	g	M	m	P	m	P	M	P	m	#m	#m	#p
P1/M	p	M	p	m	p	P	m	M	m	#p	#p	#m
MP1	#p	P	g	#m	#m	P	p	P	m	#m	#p	#p
M/P2	m	P	m	m	p	M	m	P	p	#m	#m	#p
P2/M	m	#P	m	m	p	p	p	M	p	#m	#m	#p
MP2	#p	G	m	P	m	p	p	M	m	#p	#p	#m
M/P3	g	P	p	#m	m	m	p	P	m	#m	#m	#m
P3/M	p	M	m	P	m	m	m	P	m	#m	#m	#m
MP3	g	P	#p	P	#m	m	p	M	p	#m	#p	#m
M/P1 *	e	G	p	G	p	m	g	P	m	m	m	p
P1/M *	g	G	p	P	m	p	p	P	m	m	m	m
MP1 *	g	E	#p	G	m	g	p	M	p	p	m	m
M/P2 *	m	M	p	m	p	m	m	M	p	p	m	g
P2/M *	p	P	g	m	p	m	m	M	m	m	m	p
MP2 *	g	E	p	m	p	p	m	P	p	g	p	p
M/P3 *	e	G	g	P	m	m	p	M	m	p	p	m
P3/M *	g	p	g	m	m	p	m	M	m	p	m	m
MP3*	e	g	p	m	p	m	p	M	p	p	m	p

RESULTS AND DISCUSSIONS

Starting from the information provided by Park's work [20] (who extracts, purifies and separates polysaccharides from *Fomes fomentarius*), we extracted, purified and separately collected polysaccharides from *Fomes fomentarius* harvested from the native Romanian flora. Some changes were made as follows: after the fruiting bodies of *Fomes fomentarius* were converted into powder, 300 g were extracted with hexane (to remove lipids) and then with acetone (to remove some of the colored compounds) and finally with water. After purification and isolation, chromatography is performed on Sepharose. In the first stage, during elution with a 0.01 M Tris solution adjusted to pH 7, the migration of neutral polysaccharides takes place, after which, during elution with NaCl (0.1 M, 0.4 M and 1 M, respectively), the elution of acid polysaccharides occurs. The eluates were tested for the presence of carbohydrates using the $PhOH/H_2SO_4$ method according to a well-established protocol [18]. Graphical representation of the absorbance measured at λ=490 nm as a function of the number of fractions leads to an elution profile, which is shown in Figure 1 (see Experimental: UV spectrophotometry).

In contrast to the previous works, our chromatogram reveals three fractions at neutral/low alkaline pH elution and two fractions at acidic pH elution. The three fractions were isolated, lyophilized, dialyzed (against water) and finally lyophilized. FT-IR analysis of each fraction did not reveal the carbonyl valence vibration (1700-1800 cm^{-1}), so it was concluded that it does not contain uronic acids or acetyl residues. The three fractions were named NP1, NP2 and NP3 (from *neutral polysaccharides*). The FT-IR spectrum of NP2 fraction is shown in Figure 2.

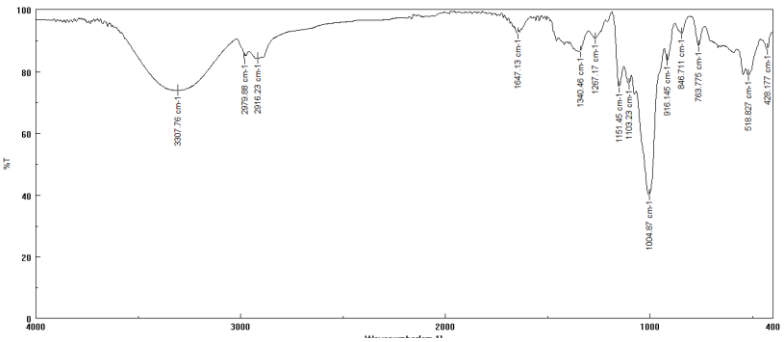

Fig. 2. FT-IR spectra in attenuated total reflectance (ATR) mode of the NP2 fraction in the 4000–400 cm^{-1} domain

Using the mass spectrometry to investigate the structure of natural and chemically modified polysaccharides is always a challenge, which is rewarded when good quality spectra offer a large number of details regarding the composition of glycans, the type of connections, and the alternation mode of the constituent monosaccharides [21-26]. The mass spectrometric analysis of the NP2 fraction required several attempts to find the proper conditions (matrix type, matrix concentration, ionization promoters, sample concentration, application order on targets etc.) that would generate a good ionization and would give a high quality spectrum. These attempts, as well as the related explanations, are given in Table I. The mass spectrum of NP2, under the specified conditions, is shown in Figure 3 (A-C).

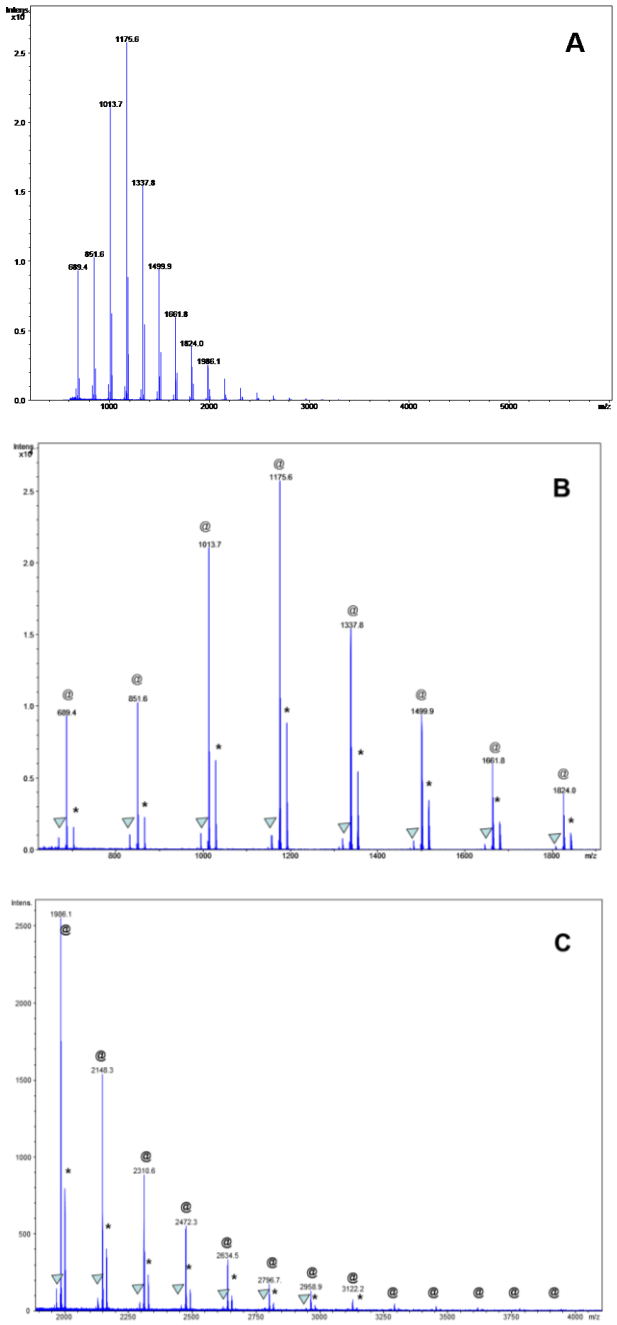

Fig. 3. Positive MALDI-TOF-MS of the NP2-FF sample in reflectron mode A) *m/z* 600- 4500; B) *m/z* 600-1900; C) *m/z* 1900-4200; $ = [Hex$_n$ + Na^+]$^+$;Δ =[Hex$_n$ + Na^+-H2O]$^+$; *= [Hex$_n$ + K^+]$^+$

The spectrum analysis immediately reveals the 162 Da "step", which indicates the existence of a polysaccharide, more specifically of a polyhexose. The absence, on the whole measuring range (*m/z* 600-5000) of a weight loss of 146 Da, suggests that neither rhamnose nor fucose are present. The assignment of all peaks from the mass spectrum presented in Figure 3 (A-C) is given in Table II.

Table II Assignment of the ions observed in (+)MALDI TOF spectrum of NP2

Nr.	m/z=	Char-ge	Assignments	Nr.	m/z=	Char-ge	Assignments
1.	3933.4	+1	Hex24 +Na^+	32	2164.3	+1	Hex13 +K^+
2	3787.4	+1	Hex23 +K^+	33	2148.3	+1	Hex13 +Na^+
3	3771.3	+1	Hex23 +Na^+	34	2130.3	+1	Hex13 + Na^+-H_2O
4	3753.2	+1	Hex23 + Na^+-H_2O	35	2002.1	+1	Hex12 +K^+
5	3625.2	+1	Hex22 +K^+	36	1986.1	+1	Hex12 +Na^+
6	3609.1	+1	Hex22 +Na^+	37	1968.1	+1	Hex12 + Na^+-H_2O
7	3591.1	+1	Hex22 + Na^+-H_2O	38	1840.1	+1	Hex11 +K^+
8	3462.8	+1	Hex21 +K^+	39	1824.0	+1	Hex11 +Na^+
9	3446.9	+1	Hex21 +Na^+	40	1806.0	+1	Hex11 + Na^+-H_2O
10	3428.7	+1	Hex21 + Na^+-H_2O	41	1677.8	+1	Hex10 +K^+
11	3300.6	+1	Hex20 +K^+	42	1661.8	+1	Hex10 +Na^+
12	3284.6	+1	Hex20 +Na^+	43	1643.7	+1	Hex10 + Na^+-H_2O
13	3266.5	+1	Hex20 + Na^+-H_2O	44	1515.9	+1	Hex9 +K^+
14	3138.2	+1	Hex19 +K^+	45	1499.9	+1	Hex9 +Na^+
15	3122.2	+1	Hex19 +Na^+	46	1481.8	+1	Hex9 + Na^+-H_2O
16	3104.3	+1	Hex19 + Na^+-H_2O	47	1355.8	+1	Hex8 +K^+
17	2974.9	+1	Hex18 +K^+	48	1337.8	+1	Hex8 +Na^+
18	2958.9	+1	Hex18 +Na^+	49	1319.7	+1	Hex8 + Na^+-H_2O
19	2940.9	+1	Hex18 + Na^+-H_2O	50	1191.6	+1	Hex7 +K^+
20	2812.7	+1	Hex17 +K^+	51	1175.6	+1	Hex7 +Na^+
21	2796.7	+1	Hex17 +Na^+	52	1157.7	+1	Hex7 + Na^+-H_2O
22	2778.8	+1	Hex17 + Na^+-H_2O	53	1029.7	+1	Hex6 +K^+
23	2650.5	+1	Hex16 +K^+	54	1013.7	+1	Hex6 +Na^+
24	2634.5	+1	Hex16 +Na^+	55	995.6	+1	Hex6 + Na^+-H_2O
25	2616.5	+1	Hex16 + Na^+-H_2O	56	867.7	+1	Hex5 +K^+
26	2488.4	+1	Hex15 +K^+	57	851.6	+1	Hex5 +Na^+
27	2472.3	+1	Hex15 +Na^+	58	833.6	+1	Hex5 + Na^+-H_2O
28	2454.2	+1	Hex15 + Na^+-H_2O	59	705.4	+1	Hex4 +K^+
29	2326.6	+1	Hex14 +K^+	60	689.4	+1	Hex4 +Na^+
30	2310.6	+1	Hex14 +Na^+	61	671.3	+1	Hex4 + Na^+-H_2O
31	2292.5	+1	Hex14+ Na^+-H_2O			+1	

It can be clearly seen in the spectrum that only three ion series, i.e. [Hex_n + Na^+], [Hex_n +K^+] and [Hex_n+Na^+-H_2O], are present. It remains to be investigated whether we are dealing with a homoglycan or a heteroglycan. The total acid hydrolysis of an NP2 sample was carried out, which, after processing, was analyzed by TLC using galactose, mannose and fucose as references. The analysis of chromatograms from Figure 4 (A-C) clearly shows that NP2 is a $(Gal)_x(Man)_y$ heteropolysaccharide. In order to be able to estimate the relationship between the two components, the NP2 hydrolysate separation was performed on the HTPLC plate, which was followed by visualization and densitometry analysis of the chromatogram.

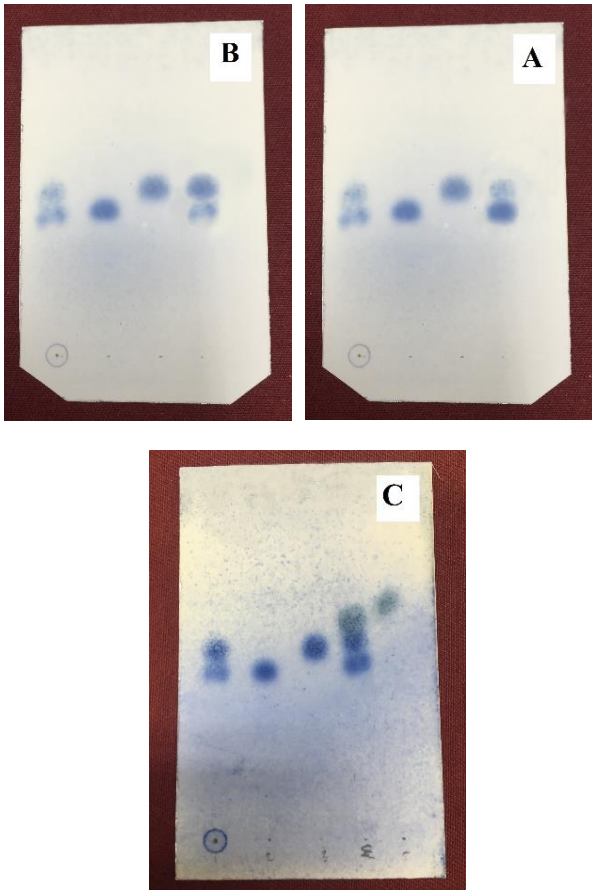

Fig. 4. TLC chromatograms of acidic hydrolisated sample NP2-FF. **A**) *Identification of galactose*: Line1-acidic hydrolysate of NP2, Line 2: standard galactose; Line 3: standard mannose; Line 4: acidic hydrolysate of NP2 + Gal; **B**) *Identification of mannose* Line1-acidic hydrolysate of NP2, Line 2: standard galactose; Line 3: standard mannose; Line 4: acidic hydrolysate of NP2 + Man; **C**) Absence of fucose: Line1-acidic hydrolysate of NP2, Line 2: standard galactose; Line 3: standard mannose; Line 4: acidic hydrolysate of NP2 + Fuc; Line 5: standard Fucose; Elution: AcOEt:1-PrOH;AcOH:H_2O= 4:2:2:1 (v/v); visualization: $Ce(SO_4)_2$:$(NH_4)_6Mo_7O_{24}.4H_2O$:H_2SO_4:H_2O = 1:5:10:90 (w) then 5 min to 120 °C.

The densitogram taken at λ=580 nm is shown in Figure 5. From the analysis of the ratios of the areas of the two components, galactose and respectively mannose, we can conclude that the linear or branched heteropolymer is a galactomannan with the presumptive formula of $[(Gal)_{18}(Man)_5]_n$

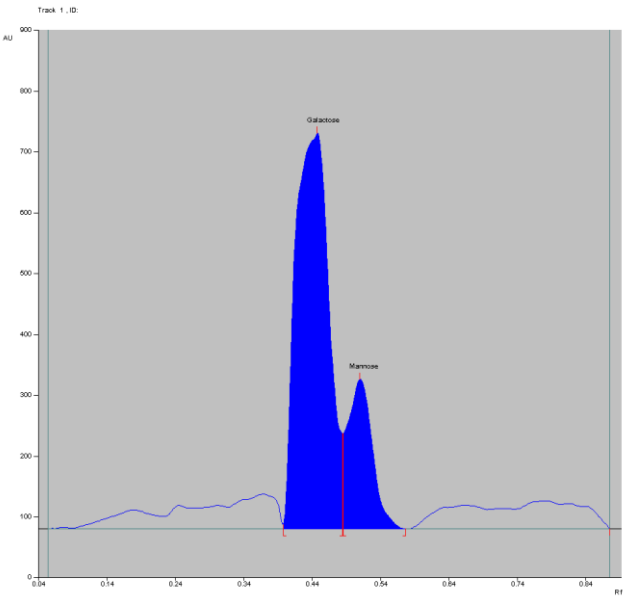

Fig. 5. Densitometric chromatogram of hydrolyzed NP2 fraction after development and vizualisation. Scanned at 580 nm. The HPTLC plate coated with Silica gel 60 F_{254} was developed using the mobile phase ethyl acetate: 1-propanol:anhydrous acetic acid:H_2O = 4:2:2:1 (v/v) and visualized with $Ce(SO_4)2$: $(NH_4)_6Mo_7O_{24}.4H_2O$: H_2SO_4 : H_2O = 1:5:10:90 (w) then 5 min to 120 °C.

CONCLUSIONS

The analysis of the polysaccharide content from *Fomes fomentarius*, harvested from the Romanian spontaneous flora, by combined methods (chemical and spectral), reveals the existence of a neutral polysaccharide, which is a galactomannan and which does not contain any rhamnose or fucose residues, in contrast to what was found in other previous research regarding polysaccharides from this mushroom. A judicious choice of sample content, matrix type, ionization promoters and application mode allowed for the best ionization method to be established, which allowed for high accuracy and quality MALDI-TOF spectra.

ACKNOWLEDGEMENT

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INVESTIGAREA POLIZAHARIDELOR NEUTRE PROVENITE DIN *FOMES FOMENTARIUS*: UN STUDIU PRELIMINAR

REZUMAT

Prin combinarea analizei chimice și spectrale a conținutului de polizaharide din ciuperca *Fomes fomentarius*, recoltată din flora spontană, se pune in evidenta prezența unei noi polizaharide neutre. Acest glican este un galactomanan liniar sau ramificat, care nu conține resturi de fucoză, spre deosebire de cele descoperite în cercetările anterioare privind polizaharidele neutre izolate din această specie.

Cuvinte cheie: *Fomes fomentarius,* MALDI-TOF-MS, Glican

MANAGEMENT OF EPISTAXIS IN NASAL TUMORS AND HEART DISEASES AT THE UNIVERSITY HOSPITAL OF TIMISOARA

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ABSTRACT

Background and aim. Epistaxis is a frequently disease, affecting up to 60% of the population and is the common reason for healthcare utilization.

Methods. The study includes patients presenting to the E.N.T department of the University Hospital of Timisoara from January 2014 to October 2018 with anterior and / or posterior epistaxis. Demographic factors (age, rase, gender), medication use (anticoagulants and anti-aggregant medication) and several comorbidities were analyzed as potential predictors of episodes.

Results. A total of 346 patients were identified with a total of 456 individual epistaxis episodes over 4 years. Multivariate analysis identified nasal tumors, hypertension, overdose of anticoagulant medication and seasonal variations as predictors of higher number of cases.

Conclusions. Epistaxis occurred more frequently in older patients with arterial hypertension. Allergic rhinitis, chronic sinusitis, nasal tumors and hypertension are associated with increased epistaxis incidence.

Keywords: epistaxis, nasal tumor, hypertension, nasal packing, cauterization, anticoagulants.

INTRODUCTION

Epistaxis is an otolaryngologic affection known since antiquity and the term is derived from the Greek, epistazein (epi – above, over; stazein – to drip)[1]. Epistaxis limited to nasal bleeding was introduced by the Englishman Cullen in 1785 and the Frenchman Pinel in 1818 and later became widespread in medical language [2].

This is a frequently disease and it is estimated from a Scandinavian survey in 1974 of 410 people that up to 60% of the population will one episode of epistaxis in their lifetime and 6% will seek medical attention[2]. This is a frequently disease and the Scandinavian study in 1974 of 410 people is evaluated that up to 60% of the population will have at least one episode of epistaxis in their lifetime and 6% will need health care utilization [2].

A US health examination survey from 1972 of 6672 adults revealed a 7% to 14% incidence of epistaxis [2]. Incidence from most reports from Europe and America is about 10%-15% of the population [3-5]. Incidence of epistaxis in a US health investigation study from 1972 of 6672 people is estimated between 7% and 14% [2]. The Europa and America reports revealed a 10%-15% incidence of the epistaxis.

Although epistaxis may occur at any age or at any time and in any season, it is a common complaint in the pediatric age group and the winter months but has been shown to have bimodal age range presentation in reports from North America and Europe [3-6]. Reports from North America and Europa demonstrated that epistaxis may occur at any age or at any time and in any season, it is a common disease in the pediatric age group and the winter months but has been shown to have bimodal age range presentation [3-6].

It is a frequent otolaryngologic emergency, which in serious cases will need a full complement of resuscitative measures to stabilize the patient and prevent or address hypovolemic shock [3-7]. Knowledge of history of epistaxis in a patient will assist the physician or surgeon in taking precautions while planning any treatment modality be it medical or surgical. Failure to take these precautions may lead to unpleasant consequences [3-8]. In serious cases of epistaxis will need resuscitative measures to stabilize the

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patient and prevent hypovolemic shock [3-7]. Previous medical history of epistaxis in a patient will direct the physician or surgeon to take the precautions and to choose medical or surgical treatment. Failure to take these precautions may lead to unpleasant consequences on the health of the patient [3-8].

The etiologies includes local factors, such as trauma or direct irritation and systemic factors. Yüksel et al. [4] found that the evidence available was insufficient to prove a significant association between hypertension and epistaxis. Many cases of epistaxis are prevented with the moisturizing nasal mucosa, avoidance of direct irritation or trauma, and nasal spray with oxymetazoline and epinephrine. The severity of epistaxis ranges from a minor symptoms to hemodynamically significant events, including death in rare cases. When prevention methods fail, recourse to the compression, vasoconstrictor nasal sprays, chemical or electrical cauterization, hemostatic agents and nasal packing. The primary aim of this study is to assess the factors favoring the occurrence of epistaxis according to age and associated pathologies within the patients who presented to the Emergency Room at the E.N.T. Department of the University Hospital of Timisoara. Secondary aims include assessing the therapeutic options depending on the severity of the bleeding.

METHODS

We conducted a retrospective study on sample which was obtained by consecutive sampling of all admissions for epistaxis at the Ear, Nose and Throat (ENT) Department of the University Hospital of Timisoara, in the period between January 2014 and October 2018. Our centre it is a tertiary care hospital which has 47 beds for ENT department. The study contain 346 cases that were analyzed in terms of demographics, clinical aspects and treatment. In the statistical analysis, we used measurements of central tendency and of dispersion for quantitative variables (age, blood pressure values, and length of hospital stay and severity of epistaxis) and absolute and relative frequencies for qualitative variables (gender, society, location of the epistaxis, plugging, antiaggregation, anticoagulation, hypertension and sphenopalatine artery cauterization). The study was accomplished with the approval of the ethics committee of the University of Medicine and Pharmacy "Victor Babes" Timisoara.

Patient selection

346 patients were selected for the study. The study inclusion criteria were: male and female patients of any age – patients admitted to the ENT department with a diagnosis of serious spontaneous epistaxis requiring at least one nasal pack; patients with minor epistaxis easily managed by first aid measures and/or immediately successful local treatment (cauterization), posttraumatic epistaxis (including iatrogenic epistaxis after nasal surgery) and patients with nasal tumors.

Management of epistaxis

Management of epistaxis ranged from unilateral nasal packing to endoscopic surgery depending on the severity of epistaxis. Patients with systolic blood pressure (BP) higher than 160 mmHg also received Enalapril LPH 10 mg to control BP. Anterior nasal packing was performed with Polyvinyl Alcohol sponge (MEROCEL-Standard nasal dressing) or compression belts with antihemostatic (Adrenostazin or Etamsilat); anterior and posterior nasal packing was performed with a double balloon nasal catheter or buffer nasopharynx compression. Nasal packs were removed after 48-72 h if the patient did not re-bleed. In cases of re-bleeding after removal of nasal packs, nasal packing was repeated. Sphenopalatine artery cauterization was indicated in the case of anemia, persistent bleeding after 2 or 3 nasal packs and after the tumor excision.

Study protocol

A history of hypertension was defined as patients treated with antihypertensive drugs. Patients with a self-reported history of epistaxis or who had been already hospitalized for at least one episode of epistaxis were considered to have a history of epistaxis. The mean of all BP measurements during hospitalization was determined, and BP that was difficult to control was defined as BP higher than 140/80 mmHg. Epistaxis was classified as grade 1 (serious) or grade 2 (severe). Serious epistaxis was defined by the need for medical management requiring hospitalization of the patient for epistaxis, anterior nasal packing, under 5 days of hospitalization. Severe epistaxis (grade 2) was defined as follows: length of hospital stay more than 5 days, nasal packing using double balloon nasal catheter or buffer nasopharynx compression, two or more nasal packs, presence of hematologic consequences: anemia (hemoglobin\10 g/dl) and/or blood transfusion, patients treated by cauterization of sphenopalatine artery.

Statistical Analysis

Data are presented as absolute numbers, percentages and means where appropriate. A descriptive analysis was performed with calculation of mean and medians for continuous variables and percentages for categorical variables. Multiple ordered logistic regression models and linear regression models were constructed to evaluate the association between covariates and epistaxis severity. Values were considered statistically significant for $P < 0.5$. All statistical calculations were performed using CHI SQUARE test.

RESULTS

Of the study subjects, 58.84% were men and 41.16% were women, with a male/female ratio of 1.42/1 (odds ratio=1.4296), although this ratio varies by age group. The median age for the patients with epistaxis was 75 (71-80) years with a range of 11-98 years (Figure 1) and 60 (50-70) years in nasal tumor cases. Of the total, 83.53% were aged over 50 years. According to the anatomical location, the cases of epistaxis were distributed into posterior (8.09%; 28/346) and anterior (91.91%; 318/346).

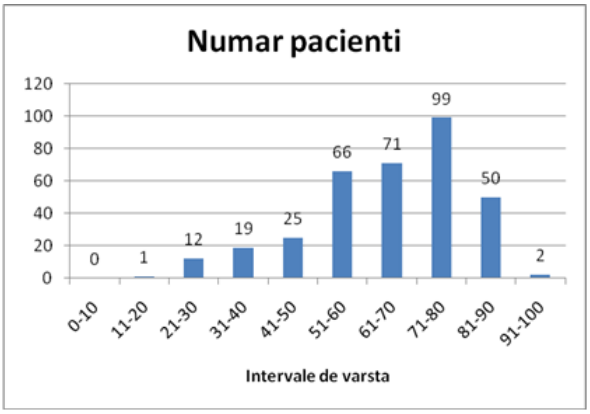

Fig. 1. The median age for the patients with epistaxis was 75 (71-80) years with a range of 11-98 years.

Pathologies incriminated in nosebleeds were: hypertension (84.39%, 292/346), ischemic heart disease (21.68%, 75/346), atrial fibrillation (12.72%, 44/346), diabetes (12.43%, 43/346), nasal tumors (8.38%, 29/346), viral (5.49%, 19/346), trauma (1.73%, 6/346) and post-surgery (0.87, 3/346). 0.87% of all the patients showed no pathology (Figure 2).

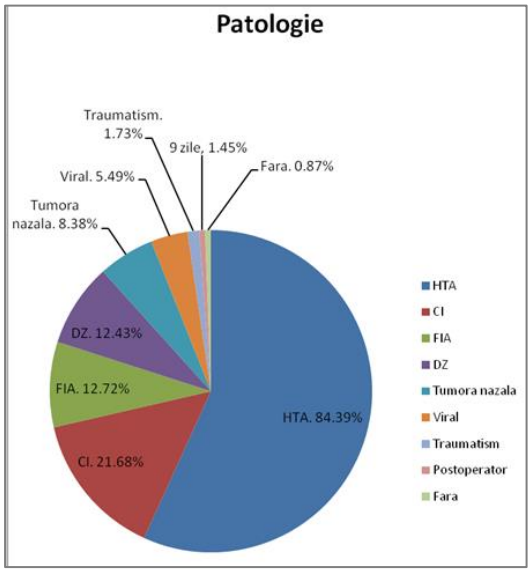

Fig. 2. Pathologies incriminated in nosebleeding.

Also, we observed a predominance of hospitalized epistaxis in the months of March and January, with a decrease in summer periods (Figure 3).

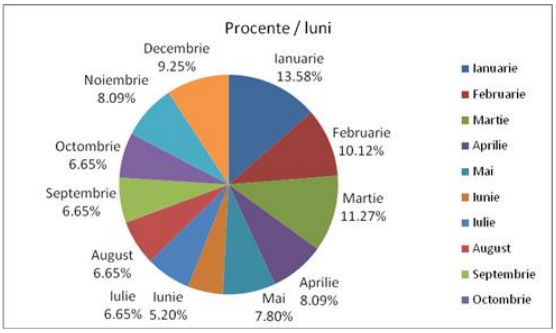

Fig. 3. Correlation between the season and the appearance of nasal bleeding.

Associated local factors were much less frequent than systemic factors. Arterial hypertension was the most prevalent and was present in 84.39% (292/346) of cases, while 43.64% of patients (151/292) were aged over 71 years. Anti-aggregation accounted for 19.08% (66/346) and anticoagulation for 17.92% (62/346). Nasal bleeding occurred in 8.41% (29/346) of patients with nasal tumors which was performed the surgery (Figure 4).

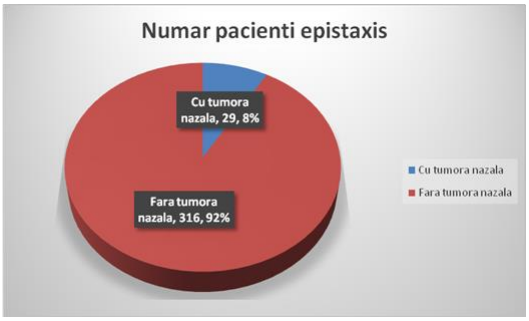

Fig. 4. Number of patients with nasal bleeding in the nasal tumor cases.

In this cases the median age was 60 (50-70) years and the hospitalization days of these patients was on average 8 days (Figures 5 and 6).

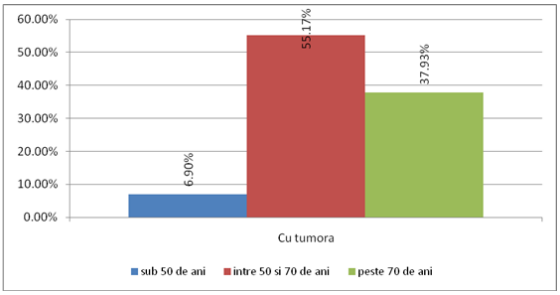

Fig. 5. Correlation between the age and tumors.

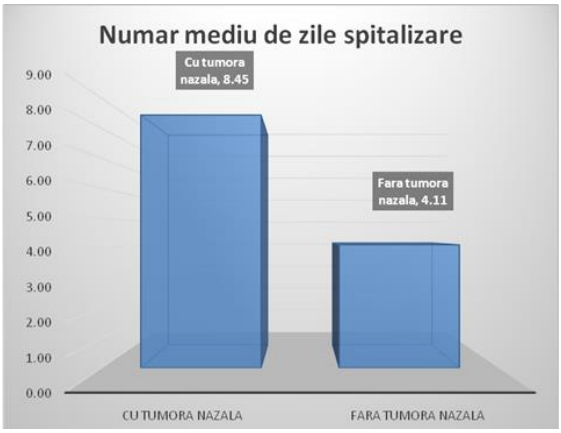

Fig. 6. Days of hospitalization at the tumors pathology.

According to the severity of epistaxis, nasal tumors were classified in grade 2 of severity and the rest of pathologies in grad 1. In nasal tumor cases we observed that posterior epistaxis (59%, 17/29) was more frequently than anterior epistaxis (41%, 12/29) (Figure 7). Management of epistaxis in our patients included 4 methods, starting with first aid, than anterior nasal packing with compresion belts or MEROCEL, posterior nasal packing and nasal cauterization.

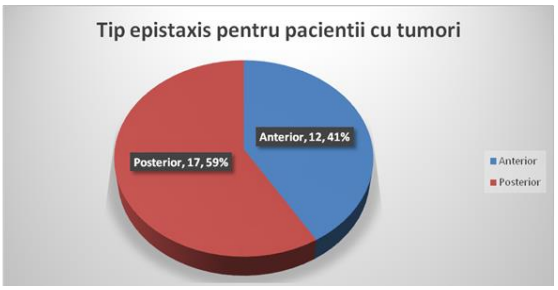

Fig. 7. The posterior epistaxis (59%, 17/29) was more frecvently that anterior localization (41%, 12/29).

In 82.66% of cases we practiced anterior nasal packing and 7.80% of cases were treated by posterior nasal packing. We have identified spontaneously stopped bleedings in 9.54% of total cases (Figure 8).

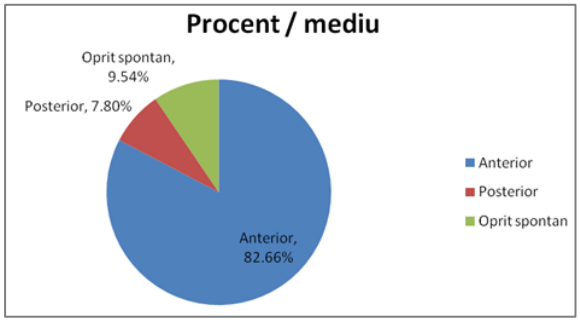

Fig. 8. Anterior nasal packing was more used than posterior packing and in 9.54% of cases the bleedings were spontaneously stopped.

At the anterior and/or posterior nasal packing refractory we have used arterial cauterization. Sphenopalatine artery cauterization was performed at a rate of 59% to stop the nasal bleeding in patients with nasal tumors (Figure 9).

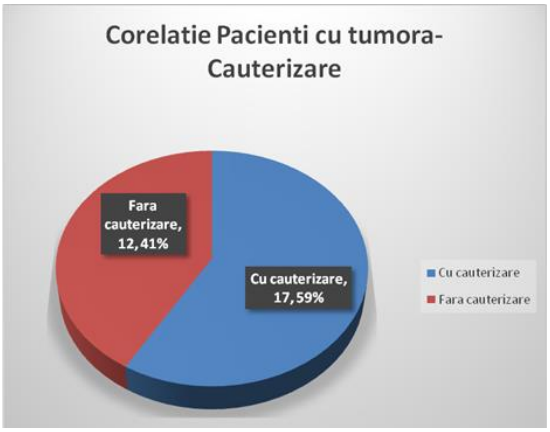

Fig. 9. Correlation between tumors and arterial cauterization.

DISCUSSION

Epistaxis is one of the most common emergencies of our specialty. The male predominance of epistaxis has been well documented by most authors. It is a constant feature in the literature, which in our series was reflected across all age groups until the eighth decade of life and the result is statistically significant ($p=0.0010$, $p<0.5$). Epistaxis incidence by gender and age distribution was described by Tomkinson et al. [9] in 1997, with a male predominance between the ages of 20 and 49 years. However, this difference by gender was not present after the age of 50 years in his study. From this age, the incidence in men was similar to that in women, a fact that the author relates to the possible protective effect of estrogens in pre-menopausal women. It is a condition that can affect all age groups, but there is an increased incidence in the population above age 50. In 1974, Juselius reported that up to 71.4% of his patients exceeded this age. This finding was later confirmed by other authors. Walker et al.[10], in their extensive series of 21,770 admitted epistaxis cases, reported a median age of 70 years, in which approximately 75% of patients were over 40. The classification by decades in our series showed an ascending distribution, and the vast majority of cases were over 50. Another epidemiological datum in the literature has been the environmental and seasonal incidence of nasal hemorrhages. We recorded a non-uniform distribution with a higher incidence during the months of January and March, thus supporting the seasonal variability described in classic references. Danielides et al. [11] showed a significant association between weather changes and the incidence of epistaxis, with the maximum

and minimum daily temperatures and the pressure of water vapour being the most influential environmental factors. A greater influence of the environment during the coldest and driest periods of the year has also been highlighted. However, there is no full agreement in this respect and other authors such as Bray et al. [12] have failed to demonstrate this relationship.

In our series, epistaxis with anterior local source accounted for a high percentage at the patients with heart pathologies. The posterior epistaxis predominated over the anterior in the nasal tumors cases. The high percentage of epistaxis with a posterior location is evident in the case of a hospitalized population, which is eight days in most of the cases and which is also associated with nasal tumors ($p=0.35$, $p<0.5$). In the work of Pino V. et al. [13], which evaluated the etiopathogenesis and treatment of cases of epistaxis admitted between 1990 and 2000, the percentage of posterior epistaxis slightly exceeded 70%. Local factors associated were present in 11% of cases, with a predominance of nasal tumors, followed by local trauma and infections of the upper airway [13]. The vast majority of patients presented an associated systemic disease, primarily in the form of hypertension, ischemic heart disease, atrial fibrillation and diabetes. Despite this, the descriptive design of the study prevents us from stating that these were the direct causes of bleeding. There are many publications that have attempted to explain the influence of arterial hypertension in epistaxis. Contrary to what is classically considered, there is, to date, insufficient evidence to say that arterial hypertension behaves as a risk factor. Most studies do not permit an assessment of this association as they are case series without a control group. Moreover, the impossibility of knowing the figures of blood pressure prior to bleeding and the inability to know whether these are a cause or an effect in patients with nasal hemorrhage greatly impedes their study. Knopfholz et al. [14] studied a series of 121 patients with epistaxis and a prior history of hypertension. They investigated the blood pressure values during episodes of bleeding and the incidence of epistaxis according to the severity of hypertension, classifying their sample into three groups. They found no differences between baseline blood pressure figures in routine checks and the figures recorded during active bleeding. Neither were the cases of epistaxis more frequent in patients with higher levels of hypertension. Neto et al. [15] failed to confirm a definite association between these two parameters. They argue that the real factors contributing to bleeding is sustained hypertension (for more than 5 years), because it produces a series of arteriosclerotic degenerative changes in the vessel walls, weakening them and thus making it easier for them to break. There are very few publications that have continued this avenue of research. A high proportion of our patients presented abnormalities of hemostasis by anti-aggregation or anticoagulation due to drugs, which could indicate that drugs altering hemostasis influenced patients hospitalized for epistaxis. The relationship between anticoagulant drugs and epistaxis has been poorly documented in the literature. Its range of therapeutic hypo-coagulability is measured by its international normalized ratio (INR); its normality index values typically range between 2.5 and 3.5, depending on the pathology being treated. The classic belief considering that poor anticoagulation control carried an increased risk of epistaxis was challenged by Garcia Callejo et al. [16] in a 1990-1995 study. In their extensive series of 1410 anticoagulated patients, nasal hemorrhages were more prevalent in patients with correct anticoagulation control, and only 20% of patients with nasal bleeding presented altered INR levels [16]. In our series, the anticoagulants were not involved in the occurrence of epistaxis (17.92%, 62/346). Most authors agree that, in these patients, the average hospital stay is longer than that for patients not requiring anticoagulation, but this is a fact that Denholm et al. [17] could not confirm. The association between consumption of non-steroidal anti-inflammatory drugs (NSAIDs) and epistaxis is better documented [17]. In 1990, Watson et al. [18] had already confirmed this association, showing that 32% of patients admitted for epistaxis in their study were anti-aggregated, compared to 6% who required hospitalization for other causes. In our study, anti-aggregated use were associated with the nasal bleeding in 19.08% of cases. Aspirin was the most used and its one of the most potent platelet anti-aggregation agents. It interferes with the metabolism of arachidonic acid, producing an irreversible inhibition of the synthesis of cyclooxygenase. It thus promotes a permanent platelet dysfunction for 5-7 days, which is the average life of a platelet. This is one of the main aspects that differentiate it from other NSAIDs, as the latter have shorter duration and potency because the inhibition they generate on cyclooxygenase is variable in level and reversible. Spenopalatine artery cauterization was performed to stop the nasal bleeding only in patients with nasal tumors.

CONCLUSIONS

This first Romanian report on the predictors of epistaxis disorders showed that the typical pattern of patients admitted for nasal bleeding is a preferably male patient, of middle or advanced age with an anterior location and who displays some associated comorbidity or treatment. In our study, the only variable that behaved as a risk factor for bleeding was the posterior location of the epistaxis which occurred more in patients with nasal tumors. In these cases, patients require several days of hospitalization and severity of epistaxis is higher than in patients with other associated pathologies.

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MANAGEMENTUL EPISTAXISULUI ÎN TUMORI NAZALE ȘI AFECȚIUNI CARDIACE LA SPITALUL UNIVERSITAR DIN TIMIȘOARA

REZUMAT

Background și scop. Epistaxisul este o boală frecventă, care afectează până la 60% din populație și este un motiv uzual de adresabilitate la sistemele de sănătate.

Meode. Studiul include pacienți care s-au prezentat la departamentul ORL al Spitalului Universitar Timișoare în perioada ianuarie 2014 – octombrie 2018, prezentând epistaxis anterior și/sau posterior. Factorii demografici (vârstă, rasă, gen), medicamentele utilizate (anticoagulante sau medicație anti-agregantă) și alte comorbidități au fost analizate ca potențiali predictori ai noilor episoade.

Rezultate. Au fost incluși 346 pacienți în studiu, din totalul de 456 pacienți cu episoade de epistaxis în ultimii 4 ani. Analiza multi-variant a identificat predictorii pentru un mare număr de cazuri: tumori nazale, hipertensiune, supradoză de medicamente anticoagulante și variațiile sezoniere.

Concluzii. Epistaxisul apare mai frecvent la pacienții vârstnici cu hipertensiune arterială. Rinita alergică, sinuzita cronică, tumorile nazale și hipertensiunea sunt asociate cu o creștere a incidenței epistaxisului

Cuvinte cheie: epistaxis, tumoră nazală, hipertensiune, packing nazal, cauterizare, anticoagulant.