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CONTENTS

CUPRINS

OBSTETRICAL HEMORRHAGIC SYNDROME IN PLACENTAL INSERTION ABNORMALITIES

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ABSTRACT

Aim and objectives: The main purpose of this paper is to make an accurate and succinct evaluation of the obstetrical hemorrhagic syndrome in placental insertion abnormalities. Obstetric hemorrhage is associated with an increased risk of severe maternal morbidity and mortality. Postpartum hemorrhage is the most common form of obstetric hemorrhage, and worldwide, a woman dies from massive postpartum hemorrhage about every few minutes.

Material and Methods: The present research study, performed both retrospectively and prospectively, was conducted between October 2014 and March 2019, on a study group consisting of 82 selected pregnant patients diagnosed with placental insertion abnormalities.

Results: From the point of view of the studied group, made up of the 82 patients who presented postpartum hemorrhages, a number of 21 patients, representing a percentage of 26% had severe hemorrhages, over 1000 ml, while a number of 61 patients, representing a percentage of the studied group of 74% had minor bleeding.

Conclusions: Specific statistical analysis indicated that age, number of births, number of abortions, history of vaginal birth, weeks of pregnancy, depth of relationship between placenta and uterine muscle wall, and blood transfusion during birth were independent risk factors for severe postpartum hemorrhage.

Keywords: obstetrical hemorrhagic syndrome, placenta praevia

INTRODUCTION

Obstetric hemorrhage is associated with an increased risk of severe maternal morbidity and mortality [1,2]. Postpartum hemorrhage (PPH) is the most common form of obstetric hemorrhage, and worldwide, a woman dies from massive postpartum hemorrhage about every 4 minutes [3,4].

In addition, after hemorrhage, many women experience severe morbidity, such as multiple organ failure, complications of multiple blood transfusions,

peripartum hysterectomy, and unintentional pelvic organ damage, loss of fertility, and psychological sequelae, including posttraumatic stress disorder [5,6].

The placenta is considered an important organ that evolves with the implantation of the blastocyst throughout pregnancy [7]. This special organ plays an essential role in functions such as nutrition, excretion, immune and endocrine function [8]. The physiological placenta is a round or oval organ, which attaches to the uterine wall and is about 22 cm in diameter, about 2-2.5 cm thick and weighs about 1/6 of the fetal weight at birth [9]. Thus, a normal development of the placenta is important for a normal embryonic and fetal development[10]. Consequently, placental abnormalities can range from structural abnormalities to functioning disorders to implantation abnormalities [11].

Approximately 830 women die every day worldwide as a result of pregnancy or birth-related complications, generally caused by obstetric hemorrhages, which remain a major cause of maternal morbidity and mortality [12]. A systematic review by the World Health Organization (WHO) of the global causes of maternal deaths from 2003 to 2019 found

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that bleeding is the leading direct cause of maternal mortality followed by hypertensive disorders [13]. Overall, bleeding accounted for 27.1% of all maternal deaths worldwide [14].

In this context, I consider useful a study to analyze the risk factors that were correlated with severe postpartum hemorrhage in probably the most common placental pathology associated with obstetric hemorrhagic syndromes.

AIM AND OBJECTIVES

The main personal motivation for which, together with my PhD coordinator, I decided to conduct this study was that postpartum hemorrhage is the leading cause of death in pregnant women in our country and, unfortunately, I believe that for some cases, with a management and a well-developed and developed guide, at least some of which could be saved [15].

Another very important aspect that led me to conduct this research was that the placenta praevia is globally but also nationally, in Romania, the main reason for postpartum hemorrhage, although it has been proven that this situation does not always lead to severe postpartum hemorrhage [16].

Most likely, accurate assessment of the risks of severe postpartum hemorrhage in the placenta praevia is extremely important to reduce mortality in pregnant women.

MATERIAL AND METHODS

The present research study, performed both retrospectively and prospectively, was conducted between October 2014 and March 2019, on a study group consisting of 103 selected pregnant patients diagnosed with placenta praevia. Patients with surgical disease, fetal hypoplasia, and incomplete clinical and ultrasound information were excluded, resulting in 82 patients included in the study.

The cases included in the study groups were selected from the case studies of the Obstetrics-Gynecology Department of the "Caritas" Hospital Roșiori de Vede, the private practice of the Obstetrics-Gynecology office "Dr. Caravețeanu Dragoș Cristian" and private practice of 2 other Obstetrics-Gynecology offices in Teleorman County.

This study was conducted entirely in accordance with existing regulations in the field of scientific research, provided in both European and Romanian legislation but also according to the rules of the University of Medicine and Pharmacy of Craiova and by approving the Ethics Commission of the Senate of the University of Pharmacy from Craiova.

Based on the postpartum hemorrhage management and prevention protocol issued by the Obstetrics and Gynecology Associations of Europe and Romania, blood loss was assessed using a combination of gauze weighing methods and blood clot collection.

In this study, postpartum blood loss was measured at the beginning of the skin incision for cesarean section or at the beginning of perioperative vaginal bleeding for cesarean section or at the beginning of parturition for vaginal birth. Measurements were obtained for 24 hours after surgery or vaginal birth. Blood loss over 1,000 ml was defined as severe postpartum hemorrhage and was the only clinical observation in this study.

The diagnosis for placenta praevia was made after 28 weeks of pregnancy using ultrasonography.

Clinical suspicion of placenta praevia was maintained in the following cases: pregnant patient with vaginal bleeding at a minimum time after 24 weeks of amenorrhea, unprovoked associated with the absence of painful uterine contractions, with a normal uterine tone present, with oo dystocic presentation, with fetal heartbeats present and normal.

Statistical processing of results and comparative data analysis was performed using the following softwares: Microsoft Excel version 2007, Anova One Way, respectively SPSS version 22.0. Sublots analyze was performed by statistical indicators: weight, median, standard deviation. Testing the consistency of the samples was performed using the independent Student t-test, bilateral, and testing the combination of parameters by using the Chi square test of association. Statistical differences of the test were representative when the p value was <0.05 at a confidence interval of 95%.

RESULTS

Out of a total of 82 patients enrolled in the study, a number of 6 had a vaginal birth, representing a percentage of 7.31%, while a number of 76 pregnant women included in the study group, representing a percentage of 92.69 % gave birth by cesarean section, the differences being given both by the recommendation of the attending physician and by the guides of the Obstetrics and Gynecology associations. Analyzing the data that characterize the main characteristics of the study group, it is observed that, regarding the areas of origin, a predominance of patients from urban areas, namely 61 from the study group, representing 74.39%, the mean age was 31 years (± 12 years), the level of education was above the high school (post-high school studies, bachelor's, master's) for at least 31% of the patients included in the studied group, the duration of gestation was in most patients included in the study under 39 weeks (over 90%) and only a number of 8 patients had over 39 weeks of pregnancy.

As associated conditions, a number of 6 patients in the study group (representing 6.37% of the total) had high blood pressure during pregnancy, the rest of the patients included in the study were normotensive, preeclampsia

was classified when blood pressure was recorded < 140/90 mmHg at the first prenatal consultation, but with hypertension and proteinuria (> 0.3 g protein / 24h) after at least 20 gestational weeks, in this case registering a number of 14 patients from the studied group (representing 17.07% of the total).
Also, 1 patient from the studied group presented high values of fasting blood glucose and subsequently, by consultation in the outpatient clinic, was diagnosed with gestational diabetes, being followed throughout pregnancy and postpartum. From the BMI calculation, there were values characteristic of obesity (over 30 BMI values) in a number of 13 patients in the studied group (representing 15.85% of the total), the rest of the patients having values of body mass index below 29.9.
Regarding the presence of abortion in the antecedents, 13 patients from the studied group (representing 15.85% of the total) mentioned this information at the specialized control, the rest of the patients not having an abortion in the antecedents or not declaring this.
Also, in connection with the loss of a previous pregnancy, 4 patients from the studied group (representing 4.88% of the total) reported the loss of a pregnancy, while 1 patient from the studied group (representing 1.22% of the total) evoked the loss of two previous pregnancies. In contrast, no patients reported losing more than two pregnancies.
From the personal pathological antecedents, it was also highlighted that 2 patients from the studied group (representing 2.44% of the total) had venous thrombosis.
As mentioned above, out of a total of 82 patients enrolled in the study, a number of 6 had a vaginal birth, representing a percentage of 7.31%, while a number of 76 pregnant women included in the study group, representing a percentage of 92.69% gave birth by cesarean section. All of these patients were initially diagnosed with the lower placenta inserted.
From the point of view of the studied group, made up of the 82 patients who presented postpartum hemorrhages, a number of 21 patients, representing a percentage of 26% had severe hemorrhages, over 1000 ml, while a number of 61 patients, representing a percentage of the studied group of 74% had minor bleeding. There are about three times as many patients with severe postpartum hemorrhage.
Regarding the age of pregnant patients included in the study group, in the case of patients with severe postpartum hemorrhage, the mean age was, according to statistics, about 32 years, with extremes of 23 years and 41 years, respectively, in while the age of pregnant patients included in the study group who had minor postpartum hemorrhage, the mean age was approximately 29 years, with extremes of 19 years and 38 years, respectively. There is a difference of about 3 years in favor of patients who had severe bleeding, the difference being maintained even in patients who had the extreme ages of the group.
Regarding the duration of pregnancy, the statistical calculation showed that depending on the duration of pregnancy (more or less than 38 weeks of pregnancy) a number of 59 patients, representing 96.72% of all those who had minor postpartum hemorrhage (61 patients) had a gestation period of less than 38 weeks of pregnancy, while in the case of pregnant patients included in the study group who had severe postpartum hemorrhage, a group of 19 patients (90.48% of the total 21 patients) had a gestation period of less than 38 weeks of pregnancy.
There is a slight difference of about 6 percent in favor of patients with minor bleeding, but without a strong statistical significance. We also observed a higher percentage in favor of patients born by caesarean section and had major postpartum hemorrhage, both to patients with natural birth and major postpartum hemorrhage, and to both subcategories (natural birth and cesarean section) within patients with minor bleeding studied.
From the total number of patients studied, consulting the patient observation sheets, with the patients' history but also performing the clinical examination and the anamnesis with heredocolateral and personal pathological antecedents, we noticed that only a number of two patients (2.44% of all patients included in the group). respectively 9.52% of all patients with severe bleeding) have a history of surgical complications in the last 15 years.
In the statistical calculations we also took into account a whole series of other clinical indices in the prediction model of postpartum hemorrhages, among which:
- history of premature birth - 2 patients with minor hemorrhage, representing a percentage of 3.28%, respectively a patient with severe hemorrhage, representing a percentage of 4.76%;
- history of placenta praevia - no patient with minor hemorrhage, respectively a patient with severe hemorrhage, representing a percentage of 4.76%;
- the presence of uterine fibroids - no patient with minor hemorrhage, respectively a patient with severe hemorrhage, representing a percentage of 4.76%.

DISCUSSION

As previously shown, placenta praevia is a major risk factor for severe postpartum hemorrhage. With the increase in the number of cesarean operations worldwide, as well as the consequent increase in the incidence of placenta praevia, a significant increase in severe postpartum hemorrhage has been documented. The definition of severe postpartum hemorrhage, as mentioned in the guidelines of obstetric and gynecological societies, differs between different organizations and continents [5].
The definition of blood loss in severe postpartum haemorrhage varies between different country guidelines, ie over 1,500 mL, 2,000 mL, 30-40% of whole blood volume or over 4 transfusion blood units (800 ml) [13].

In this study, severe postpartum hemorrhage was defined as blood loss over 1,000 ml. The management and prevention of postpartum haemorrhage published by the profile associations states that when postpartum blood loss exceeds 1,000 ml, special measures should be implemented to save patients' lives.

These include timely referrals to a superior clinic, uterine artery embolism or total hysterectomy, as well as intensive care. We did not define postpartum hemorrhage based on the volume of blood transfusion, because it is associated with the state of anemia, characterized by the value of hemoglobin [16].

In this study, risk factors for severe postpartum hemorrhage included age, number of pregnancies, number of births, number of miscarriages, history of vaginal birth, history of cesarean section, surgical complications, body mass index before pregnancy, number of fetuses, number of weeks at termination of pregnancy, the presence of uterine fibroids, the types of placenta praevia, the depth of the placenta that invades the uterine muscle wall and blood transfusions [17,18].

The possibility of complicated pregnancies is high and the risk of severe postpartum hemorrhage is also increased. In addition, multiple births are considered risk factors for postpartum hemorrhage. Pregnancy duration has also been shown to be an independent risk factor for severe postpartum hemorrhage. Because multiple births could lead to changes in myometric contraction, it is thought to be a significant factor for severe postpartum hemorrhage [13].

We found correlations with cesarean section and uterine surgical complications with severe postpartum hemorrhage in the placenta praevia, which was consistent with a number of previous studies. The history of cesarean section and uterine surgical complications are considered risk factors for the implantation of the placenta praevia and is frequently associated with severe postpartum hemorrhage [19].

In addition, the body mass index calculated before pregnancy, the number of fetuses and the presence of uterine fibroids are associated with severe postpartum hemorrhage, but were not independent risk factors for severe postpartum hemorrhage. Interestingly, we found that a history of vaginal birth was an independent protective factor for severe postpartum hemorrhage. This could be explained by the subsequent relaxation of the cervix after vaginal birth.

CONCLUSION

This study suggests that placenta praevia is a major risk factor for severe postpartum hemorrhage. With the increase in the number of cesarean operations nationwide, the consequent increase in the incidence of placenta praevia has led to a significant increase in severe postpartum hemorrhage. Specific statistical analysis indicated that age, number of births, number of abortions, history of vaginal birth, weeks of pregnancy, depth of relationship between placenta and uterine muscle wall, and blood transfusion during birth were independent risk factors for severe postpartum hemorrhage.

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SINDROMUL HEMORAGIC OBSTETRICAL ÎN ANOMALIILE DE INSERŢIE PLACENTARĂ

REZUMAT

Scop și obiective: Scopul principal al acestei lucrări este de a realiza o evaluare precisă și succintă a sindromului hemoragic obstetrical asociat anomaliilor de inserție placentară. Hemoragia obstetricală este asociată cu un risc crescut de morbiditate și mortalitate maternă severă. Hemoragia postpartum este cea mai frecventă formă de hemoragie obstetricală și, la nivel mondial, o femeie moare din cauza unei hemoragii masive postpartum aproximativ la fiecare câteva minute.

Material și metode: Prezentul studiu de cercetare, realizat atât retrospectiv cât și prospectiv, a fost realizat între octombrie 2014 și martie 2019, pe un grup de studiu format din 82 de paciente însărcinate diagnosticate cu anomalii ale inserției placentare.

Rezultate: Din punctul de vedere al grupului studiat, format din cele 82 de paciente care au prezentat hemoragii postpartum, un număr de 21 de paciente, reprezentând un procent de 26% au avut hemoragii severe, peste 1000 ml, în timp ce un număr de 61 de paciente, reprezentând un procent din grupul studiat de 74% au avut sângerări minore.

Concluzii: Analiza statistică specifică a indicat faptul că vârsta, numărul nașterilor, numărul avorturilor, istoricul nașterii vaginale, săptămânile de sarcină, adâncimea invaziei placentare spre peretele muscular uterin și transfuzia de sânge în timpul nașterii au fost factori de risc independenți pentru hemoragia severă postpartum.

Cuvinte cheie: sindrom hemoragic obstetrical, placenta praevia

ON THE MECHANISMS UNDERLYING IMMUNOMODULATORY EFFECTS OF SOME INDIGENOUS PLANTS

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ABSTRACT

Immunity is the organism's defense system that confers protection against diseases carried by pathogens such as bacteria, viruses and fungi, working as a barrier against these agents. Vegetal extractive solutions (aqueous or organic) may influence the immune system through its activity stimulation (in this case, the extractive solution has immunostimulant properties) or depression (the extractive solution is an immunosuppressive agent). The spontaneous flora includes many species well known for their effect on immune system, this article reviewing some cellular and physiological mechanisms presumed to be responsible for the immunomodulatory, anti-inflammatory or anticancer potential of indigenous plants.

Keywords: immunomodulatory, innate and adaptative immune system, anti-inflammatory, indigenous plants.

INTRODUCTION

The immune system is the main defense mechanism against infectious diseases and inflammation produced by bacteria, fungi, viruses and other non-self structures. The innate immune system is the first defense line against external pathogen agents, encountered from the most primitive life forms such as unicellular organisms to mammalians, including humans (9). For humans, the first defense line against non-self pathogens is represented by biological structures like skin, mucous membranes, enzymes (as lysozyme and phospholipase A) present in tears and saliva, mucous present in the gastrointestinal or pulmonary tract and gastrointestinal flora. Once pathogens have crossed these barriers, humoral (complement system, lactoferrin, transferrin) and cellular (mast cells, natural killer cells, phagocytes, like macrophages, neutrophils and dendritic cells) means of defense occur (50).

The adaptive immune system is composed of highly specialized cells (T and B lymphocytes) that eliminate the antigen or prevent pathogens' growth. They provide the adaptive immune system with specific properties like immunological memory, that leads to a stronger response to a subsequent contact with the pathogen, and antigenic specificity, that leads to a signal only in contact with a certain antigen (23). The protection against pathogens is assured not only through specialized cells, but also through humoral components such as immunoglobulins secreted by B cells (50). The immune system anomalies can evolve to immunopathological conditions: autoimmune diseases, inflammatory diseases and cancer (20).

Immunomodulators are agents that adjust the immune system activity being able to prevent, ameliorate or cure an immunological disease. They interact with different parts of the immune system, so they stimulate or inhibit different types of cells and humoral mediators involved in the immune system, such as lymphocytes, macrophages, NK cells, cytokines (36). Immunomodulators can be classified as immunostimulants, drugs that increase the immunity in infectious diseases, tumors, primary and secondary immunodeficiency, and immunosuppressants, drugs that decrease the immune response, so they are used in autoimmune diseases, inflammatory diseases and organ transplant (26, 51).

Immunomodulators, especially synthetic ones, haven't only pharmacodynamic effects, but also pharmacotoxicological ones (synthetic immunosuppressants can cause gastric irritations, hepatitis, pancreatitis, nephropathy, myelotoxicity) (8). Synthetic immunostimulants can also present side effects like acute phase reaction, release of proinflammatory

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cytokines, humoral lysis syndrome, autoimmunity and high vascular permeability with interstitial edema (44).

This is the main reason why many researches are focused on pharmaceutical products of vegetal origin.

Vegetal immunomodulators may increase or decrease both the innate and adaptative immune response, but unlike those obtained through synthesis, they are more affordable (especially the endemic ones) and many of them with lower pharmacotoxicological potential (1). Because of these advantages, the modulation of the immune response through plants products represents a highly studied subject by researchers from all around the world.

This paper's aim is to review aspects regarding plants from the spontaneous flora discussed for their immunomodulatory potential, mainly the active compounds, their mechanism of action, diseases that can be treated and cured using vegetal extracts.

We will shortly present the main indigenous families containing plants discussed for their immunomodulatory potential.

Aristolochiaceae family includes well known plants, such as *Aristolochia clematitis*, birthwort, a ruderal plant whose aerial part contain aristolochic acid, a substance when used for external purposes, in small potential, used to relief the symptomatic pain in arthritis (2). Its mechanism of action is common with the one of the nonsteroidal anti-inflammatory drugs, at a dose of 10 mg/ml inhibiting both subtypes of cyclooxygenase (COX) (40).

doses, presents anti-inflammatory properties (in folk medicine it is used to treat arthritis, rheumatism, gout, chronic inflammatory and dermatological conditions), but in high doses, due to continuous internal use it may be carcinogenic, particularly at kidney level (29). That is why this is a high interest subject for researchers worldwide, they even talk about an endemic Balkan nephropathy, as a consequence of the internal administration of *A. clematitis* for a long period of time, that affect lots of rural populations (48). The anti-inflammatory mechanism of aristolochic acid consists in phospholipase A2 inhibition (after i.m. /i.p. administration), so it inhibits the synthesis of pro-inflammatory prostaglandins (41). A double-blind study realized on healthy volunteers has shown that aristolochic acid increases peripheral granulocytes' phagocytic activity after administration of a 0.9 mg/day dose, for ten consecutive days, highlighting its immunostimulant activity (32).

Another representative plant from this family is *Asarum europaeum* L., hazelwort, plant that is commonly found in deciduous forests. The rhizome and aerial parts are used for external purposes because they have a high concentration of volatile oil rich in azarone, a phenylpropanoid compound with anti-inflammatory

Results of the main researches regarding the mechanism of action for some indigenous plants of the 13000 species of the *Asteraceae* family are presented in Table 1.

Table 1. Potential mechanisms of the immunomodulatory action for some representative plants of the *Asteraceae* family

Species	Part used	Type of extract	Mechanism of action
Achillea millefolium L. (Yarrow)	Flowers	Aqueous	*In vitro*, on LPS stimulated RAW 264.7 macrophages: - Decrease of NO synthesis and iNOS expression; - Increase of IL-1β and TNF-α synthesis (12)
Arctium lappa L. (Burdock)	Roots	Butanolic	*In vitro*, on RBL-2H3 cells: - Decrease of IL-4 and IL-5 synthesis; - Decrease of NF-kB synthesis (46)
Arnica montana L. (Mountain arnica)	Flowers	Hydro-alcoholic	*In vivo* experiment on Wistar rats: - Increases the B and T cells synthesis; - Increase of ICAM-1 synthesis (28)
Artemisia absinthium L. (Absinthe)	Leaves	Aqueous	*In vivo*, on rats: -Decreases the synthesis of IL-1 and TNF-α (6)
Calendula officinalis L. (Marigold)	Flowers	Aqueous	*In vitro*, on tumor cells lines: -Inhibits proliferation of tumor cells (leukemia, fibrosarcoma, breast cancer, pancreatic, lung, colorectal cancer); *In vivo*: -Growth inhibition of melanoma cells (25)

Cichorium intybus L. (Chicory)	Flowers	Ethanolic	*In vivo*, on rats: -Stimulates the activity of NK cells; -Increase of IFN-γ synthesis; -Increase of phagocytic activity (31)
Inula helenium L. (Horse heal)	Roots	Ethanolic	*In vitro*, on alveolar macrophage NR 8383: -Decreases the synthesis of IL -1,-6, TNF-α (19)
Matricaria recutita L. (Chamomile)	Flowers	Aqueous	*In vitro*: -Inhibits COX and 5-LOX activity, decreasing the levels of prostaglandins and leukotrienes; *In vivo*, on rats' skin: -Antiinflammatory effects against inflammation produced by croton oil (14)
Tagetes patula L. (French marigold)	Flowers	Aqueous	*In vitro*: -Decreases the synthesis of oxygen reactive species; -Decreases CD4+ T cells synthesis; -Decreases TNF-α synthesis; *In vivo*, on rats: -Decreases IL-1β synthesis (22)
Taraxacum officinale L. (Dandelion)	Aerial parts	Methanolic	*In vitro*, on RAW 264.7 macrophages: -Decreases the synthesis of TNF-α, IL-1β, -6, PGE2 (33)

Colchicine is a protoalkaloid well known for its immunomodulatory and antitumor activity, that is the reason why it is being used in rheumatic diseases, gout, Mediterranean fever and malign conditions. It is isolated from *Colchicum autumnale's* seeds, commonly known as autumn crocus, an indigenous *Colchicaceae* family's plant.

Colchicoside and demecolcine (the later an antimitotic less toxic, isolated from bulbs and flowers, used as a cytostatic, Colcemid, in myeloid leukemia) are isolated from crocus the same (34). All these alkaloids inhibit tubulin polymerization, so they block cellular division and also leukocytes' migration to urate crystals and phagocytosis (43).

Hippophae rhamnoides L., sea buckthorn, member of the *Elaeagnaceae* family, is one of the most appreciate plant with immunomodulatory potential and grow spontaneous in Romania. Its fruits are rich in vitamins (ascorbic acid, tocopherols, carotenoids), flavonoids, carbohydrates, proteins, some organic acids (palmitoleic and palmitic acids identified in the mesocarp), fruits oil being used for wound healing in the traditional medicine, based on the anti-inflammatory, cicatrizing and rejuvenating properties (49). As it results from studies that evaluate fruits and leaves extracts effect on proinflammatory cytokines production (IL-2, -4, IFN-γ), antibodies level and antioxidant potential, one can assume that not only sea buckthorn fruits have immunomodulatory potential but also the leaves (17).

Fabaceae family counts numerous indigenous species discussed for their immunomodulatory properties (Table 2).

Table 2. Potential mechanisms of the immunomodulatory action for some representative plants of the *Fabaceae* family

Species	Part used	Type of extract	Mechanism of action
Baptisia tinctoria L. (Wild indigo)	Roots	Aqueous	*In vitro*, in cultures of alveolar macrophages: - Activates macrophages and increase IL-6 and TNF-α production (13)
Galega officinalis L. (Galega)	Aerial parts	Dichloro methane	*In vitro*: - Inhibits NO synthesis in RAW 264.7 macrophages stimulated with LPS; - Cytotoxic activity on A-549 pulmonary adenocarcinoma cells and U-87 glioblastoma (27)

Glycyrrhiza glabra L. (Licorice)	Roots	Aqueous	*In vivo*, in rats: - Inhibits colon carcinoma cell growth; - Activates CD4+ and CD8+ lymphocytes; - Increase of antitumoral factors synthesis (IL-2,-6,-7); - Decrease of TNF-α synthesis (7)

Trigonella foenum grecum L. (Fenugreek)	Seeds	Ethanolic	*In vitro*, on monocytes cultures: - Decrease of proinflammatory cytokines (IL-1α,-1β, -2, -6, TNF-α) levels; - Decrease lipid peroxidation, malondialdehyde level; - Increase of SOD and CAT activity, GSH level (10).

Malvaceae family counts over 4200 plant species, some of them well known for their anti-inflammatory, immunomodulatory, expectorant and antibacterial effects. One of these is *Althaea officinalis* L. studied for its immune stimulant and anti-inflammatory activity because its great content of mucilage and flavonoids. *In vitro* studies have been proved that roots aqueous extract stimulates phagocytosis and leukotrienes release from neutrophils, as well as the release of Il-6 and TNF-α cytokines from human monocytes (5). Hypolaetin-8-glucoside have proved its anti-inflammatory and analgesic effects on rats, being even more potent than equal doses of phenylbutazone in the suppression of acute phase carrageenan-induced inflammation without producing gastric ulcerations, unlike phenylbutazone. Hypolaetin-8-glucoside have proved to be more potent than troxerutin in decreasing the capillary permeability induced by histamine in rats (16).

Another immunostimulatory plant is *Nigella sativa* L. (black caraway), from the *Ranunculaceae* family, whose seeds are widely used mainly in respiratory viral infections, thanks to the high level of quinines (thymoquinone, thymohydroquinone, dithymoquinone and thymol) (3). They also contain α-hederin, an anticarcinoma saponin, whose efficiency have been proved *in vitro*, on A549 (pulmonary cancer), Hep2 (laryngeal carcinoma), HT-29 (colon cancer) and MIA PaCa-2 (pancreatic cancer) cells (45). It has been proved that aqueous extract of *N. sativa* stimulates splenocytes proliferation in a dose dependent manner and also the antitumoral activity of NK cells against lymphoma YAC1 cells. It also inhibited macrophages secretion of IL-6, TNF-α, NO, Th1 cytokines (IFN-γ, IL-2) and also it increased the secretion of Th2 anti-inflammatory cytokines (IL-4, -5, -6, -10) (38). *N. sativa* extracts have been intensively studied not only for their immunomodulatory effects, but for the antioxidant, antihistamine, anthelminthic, anticancer, antibacterial, antihypertensive, hepatoprotective, uricosuric, antidiabetic and anti-sterility effects (3).

Among the plants of the *Papaveraceae* family we can name *Chelidonium majus* L., also called greater celandine, a spontaneous Romanian plant often used for its therapeutic properties. Both the aerial parts and the roots, from which there are realized crude extracts or purified products, are being used for medical purposes. Their therapeutic benefits (among which anti-inflammatory, antioxidant, immunomodulatory and antitumoral) are related to the high level of isoquinoline alkaloids such as chelidonine, sanguinarine, berberine (18). Their immunomodulatory potential has been proved in a plethora of *in vitro* studies (a protein-bound polysaccharide extracted from *C. majus* showed mitogenic activity on spleen and bone marrow cells and increased the number of granulocyte macrophage-colony forming cells) (47) and also *in vivo* studies (*C. majus* methanolic extract decreased the production of TNF-α, IL-6, IFN-γ, B cells and increased the proportion of CD4+ and CD25+ regulatory T cells in collagen induced arthritis in mice) (35). It is well known that *C. majus* has anticancer activity, due to the high concentration of the compounds mentioned above and also due to the lectins and polysaccharides that inhibit cell growth, too (18). The anticancer mechanism of action is complex: i) reduction of telomerase activity by chelidonine; ii) induction of cancer cell death by apoptosis and iii) arrest of mitosis through inhibition of tubulin polymerization (42). Ukrain™ (an anticancer drug whose major components are chelidonine, sanguinarine and chelerythrine alkaloids) is a well-known therapy successfully used in different types of cancer, such as breast, pancreatic, ladder, or colorectal cancer (18).

We have to mention plants of the *Salicaceae* family, like *Salix alba* L., white willow, from whose bark was first isolated salicine, a glycoside with anti-inflammatory, analgesic, antipyretic properties, widely used for rheumatic diseases, the forerunner of modern nonsteroidal anti-inflammatory medicines like aspirin (24). Its anti-inflammatory potential has been proven *in vitro* (it decreased the synthesis of PGE2, NO, TNF-α, activated the apoptosis of monocytes that produce proinflammatory cytokines) (11). Also, *Populus sativa* buds contain saligenol, compound with COX2 inhibitory potential. The aqueous extract of *Populus gemma* buds also has anti-inflammatory potential, demonstrated in various animal models, like carrageenan rat paw edema, arachidonic acid and xylene ear edema, cotton granuloma (52).

Mistletoe, *Viscum album* L., a hemiparasite indigenous *Santalaceae* family plant, presents a remarkable immunomodulatory and cytostatic activity due to its high content of lectins. It also contains viscotoxins, small proteins with antifungal properties, enzymes, amino acids, thiols, amines, polysaccharides, phytosterols, triterpenes, flavonoids, phenylpropane and minerals (15, 39). Both the mistletoe extract and its constituents present antitumor activity through different mechanisms: i) cytotoxic activity (lectins have cytotoxic activity on multidrug resistant cancer cells, as colon cancer cells); ii) increase of other anticancer drugs activit; iii) apoptosis induction and iv) interference with tumoral angiogenesis (30). Due to its immunostimulatory properties (either *in vitro* or *in vivo*, it activates the immune system's cells and induces anti-inflammatory cytokines) and also cytotoxic activity, mistletoe extract is successfully used as a therapy for patients with cancer (Isorel, Iscador, Helixor) increasing the quality of life and presenting the advantage of rare or mild side effects, such as nausea (39).

Another indigenous plant, *Urtica dioica* L., common nettle, a native Romanian plant from *Urticaceae* family, is well-known for its therapeutic properties; its leaves and roots have demonstrated anti-inflammatory and cytostatic activity, due to the contained agglutinins and lectins that have mitogenic action on T cells, especially on CD4+ helper and CD8+ cytotoxic subtypes (37). It has also been shown that it inhibits NO synthesis in macrophages stimulated with lipopolysaccharides (21). Also, flavonoid glycosides isolated from aerial parts have proved high chemotactic effect and intracellular killing activity on neutrophils (4).

CONCLUSION

Indigenous plants represent a wide source of compounds with anti-inflammatory, immunomodulatory and cytostatic properties. They exert these effects through complex mechanisms controlling the balance between immune cells and cells that aggress the normal body functioning. Many times, they are more used than synthetic drugs because they don't produce side effects, they are more affordable and show synergic action with synthetic drugs. So, immunomodulatory plants will always be an intense studied topic, not only in oriental underdeveloped countries with limited access to classic treatment, but in western developed countries, too.

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MECANISMELE CARE STAU LA BAZA EFECTELOR IMUNOMODULATOARE ALE UNOR PLANTE INDIGENE

REZUMAT

Imunitatea este mecanismul de apărare a organismului ce îi conferă protecție împotriva îmbolnăvirii, acționând ca o barieră împotriva agenților patogeni, precum bacterii, fungi, virusuri, astfel încât organismul nu contactează afecțiunea mediată de respectivul agent. Soluțiile extractive vegetale (apoase sau organice) pot influența sistemul imun, fie prin stimularea activității sale, având efect imunostimulator, fie prin deprimarea activității acestuia, în acest caz vorbindu-se despre extracte cu potențial imunosupresor. Flora spontană prezintă numeroase specii recunoscute pentru potențialul imunomodulator, scopul acestui articol fiind de a prezenta unele mecanisme celulare și fiziologice presupuse a fi responsabile de potențialul imunomodulator, antiinflamator și antitumoral al unor plante indigene.

Cuvinte cheie: imunomodulator, sistem imun înnăscut și dobândit, antiinflamator,

SIGNIFICANCE OF IMPAIRED RESPIRATORY ANATOMICAL AND PHYSIOLOGICAL DEFENSE MECHANISMS IN THE PATHOGENESIS OF LUNG DISEASE DUE TO NONTUBERCULOUS MYCOBACTERIA (LD-NTM) IN IMMUNOCOMPETENT HOSTS

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ABSTRACT
Currently, lung disease due to nontuberculous mycobacteria (LD-NTM) can become a public health problem due to the increasing proportion of immunocompromised people in society, on the one hand by increasing the incidence of HIV-AIDS infection, but especially by the widespread use of antineoplastic chemotherapeutics and monoclonal antibodies, anti-TNF and anti-IFN. As a result, the costs of diagnosing and hospitalizing these patients will be increased, putting financial pressure on public health systems in developed countries, but especially in developing ones. Also, the main goal of doctors is the early and correct diagnosis of these lung infections, especially since nontuberculous mycobacteria are ubiquitous and can contaminate any sample of pathological product collected from the patient.
Keywords: respiratory system defenses, NTM, immunocompetent hosts.

INTRODUCTION

If in the past, tuberculosis and leprosy were two common human diseases caused by mycobacteria, now bacterial infections caused by nontuberculous mycobacteria (NTM) tend to become the main mycobacterial diseases in developed countries, in part due to the occurrence of HIV-AIDS infection [1] and

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extensive use of immunosuppressive therapeutic means. NTMs include saprophytic and opportunistic microorganisms other than Mycobacterium tuberculosis and M leprae. The genus Mycobacterium contains over 200 species and 13 subspecies, which are grouped into 3 major groups: the Mycobacterium tuberculosis complex, M leprae and mycobacteria other than M. tuberculosis and M leprae (nontuberculous mycobacteria: NTM or atypical mycobacteria) [2-4]. Atypical mycobacteria, morphologically similar to the Koch bacillus (acid-fast-bacillus: AFB), however, differ from it by faster cultivation and sometimes at lower temperatures [5]. NTMs are ubiquitous organisms found in environmental sources that include drinking and natural water, as well as soil and dust **[**6**]**. Human subjects can inhale or ingest NTMs from water, aerosols or dust. NTMs are opportunistic microorganisms, in the sense that they produce diseases only in people who have pre-existing conditions of the respiratory system or the immune system **[**7**]**. Several predisposing factors for lung NTM infections are known, such as old age, immunodepressive diseases, and drugs **[**8**]**. The occurrence of lung disease (LD) caused by NTM (LD-NTM) is the result of the interaction between: exposure to the environment (example: infectious dose of nontuberculous mycobacteria, number of microorganisms and duration of exposure), microorganisms (virulence and pathogenicity) and host (genetic risk factors, pre-existing lung diseases and local and systemic immune status) **[**9-13**]**. The host organism provides antibacterial resistance through the anatomical defense mechanisms (the complex system of filtering inhaled air, respiratory epithelial integrity, mucociliary clearance) and physiological (inflammatory and immune reactions) of the respiratory system **[**14**]**.

RESPIRATORY SYSTEM DEFENSES

1.The general, non-specific pulmonary defense mechanisms (first defense barrier) include anatomical (respiratory anatomical filter, mucociliary clearance, mucus), functional (cough and sneezing), chemical (mucus, lysozyme, fibronectin), and biological mechanisms (respiratory microbiome) **[**15**]**. The **anatomical and mechanical filter** against microparticles, represented by the sinuous path of the airways, consisting of the polyseptal, polycavitary structure and dichotomous branching of the bronchi is complex. By passive diffusion, only the microparticles between 0.5 and 2 μm penetrate the bronchioles and alveoli **[**16**]**. **Epithelial cells** together with the mucociliary system represent a physico-chemical and biological barrier against microbial invasion. Epithelial cells are joined together by intercellular structures called junctions, the most important structures are the tight junctions that ensure epithelial integrity **[**17**]**. The **mucociliary apparatus**, made up of mucus and vibrating cilia, is the main system of physical purification of particles entering the airways **[**18**].** The mucus of the respiratory tract is viscous, has a complex composition, the most important components being mucins **[**19**]**. The cilia have a vibration of 1.000 – 1.500 beats/min, their movement being triggered by reflex mechanisms. **Sneezing** promotes the elimination of irritating particles from the nasal epithelium, and **coughing** facilitates the expulsion of bronchial secretions loaded with inert or biological mycoparticles to the outside, making up the functional defense mechanisms of the respiratory system **[**20**]**.
The **chemical mechanisms** are represented by substances with bactericidal effects located on the surface of the epithelium of the respiratory mucosa (mucus, lysozyme, fibronectin). **Mucus** provides additional protection by forming a semipermeable barrier that ensures the exchange of nutrients, water and gases, and waterproof at the same time for pathogens **[**21,22**]**. It intervenes in the innate immune defense through mucins, with antimicrobial and antiinflammatory properties **[**23**]**. **Lysozyme** produced by respiratory epithelial cells, bronchial glands and alveolar macrophages, has bactericidal activity and influences the secretion of IgA and complement, inhibiting the function of neutrophils, by decreasing chemotactism and oxygen radical synthesis **[**24**]**. Cellular **fibronectin**, secreted along the respiratory tract by macrophages and fibroblasts, has a role in intracellular adhesion, and plasma fibronectin has a role in opsonization **[**25**]**. Fibronectin by competition blocks the adhesion of bacteria to the cell epithelium and opposes phagocyte particles, binding to them **[**26**]**.
The microbiome of the respiratory system is acquired through a dynamic process, which begins at birth and continues throughout the entire life, showing major changes with the onset of the chronic lung disease. The microflora of the respiratory system also presents topographic variations **[**27**]**. The human lungs are a low nutrient source for the microorganisms, compared to the intestinal tract where there is an exaggerated endogenous microflora **[**28**]**. The phenomena that contribute to the composition of the lung microbiota are immigration of the bacteria (by inhaling bacteria from the inspired air, direct dispersion along the mucosal surface and microaspiration), elimination of the bacteria (activity of the mucociliary apparatus that brings out the microorganisms from the depth of the lungs) and relative replication rate of these germs **[**29**]**. The intrapulmonary factors restricting the development of lung microbiome are blood oxygen, blood flow, local pH, temperature,

disposition of the effector inflammatory cells and the epithelial cell architecture. Beside these local physiological factors, the local immune defense respiratory mechanisms prevent the colonization of the lung parenchyma [30]. Commensal bacteria are non-pathogenic and protect the airways against pathogens through the following mechanisms: they are the native competitors of the pathogenic bacteria by tending to occupy the same ecological niche in the human body and are able to produce antibacterial substances called bacteriocins that inhibit the growth of pathogens. The genera Bacillus, Lactobacillus, Lactococcus, Staphylococcus, Streptococcus and Streptomyces are the main producers of bacteriocins in the respiratory tract [31]. **Chronic lung disease**, in all its clinical forms, besides the architectural changes, will also cause significant changes in the microbial ecosystem of the lung [32). Some authors have shown that in the bronchoalveolar lavage fluid of these patients is an abundance of microorganisms belonging to the phyla Prevotella and Veillonella genera (from the common anaerobic oral flora), along with an increased number of lymphocytes and neutrophils [33]. Subclinical aspiration, the inability of the mucociliary apparatus to eliminate this commensal flora reaching the lower respiratory tract and the decrease of local immune mechanisms determine the development of an excessive chronic infection, characterized by a certain inflammatory phenotype in BAL (bronchoalveolar lavage) fluid [34]. Therefore, the lung microbiome intervenes in the pathogenesis of the chronic lung disease through its ability to modulate the local inflammatory responses and through the influence of the chronic lung disease [35]. Also, the patients with NTM lung infections showed a particular inflammatory phenotype on the analysis of bronchoalveolar lavage fluid, compared to patients without NTM infections [36]. Therefore, in these patients with NTM lung infections, the implications of the commensal flora reaching the lower respiratory tract could explain the heterogeneity of the clinical picture and the progression of this chronic lung disease [37].

2.The mechanisms of natural (native or innate) immune defense include the cells involved in the inflammatory reaction (neutrophilic, basophilic and eosinophilic polymorphonuclear cells, monocytes, alveolar macrophages and lymphocytes), NK cells, cytokines and the febrile process.

Alveolar macrophages, present in a percentage of 90% in the bronchoalveolar lavage fluid, have mesenchymal origin and come from the bone marrow, circulating in the blood as monocytes. In the alveoli, the macrophages may be free or may adhere to the alveolar walls [38]. Macrophages are large cells with many cellular organs (especially lysosomes and phagosomes), which are the enzymatic basis of phagocytosis and germ destruction. The complex enzymatic equipment of these cells contains: proteases, aminopeptidases, especially leucylpeptidase, esterases, acid phosphatase, ribonuclease, deoxyribonuclease, lipase, beta-galactosidase, beta-glucuronidase and lysozyme. The phagocytic activity of the macrophages is nonspecific, representing a barrier to the microbial or inert particles in the alveoli with the diameter less than 2 mm. At the surface of the membrane, they have receptors for C3 and Fc receptors for IgG, with a stimulating role in phagocytosis [39]. If the macrophages are transported on the mucociliary carpet, outside the alveolus, they become inactive. **Neutrophilic polymorphonuclear cells**, present in a proportion of 1% in the bronchoalveolar lavage fluid, come from the systemic circulation, adhere to the vascular epithelium by chemotaxis and have effective bactericidal potential due to a higher density of Fc receptors for the immunoglobulins on the membrane surface and increased production of oxygen radicals [40]. The **physiological inflammatory reaction**, which occurs during the non-specific lung defense process, actually consists of: mobilization from the bone marrow of these cells (neutrophils, monocytes, lymphocytes), chemotaxis, phagocytosis and, finally, intracellular digestion of microorganisms in the lungs. The effects of the inflammatory reaction are mediated, in particular, by complement and cytokines. The **complement system** is a key component in the success of the inflammatory response, with possible deficiencies leading to susceptibility to infections. This system consists of serum proteins (approximately 35), synthesized in the liver that circulate in inactive form, with enzymatic, cytolytic and regulatory activity that produce chemotaxis, opsonization and cytolysis of pathogens. Activation of the complement triggers the assembly of the complement's membrane attack complex (MAC). MAC consists of the complement components C5b, C6, C7, C8 and C9. The component proteins participate in the innate immune response both by destroying microbial agents and by recognizing them, so that they can be easily identified and destroyed by leukocytes. MAC increases membrane permeability and triggers the production of bioactive substances, including mediators of inflammation (oxygen free radicals) and cytokines. This mechanism has a positive effect on the elimination of pathogens and on the amplification of the inflammatory reaction. Also, the MAC promotes the expression of p-selectin, which increases the adhesion of neutrophils, platelets and monocytes, then causes the release of inflammatory mediators [41]. Based on the practical finding that the incidence of C4 fraction deficiency is much higher in patients with pulmonary tuberculosis, it was considered that C4 deficiency could

play a major role in susceptibility to lung NTM infection, especially in immunocompetent individuals [42]. A Finnish study showed a statistically significant difference in serum C4 deficiency in patients with NTM infections compared to the control group (no serum C4 eficiency) [43]. In addition, the same study showed statistically significant differences in C4 deficiency, especially in women with NTM lung infections. It has been thought that genetic variations of serum complement will cause a decrease in its ability to attack, which makes nontuberculous mycobacteria, possessing a thick lipid layer in the peripheral capsule, become resistant to this physiological defense mechanism. On the other hand, serum C4 deficiencies are also associated with certain alleles encoding the synthesis of HLA histocompatibility antigens. The frequency of these HLA alleles and haplotypes varies greatly by population and ethnicity [44,45,46]. The fact that that lung infections with NTM in immunocompetent hosts occur quite frequently in menopausal women in menopause, it was thought that the aging of the immune physiological systems could explain this predisposition to this type of mycobacterial infection. [47-51] On the other hand, these patients with NTM lung infections were found to have bronchiectasis much more often. [52, 53] The findings of this study are that serum C4 complement deficiency, associated with bronchiectasis in immunocompetent women in menopause, may be a major risk factor for lung NTM infections. **Cytokines,** substances with protein or glycoprotein structure, synthesized by lymphocytes and macrophages, act as mediators in the inflammatory reaction, serving as molecular messengers between participating cells, with a role in biological amplification. **Lymphokines** are soluble mediators, secreted by sensitized lymphocytes, with the role of increasing the phagocytic and enzymatic activity of phagocytic cells on the opsonized microorganism, as well as against other intracellular pathogens. **NK cells** represent 10-20% of the lymphocyte population and intervene in the innate immune defense of the host [54], providing protection against bacteria, viruses and cancer [55-57]. It also provides "natural" cytotoxicity through the secretion of cytokines (IFN-γ and TNF) and chemokines (MIP-1α and MIP-1β), a secretion that supports a pro-inflammatory environment [58]. NK cells also intervene in the adaptive immune response, regulate T cell activity and interact with dendritic cells (DC) [59]. The interaction of NK cells with DC affects the adaptive immune response and leads to the killing of M. tuberculosis [60]. The role of NK cells in the pathogenesis of lung infections with NTM was demonstrated, initially in mice, in which NK1.1 cell depletion was shown to exacerbate NTM-induced pathogenesis by reducing macrophage phagocytosis, dendritic cell proliferation, cytokine production and granuloma formation. Similar pathological phenomena have been observed in mice with IFN-γ deficiency after NTM infection. Also, recombinant IFN-γ infection in IFN-γ-deficient mice significantly improved immunity against NTM lung infection. These results indicate that stimulation of IFN-γ production by NK cells may play an important role in activating and enhancing innate and adaptive immune responses in the early stages of NTM lung infection. On the other hand, NK cells stimulate direct mycobacterial killing and IL-12 production by macrophages. **Fever**, as a physiological, general defense mechanism of the body has a beneficial role against infections, because it delays the multiplication of sensitive microorganisms at high temperatures and accelerates the metabolism of body cells, while increasing the immune response and the process of phagocytosis [61].

3.The specific immune defense mechanisms are mediated both by the lymphoid tissue cells associated with the respiratory mucosa and by the cells of the lymphoreticular system, which intervene in the general defense mechanisms (all the immune function cells that are present in the circulation, lymph nodes and spleen). From a topographical, morphological and immunological point of view, the respiratory system is divided into a cranial portion corresponding to the bronchi (where the secretory immune system predominates) and a distal portion represented by alveoli, where the alveolar macrophages play an important role in cell-mediated immunity. **Bronchus-associated lymphoid tissue (BALT)** is composed of lymphoepithelial structures, in which the lymphocytes come in direct contact with the bronchial and bronchiolar epithelium, participating in the local pulmonary immune response (secretory IgA formation) [62]. The immunoglobulins, which participate in the anti-infective defense of the lungs, come from the local and systemic immune compartments. Secretory IgAs have an antibacterial, antifungal and antiviral role, due to an agglutination reaction and a reduction in the ability of the bacteria to attach to the mucosal surface, as well as a role in neutralizing toxins [63]. The other immunoglobulins (IgM, IgG, IgD, IgE), present in lower concentrations in bronchial secretions and bronchoalveolar lavage fluid, are involved in: bacterial agglutination, complement fixation and lysis of gram-negative bacteria (IgM, present only in those with IgAs deficiency); bacterial agglutination, complement activation and germ opsonization for the alveolar macrophages (Ig G); bacterial antigen recognition and antibody synthesis (IgE); increasing bacterial clearance, by releasing vasoactive amines during the anaphylactic reaction, but also by inhibiting the penetration of antigen into the mucosa (IgE). Through the BALT system, the lung is a unique immune organ, and the immune events at this level are not necessarily

reflected in parallel changes in systemic immunity. Therefore, it is not accurate to draw conclusions about the normality or abnormality of the pulmonary immune status, only in base of the serum factors or the circulating blood cells [64].

CONCLUSION

In conclusion, all pre-existing bronchopulmonary diseases cause alterations in local immune physiology and anatomical lesions of the respiratory mucosa, which explains the predisposition of the immunocompetent persons to the lung infections with NTM.

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SEMNIFICATIA ALTERARII MECANISMELOR DE APARARE ANATOMICE SI FIZIOLOGICE RESPIRATORII IN PATOGENEZA BOLII PULMONARE DATORATE MICOBACTERIILOR NONTUBERCULOASE LA GAZDELE IMUNOCOMPETENTE

REZUMAT

In prezent, boala pulmonara datorata NTM (BP-NTM) este o problemă de sanatate publica datorita cresterii proportiei în comunitate a gazdelor umane imunocompromise, pe de o parte prin cresterea incidentei infectiei HIV-SIDA, dar mai ales prin folosirea pe scara larga a chimioterapicelor antineoplazice si a anticorpilor monoclonali (anti-TNF si anti-IFN). Ca urmare, costurile cu diagnosticarea si spitalizarea acestor pacienti vor fi din ce in ce mai mari, punandu-se o presiune financiara asupra sistemelor de sanatate publica din tarile dezvoltate, dar mai ales, din cele aflate in curs de dezvoltare. In prezent, un deziderat important al medicilor este diagnosticarea precoce si corecta a acestor infectii pulmonare, cu atat mai mult cu cat micobacteriile nontuberculoase sunt omniprezente si pot contamina orice probă de produs biologic recoltat de la bolnav.

Cuvinte cheie: aparare a sistemului respirator, NTM, gazde imunocompetente.

ASSESSMENT OF SERUM LEVELS OF THE ANTI-INFLAMMATORY CYTOKINE IL13 IN OBESE AND TYPE 2 DIABETES PATIENTS

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ABSTRACT
Aim: To study the circulating levels of IL13 in obese and diabetic patients. Methods: The study group included 69 patients from an outpatient clinic, divided in four subgroups: obese without diabetes (O) (n= 24), obese with diabetes (OD) (n= 24), non obese diabetics (NOD) (n= 9) and controls, lean individuals without diabetes (C) (n= 12). Several clinical (age, sex, weight, BMI, abdominal and hip circumference, blood pressure), laboratory (blood glucose, glycated hemoglobin, serum proteins and protein electrophoresis, lipid metabolism parameters, urea and creatinine, CRP and IL13 serum levels) were assessed in the study group and separately in each subgroup. Statistical analysis was performed using the appropriate statistic tests. Results: IL13 was significantly higher in the OD versus the C group (27.61±9.72 pg/ml vs 20.69±5.89 pg/ml, p= 0.03). There was a moderate positive correlation between IL13 and abdominal circumference (correlation coefficient r_s 0.416, p=0.0004). In multivariate regression analysis, abdominal circumference and IL4 were significant predictors of IL13 (regression model p=0.0086). No statistically significant differences in IL13 levels were found between the NOD and the control groups. Conclusions: Abdominal obesity was associated with higher levels of IL13 which was negatively correlated with C-reactive protein level. Diabetes in non obese individuals was not associated with alterations in the IL13 levels.
Keywords: serum level IL13, anti-inflammatory cytokines, obesity, diabetes

INTRODUCTION

White adipose tissue (WAT) is composed of adipocytes, as well as of a non-adipocitary stromo-vascular fraction that includes undifferentiated cells, preadipocytes, fibroblasts, inflammatory cells and various amounts of vessels and nerves; adipocytes and pro-inflammatory resident immune cells populations, primarily macrophages and lymphocytes. These cells secrete several pro-and antiinflammatory cytokines, called adipokines, accounting for the main part of the so called adipose secretoma (43). These molecules are implicated in various physiological processes: energy consumption and storage, temperature accommodation and in immunity.

In the obese subjects, the adipose tissue is not only overdeveloped, but the immune cells inside the stromo-vascular fraction also rise in number and change in phenotype. Consequently, the secretion of some adipokines is deregulated, mainly in the visceral location of adipose tissue, where apparently the infiltration of macrophages is more important (19, 39, 13); a chronic low intensity inflammation status is established. Although subclinical, this mild inflammation, defined as metabolic inflammation (25) is associated with important systemic consequences such as type 2 diabetes mellitus, (DM) metabolic syndrome, (Mets) cardiovascular diseases and certain types of cancer (35, 2, 17, 29, 15). It has also been shown that the dysfunction of the subcutaneous adipose tissue is involved in the pathogenesis of Mets (7).

The pro-inflammatory pathway in Mets is notably induced by lymphocyte T helper Th1-Th2 polarization with consequent derived cytokine synthesis. Th1

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cytokines
(IL-12, IL-2 and interferon gamma (IFN-γ)) determine the pro-inflammatory M1 phenotype of macrophages. Macrophages undergoing the alternative activation by Th2 cytokines (IL-4 and IL-13), M2a macrophages (26) trigger a different immune response (28). The Arginase-1 gene expression is the signature of the alternative activation (23, 28, 42).
These two phenotypes of macrophages have been well studied in obese mice: the majority of the macrophage population consists of the M1 cells, characterized by high levels of pro-inflammatory cytokines, such as TNF-alpha, production of INOS (inducible nitric oxide synthase) and oxygen reactive species and by a strong microbicidal and tumoricidal activity (40). In the lean mice, most of the macrophages, with the M2 phenotype, are characterized by the expression of genes encoding anti-inflammatory proteins such as arginase -1, IL-10, considered to be involved in parasite immunity, tissue remodeling and tumor progression (6, 23, 40).
The source of the IL-13 cytokines in the adipose tissue is still under debate. There are discrepancies between the *in vitro* and *in vivo* studies, as well as between the animal and human models of alternative macrophage activation.
In mice, the source of IL-13 are the eosinophils that accumulate in the adipose tissue: their number in the perigonadal adipose tissue positively correlated with the arginase 1-positive macrophages (45). The adipocytes themselves (18) could be a source of IL-13. In mice, exogenous administration of other interleukins (IL-25 and IL-33) leads to a marked rise of the IL-4 and IL-13 levels (38).
In animal models, the paradigm of Th1/Th2 cytokines triggering the M1/M2 activation seems to be the clue of the onset and progression of inflammation in the metabolic syndrome.
Few studies are available on circulating IL-13 in obese individuals (15, 32) and very few papers exist on the relationship between these cytokines and diabetes (24). In a previous study, we showed the increase of IL-4 levels in obese and obese diabetic patients and its correlation with biochemical parameters (5).

AIMS

In the current study, we aim to determine the relationship between IL-13 plasma levels and adipometric and metabolic parameters in obese and obese diabetic human subjects, and to check the correlation between IL13 and IL4.

MATERIAL AND METHODS:

Subjects. The study included 69 subjects with complete anthropometric, adipometric and biochemical examinations recruited from the patients of the outpatient clinic Binisan Medical Center, Dragasani.
Patients presenting with medical emergencies, acute and chronic infections, parasitosis, malignancies and those treated with drugs impacting the immune system were excluded. Informed consent was obtained from every participant and the study was approved by the Ethics Committee of the University of Medicine and Pharmacy from Craiova, Romania.
Subjects were divided into four subgroups:

- the O subgroup (overweight and obese), n = 24, including overweight or obese subjects (body mass index (BMI) ≥25) without diabetes. Six obese or overweight individuals previously not known as diabetics and discovered with diabetes during the study were further assigned to the OD group;
- the OD subgroup (overweight and obese diabetics), n = 24, patients with diabetes and a BMI ≥25;
- the NOD subgroup (non-obese diabetics), n = 9 lean individuals, known with diabetes and a BMI <25;
- the C subgroup (controls), n=12, with a normal body weight (BMI<25) and without diabetes.

Anthropometric and adipometric measurements. Sex, age, height, body weight, waist and hip circumference, systolic and diastolic blood pressure, body mass index were registered.
Height (cm) was measured using a stadiometer and body weight (kg) was recorded with a calibrated digital scale with subjects in underwear. Waist circumference (WC, cm) was measured at the midpoint between the last rib and the upper point of the iliac crest. Hip circumference (HC, cm) measurement was taken at the widest lateral extension of the hips. Blood pressure was measured after 5 minutes rest using a sphygmomanometer before venipuncture for blood sample collection. BMI (kg/m^2) was defined as body weight divided by the square of height.
Biochemical measurements.
Blood samples were collected after an overnight fast, early in the morning. For each subject, three vacutainers were drawn: two without anticoagulant, with separating gel, to obtain serum samples for biochemical parameters detemination and one with K3EDTA in order to measure hba1c level.
After blood clotting, for at least 30 minutes, the vacutainers were centrifuged at 1000 rpm for 10 minutes and serum was immediately aliquoted in polypropylene tubes.
After aliquoting, samples were either processed for serum biochemistry or stored at -80^{o}c for cytokine measurement.

For each patient, two vacutainers without additives, with separator gel to obtain serum and one K3 EDTA vacutainer were collected.

After aliquoting, samples were processed according to each particular analysis, and tested using the following analytical methods:

For the serum assesment of biochemical parameters, the methods used were:

GOD PAP method for glucose, the CHOD PAP method for cholesterol, the PAP GPO method for triglycerides, direct methods for HDL, the Biuret method without blank sample for total protein, the Jaffe method without compensation for creatinine, the Urea method -UV for urea, glycated hemoglobin (hba1c) by direct method, C-reactive protein by Fixed time method; serum proteins electrophoresis by the method of migration in agarose gel in the electric field; the Jaffe method without compensation for creatinine was processed. LDL-cholesterol was calculated by the Friedewald formula. Glycated hemoglobin (hba1c) by direct method, using a Biotechnica kit, lot 512503.

High-sensitivity CRP values were determined turbidimetrically using fixed-time measurement with sample blank correction using a CRP FUTURA Kit, lot 0299-2299 02102-22102 EXP 11.2016.

All Biochemical measurements were performed with Biotechnica kits, using an automated analyzer BT3500.

The method used for IL13 concentration mesurement was ELISA (Sandwich Enzyme-Linked Immunosorbent Assay), Biolegend, kit lot B194484 dedicated to the Personal LAB CLIA Fully Automated 2 Microplate Analyser CLIA\ELISA (Adaltis).

Statistical analysis

Clinical and the above mentioned biochemical parameters were registered and analysed for the entire study group and separately for each of the four subgroups.

Numeric variables were expressed as averages ± standard deviations for the normally distributed ones and as median (25-75 percentiles) for the non-normally distributed ones. Qualitative variables were expressed as percentage. Numeric variables were compared using the t test for normal distribution and the Mann Whitney test for non-parametric variables. Qualitative variables were compared using the Chi-squared test.

Correlation between parameters was studied using the Pearson correlation coefficient for parametric variables and the Spearman correlation coefficient for the non-parametric variables. Univariate linear regression and multivariate regression were used to investigate the relationship between IL-13 and the clinical and biochemical parameters.

The statistical significance threshold chosen was $p<0.05$. The statistical analysis was performed using the Microsoft Excell software and the medcalc v.16.1 software.

Results:

The results are depicted in table 1

Table 1. Study parameters in the O, OD, NOD and M groups and statistical significance p betwen study subgroups and the control group.

Parameters	Group O N=24	Group OD N=24	Group NOD N=9	Group C N=12	p1	p2	p3
CLINICAL							
Age(years)	57.41±11.94	63 (58-66.5)	61.44±9.98	28.6±5.5	**0.011**	**<0.0001**	**<0.0001**
Sex(M/total)	13/24	13/24	4/9	10/12	0.18	0.18	0.16
Weight(kg)	93.5 (79.5-104.5)	87.45±11.73	62(59.75-71)	69.33±7.26	**0.0001**	**<0.0001**	0.28
Height(cm)	170 (164.5-177.5)	167.2±9.05	169(166.25-175)	175.91±5.93	0.064	**0.0048**	0.26
BMI(kg/m^2)	29.54 (27.3-34.36)	31.24±3.07	22.42±1.47	22.36±1.53	**<0.0001**	**<0.0001**	0.93
Abdominal circumference(cm)	108.08±15.02	112.33±8.58	88.11±8.4	82.83±7.01	**<0.0001**	**<0.0001**	0.13

Parameters	Group O N=24	Group OD N=24	Group NOD N=9	Group C N=12	p1	p2	p3
Hip circumference(cm)	115±9	115.5(111-122)	99±6.06	100.91±5.05	**<0.0001**	**<0.0001**	0.43
Systolic blood pressure(mmHg)	132.2±17.21	141.41±17.15	137.44±22.22	117.83±11.2	**0.013**	**0.0001**	**0.015**
Diastolic blood pressure(mmHg)	78.5 (70-86.5)	79.3±8.78	80 (80-92.5)	68.83±10.32	0.4	**0.0032**	**0.003**
PARACLINICAL							
Total Protein (g/dl)	7.54 (7.14-7.75)	7.69±0.56	7.74±0.41	7.87±0.43	**0.018**	0.35	0.51
Albumin%	64.9 (63.05-67)	64.98±3.63	63.78±3.99	67.83±4.08	**0.04**	**0.042**	**0.04**
Alpha1%	2.2±0.31	2.43±0.46	2.67±0.59	2.17±0.63	0.12	0.18	0.09
Alpha2%	9.72±1.1	9.95±1.2	10.73±1.19	8.78±1.49	**0.04**	**0.017**	**0.0064**
Beta1%	7.45±0.83	7.85±1.14	7.91±1.07	6.5±0.62	**0.0015**	**0.0007**	**0.0015**
Beta2%	3.5 (3.25-3.8)	3.71±0.89	3.48±0.59	3.15±0.81	0.16	0.08	0.34
Gamma%	11.45 (10.4-13.75	11.05±2.35	11.4±2.35	11.54±2.53	0.82	0.57	0.9
Albumin (g/dl)	4.87 (4.64-4.97)	4.95±0.42	5.21±0.79	5.33±0.41	**0.002**	0.1	**0.07**
Alpha1 (g/dl)	0.18 (0.16-0.21)	0.18±0.03	0.21 (0.19-1.26)	0.17±0.04	0.48	0.4	0.058
Alpha2 (g/dl)	0.73±0.08	0.76±0.1	0.82±0.12	0.68±0.1	0.17	**0.035**	**0.013**
Beta1 (g/dl)	0.56±0.07	0.61±0.08	0.59±0.08	0.51±0.06	0.07	**0.0025**	**0.019**
Beta2 (g/dl)	0.27±0.05	0.28±0.07	0.26±0.04	0.25±0.07	0.33	0.23	0.57
Gamma (g/dl)	0.87 (0.79-1.04)	0.84±0.21	0.88±0.18	0.94±0.25	0.81	0.24	0.56
Serum Glucose (mg/dl)	95 (86.5-108)	150.95±40.29	155.77±63.52	89.41±6.05	0.057	**<0.0001**	**0.0018**
HbA1C%	5.78±0.54	7.2(6.65-8)	6.67±1.24	5 (4.5-5.1)	**0.0007**	**<0.0001**	**0.0008**
Total Lipids (mg/dl)	656.09±124.31	678.049±132.72	718.25±145.92	564.42±127.4	0.05	**0.019**	**0.018**
LDL (mg/dl)	136.83±46.27	128.02±49.33	141.4±36.53	115.62±43.83	0.19	0.46	0.16
HDL (mg/dl)	44.92 (38.12-51.57)	43.17 (39.43-48.13)	40.99±6.32	45.25±14.27	0.86	0.81	0.41

Parameters	Group O N=24	Group OD N=24	Group NOD N=9	Group C N=12	p1	p2	p3
VLDL (mg/dl)	18.62 (15.34-23.1)	29.45±9.78	30.04±16.17	15.82±6.29	0.16	**0.0001**	**0.013**
Total Cholesterol (mg/dl)	203.64±50.1	202.46±51.55	212.44±40.59	176.79±46.24	0.12	0.15	0.08
Triglycerides (mg/dl)	87.92 (73.95-113.96)	147.29±48.94	150.24±83.89	79.13±31.44	0.22	**0.0001**	**0.013**
IL4 (pg/ml)	0.49 (0.1-2.4)	4.72±3.8	0.51 (0.1-1.44)	0.3±0.3	0.075	**0.0003**	0.28
IL13 (pg/ml)	24.18±6.33	27.61±9.72	22.63±6.09	20.69±5.89	0.12	**0.03**	0.47
CRP (mg/dl)	2.1 (0.83-2.82)	2.75 (1.39-4.68)	0.19 (0.1-3.39)	1.68±1.85	0.26	**0.0506**	0.93
Urea (mg/dl)	34.13 (30.32-40.63)	36.38±11.56	34.6 (27.26-48.27)	30.81±7.57	0.056	0.14	0.35
Creatinine (mg/dl)	0.86±0.15	0.88±0.21	0.75 (0.69-0.76)	0.96±0.12	0.65	0.28	0.075
Creatinine clearance (ml/min)	118.62±44.13	102.81±19.82	87.27±12.08	111.4±11.14	0.26	0.96	0.1

Legend –Table 1.
Study parameters were expressed as mean ± standard deviation for normal distribution and as median (25-75 percentile) for abnormal distribution.
p1 refers to the comparisons between groups O and M, p2 refers to the comparisons between groups OD and M, p3 refers to the comparisons between groups NOD and M. Statistical tests used were t-test for normally distributed variables and the Mann Whitney test for abnormal distribution. Comparison of proportions was performed using the chi-squared test.
The assesment of IL4 values was communicated in a previous work (5) but we considered the values for comparison (Tables 2, 3).

Table 2. Corelation of IL13 with study group parameters

Parameter	Correlation coefficient (r_S)	P
Age	0.279	0.0204
Abdominal circumference	0.416	0.0004
Hip circumference	0.311	0.0092
Weight	0.206	0.0894
IL4	0.401	0.0006

There was a moderate positive correlation between IL13 and IL4 and abdominal circumference and a weak correlation of IL13 with age and weight.

Table 3. Simple regression with IL13 as a dependent variable

Parameter	etermination coefficient	P
Age	0.08	0.02
Abdominal circumference	0.17	0.0004
Hip circumference	0.1	0.0092
Weight	0.04	0.0894
IL4	0.16	0.0006

In multivariate regression analysis, abdominal circumference and IL4 remained significant predictors of IL13 (p=0.0086).The patients in the control group were significantly younger than those in the other groups.
As expected, in the O and OD groups, there were higher BMI, abdominal circumference and hip circumference . The abdominal circumference was significantly higher in the OD group versus the O group (p=0.0047), thus the OD patients were more obese than the ones in the O group. There was no significant difference between these parameters in the O versus the OD group. Systolic blood pressure was higher in all the study groups than in the control group. Diastolic blood pressure was higher in diabetic patients, in obese as well as in non obese patients.
Differences were found in the biochemical parameters as follows: the obese patients (O group) had a poorer nutritional status, with lower total protein and lower albumin.
There were signs of inflammation in the obese group, as well as in the obese and non-obese diabetics, with higher Alpha 2 and Beta 1 globulins.
As expected, the blood sugar was higher in the diabetic groups, but there was a tendency of higher blood sugar in the obese group without diabetes than in the control group. Hb A1c was significantly higher in the diabetic groups, but also, significantly higher in the obese compared to the control group.
As for the lipid profile, significantly higher VLDL and triglyceride levels were found in both OD and NOD groups versus the control group.
CRP was modestly elevated (tendency) in the OD group.
The inflammatory molecules IL4 and IL13 were significantly higher in the OD versus the C group.
Significant correlations with IL13 were found for: age, abdominal and hip circumference, weight and IL4 (Table 2).
In univariate regression analysis, significant association was found for the same parameters. In multivariate regression analysis, with IL13 the dependent variable, significant predictors of IL 13 remained abdominal circumference and IL4 (regression model p=0.0086).

DISCUSSION:

The macrophage of the adipose tissue could be considered one of the most important actors, film director and screenwriter of metaiflammation in obesity, acting by the secretion of different type of chemokines.
In the present study we performed an IL-13 serum level assessment for 69 subjects divided in four groups as we previously described, and we compared the values found with some anthropomorphic, adipometric and biochemical measurements. A statistical correlation was also made, also taking into account the results regarding the IL-4 serum values published in a previous work in same groups of patients (5).
The WAT depots in obesity suffer an important plasticity and remodelling in term of adipocyte population and the innate and secondary immune cells phenotype. A lot of research emphasize the increased number of macrophages in the stromovascular fraction of obese subjects the change of their location in adipose lobules among the adipocytes and, more important, the dramatically change of their phenotype and their effect on adipogenesis and glucose uptake (13, 7, 23, 6, 10, 16, 44).
IL4 and IL13 are glucocorticoid hormones acting as immunomodulatory cytokines, produced mainly in lean subjects by adipocytes and activated Th2 cells and are inducers for M2a polarization of macrophages (26). Studies in animal models and cell cultures proved that breaking the M2 activation leads to obesity and insulin resistance (22, 31). IL4 and IL13 are also involved in maintaining T regulator cells and eosinophils number (26, 28). The gene encoding IL13 is part of a cluster of genes on chromosome 5q, particularly close to IL4 (14).
The simultaneous variation of Il-13 and IL-4 as we also have demonstrated is probably linked to the vicinity of the genes encoding the two cytokines on the 5q chromosome, probably responding to similar activation pathways (4).
The sequence of events in the activation of immune cells in the adipose tissue under hypercaloric intake seems to be important for understanding the polarization of macrophages and their response to inflammation. The Th1 cytokines activity, leading to the M1 macrophages activation by the so-called classical way, responsible for the metainflammation in mets, is very well documented. Makki et al (25) showed that the alterations in macrophage content and polarisation state (M2-M1) occur fairy late in the progression of obesity and they are not the initiating events of inflammation. The adipose tissue of lean subjects secretes anti-inflammatory cytokines: TGFβ, adiponectine and IL10, IL4 and IL13 (25). Macrophages are the late effectors of a coordinated inflammatory response, where the initial occurrences are the accumulation of proinflammatory T cells, (CD8+ and Th1CD4+), depletion of regulatory T cells, accumulation of B cells, NK cells, eosinophils, neutrophils, and mast cells. The resulting M1 macrophages produce proinflammatory cytokines such as IFN-γ and TNF–α (37). Consecutively, the low-grade inflammation installed in the obese adipose tissue is followed by Mets, insulin resistance, arterial hypertension and asthma (37). The Th1 cells also play a critical role in the initiation, progression and rupture of atherosclerotic plaque (3).
The role of the Th2 antiinflammatory cytokines IL4 and IL13 is poorly understood and controversial and the

interest in the IL13 involvement in human obesity is recent. The alternative activation of M2 macrophages by IL4 and IL13 is involved in the type II inflammation processes, like allergy and killing and encapsulation of parasites (26). In mice, the M2 macrophage differentiation seems to be triggered by the hypercaloric diet and the initial proinflammatory state in early obesity aims to improve the insulin sensitivity (46).

Il 13 down-regulates the activity of the macrophages, inhibiting the production of inflammatory cytokines (23, 25). Higher serum concentrations of IL13 have been associated with reduced weight gain in rodents that were fed a high fat diet and they limited diet induced inflammation and insulin resistance (11, 21).

In humans, the association between IL13 on one hand and obesity and DM on the other hand is poorly understood. The present paper is one of the few studies to investigate the relationship between type 2 diabetes and anti-inflammatory cytokines. Our results confirm the findings of previous studies (3, 9, 42). Our findings add data to the body of evidence of increased Th2 cytokine serum levels in obese individuals. This could be expected, considering the cytokine secreting cells accumulation and adipose tissue expansion in obese. The patients in the OD group had higher IL13 serum levels, positively. As displayed in table 1, the obesity was more severe in the OD group than in the other study groups.

Schmidt and coworkers (37) showed that both pro-and anti-inflammatory cytokines are elevated in obese subjects and pointed out the positive correlation between physical activity and cytokine levels. Surendar et al demonstrated that both Th1 and Th 2 cytokines are upregulated in Mets (42).

Similar results (1) reported an increase in serum levels of Il-4 and Il-13 in diabetic subjects. They noted that the diabetic nephropathy is characterised by a decline of Il-33, the master regulatory of Th-2 differentiation, meanwhile the subjects with uncomplicated DM maintain elevated levels of Il-13. The authors suggest that Th2 cytokines may have a protective effect against the complications of diabetes mellitus.

The high levels of antiinflammatory cytokines in the obese individuals are probably adaptive, reducing the proinflammatory effect of several other cytokines originating from the resident immune cells in the adipose tissue.

Effects such as insulin resistance reduction in obesity have already been demonstrated (21). Whether different levels of IL-13 and IL-4 in obese humans would be able to stratify or influence the insulin resistance and the diabetes risk in these patients remains to be established. There are animal studies showing that IL4 promotes glucose tolerance, inhibits adipogenesis and promotes lipolysis in mature adipocytes (21).

In vitro studies have shown cytoprotective effects of antiinflammatory cytokines I13 and IL4 on the pancreatic beta cells (27, 36, 41).

In multivariate regression analysis, diabetes control (HbA1c) was not a significant predictor of IL-13 levels. Moreover, IL-13 and IL-4 levels were not different between the non obese diabetic group and controls, raising the hypothesis that higher IL-13 and IL-4 levels in obese diabetic patients are probably linked to obesity and not diabetes.

The fact that our study failed to show a link between diabetes control and IL13 levels may be due to the fact that diabetes was under treatment. The interleukin levels have recently been demonstrated to be reduced in patients treated with sulfonylureas, in contrast with patients on insulin, who exhibit higher inflammatory activity (20). For the present study, data on treatment was not collected.

Several clinical effects of Il l3 have been studied. It was shown to increase asthma activity and an IL13 inhibitor has been proposed in the treatment of uncontrolled asthma (34).

An important clinical and biochemical observation is the existence of metabolically healthy obese subjects, who do not suffer from the complications of obesity, displaying instead high serum levels of anti-inflammatory Th2 cytokines. They potentially represent a variant obese phenotype (12), but one must consider the age of these subjects, their genetic background and the physical activity.

An elegant experiment of Nguyen (30) demonstrated that in mice exposed to cold, the secretion of IL-4 stimulates the alternatively activated macrophages M2 in adaptative thermogenesis, both in white and brown adipose tissue. As a result, there is an increase in uncoupling respiration and energy expenditure by noradrenaline (8). Animal models have shown that adaptative thermogenesis acts as a protective mechanism against high fat diet induced obesity. The alternatively activated M2 macrophages secreting catecholamines could be a very important factor in the browning process of a subset of cells located in WAT depots, that could be switched into brown-like adipocytes, named “beige” or “bright” (44).

In agreement with the supposition that IL13 has a protective effect in obesity, our study showed a very weak correlation between the acute phase-protein CRP, IL-4 and IL-13 in the obese and obese diabetic group. CRP is one of the most important pro-inflammatory markers in atherosclerosis and type 2 DM, synthesized in response to proinflammatory cytokines such as TNF α and IL-1 (33).

Our results corroborated with the few data available

from similar research in humans suggest the protective anti-inflammatory role of IL13 cytokine in the obese diabetic and their involvement in preserving the metabolic status of these patients.

CONCLUSIONS:

Obesity is associated with higher levels of antiinflammatory cytokine IL-13. IL-4 and IL- 13 display similar values in obesity and diabetes. Diabetes was not associated in our study with alterations in IL-13 serum levels.

Further studies are necessary to understand the implication of Th2 cells in all the secondary diseases of human obesity and to link the effect to the cause, checking the phenotype of macrophages in adipose depots in both general and central obesity.

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EVALUAREA NIVELULUI SERIC AL CITOKINEI ANTI-INFLAMATOARE IL13 LA PACIENȚI OBEZI ȘI CU DIABET ZAHARAT DE TIP 2

REZUMAT

Scop: Studiul nivelului circulant al IL13 la pacienții obezi și diabetici. Metode: În studiu au fost incluși 69 pacienți împărțiți în patru subloturi: obezi non diabetici (O) (n= 24), obezi diabetici (OD) (n= 24), non obezi diabetici (NOD) (n= 9) și lot de control cu subiecți normoponderali fără diabet (C) (n= 12). Pentru toți pacienții incluși în studiu s-au înregistrat date precum vârstă, sex, greutate, IMC, circumferință abdominală și a coapselor, tensiune arterială și s-a determinat nivelul glucozei serice, hemoglobinei glicate, proteinelor totale și electroforeza proteinelor, parametrii profilului lipidic, ureea, creatinina, proteina C-reactivă și, IL13. Datele au fost analizate statistic pentru toți pacienții incluși în studiu și separat pentru fiecare sublot. Rezultate: Nivelul IL13 a fost semnificativ crescut la pacienții obezi față de lotul de control (27.61±9.72 pg/ml vs 20.69±5.89 pg/ml, p= 0.0327). S-a observat o corelație pozitivă moderată între nivelul IL13 și circumferința abdominală (coeficient de corelație r_s 0.416, p=0.0004). Analiza de regresie multivariată a arătat că circumferința abdominală și nivelul IL4 au fost predictori semnificativi pentru nivelul IL13 (model de regresie p=0.0086). Nu s-au observat diferențe statistice semnificative între nivelurile IL13 la subiecții din loturile NOD și C. Concluzii: Obezitatea abdominală a fost asociată cu nivel crescut al IL13 care s-a corelat negativ cu cel al proteinei C reactive. La subiecții non obezi, diabetul nu s-a asociat cu modificări ale nivelului seric al IL13.

Cuvinte cheie: nivelule seric al IL13, cytokine antiinflamatoare, obezitate, diabet

AN OVERVIEW ON THE USE OF PROBE-BASED CONFOCAL LASER ENDOMICROSCOPY IN MODERN MEDICAL PRACTICE

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ABSTRACT:
Confocal laser endomicroscopy is a rather novel technique used to investigate the inner mucosa during endoscopic or laparoscopic interventions. The principle was discovered several decades ago; however, only recent technological advances allowed for a sufficient miniaturization of the components, necessary to transpose the idea to reality. Probe-based confocal laser endomicroscopy was thus established as a way to obtain optical biopsies during standard endoscopic exploratory procedures. We aimed to highlight the importance of the procedure in obtaining a rapid, valid diagnosis, with sufficient accuracy, rapidity and cost savings to justify the necessary initial investment and additional expertise required for the task.
Keywords: confocal laser endomicroscopy, optical biopsy, digestive tract, respiratory system.

INTRODUCTION:

Confocal laser endoscopy is a new technique that enable a histological examination (optical biopsy) to be performed during any endoscopic procedure. This helps to improve the diagnosis and define the treatment needed for multiple digestive diseases [1].
The mode of operation of confocal endomicroscopy was first described in 1957. A fiber optic probe advances through the channel of a bronchoscope, is directed to an area of interest that illuminates with laser light (488 nm frequently used) and light reflected is redirected in the opposite direction through an orifice [2-4].
The procedure is deemed as "confocal" since the lightning source and detection system are comprised in one common focal plane [5], allowing for a focalized view of one specific region from which classical biopsies can be taken [6]. This procedure allows for rapid and precise identification of the areas of interest, facilitating prior so-called "optical biopsies" of the tissue.
After fluorescein is administered, it can be performed in vivo even during the current endoscopic technique. The use of contrasting agents – either fluorescein or other fluorescent dyes- facilitates imaging of cellular structures, including nuclear and cytoplasmic structures, thus revealing the mucosal patterns. Barret's esophagus or celiac disease and inflammatory diseases specific to the gastrointestinal tract are one of the main applications that were initially tackled through this procedure. Classical biopsies were thus facilitated and pCLE showed that in vivo imaging can be achieved at cellular level, hence broadening the range of applications to neoplastic diseases (especially malignant) of the entire digestive tract – esophagus, stomach or colon – showing good accuracy and sufficient specificity [7-15]

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AIM

The main objective of this short review is to show the importance of using laser confocal endomicroscopy in the identification of lesions in the digestive tract but also the ability of this technique in detecting malignant lung lesions, because it has the potential to fundamentally change the role of biopsy in gastrointestinal and especially lung endoscopy in the near future.

OVERVIEW OF THE ADVANTAGES OF PCLE

Even though is considered a "gold standard" diagnostic test, biopsy can be expensive in certain settings and can generate sufficient delay between the moment when endoscopy is performed and the time of diagnosis, as the transportation, preparation and interpreting of the resulting tissue sample can be prolonged by a number of factors. Even if the interventional risk is lower than other procedures and a large number of biopsies are regularly performed worldwide, it is not a procedure that can be safely recommended in some cases, due to increased risk of bleeding or the anatomical localization of the region [16]. Other restricting aspects can be related to the need to perform it repeatedly, the incertitude of the targeted area or the limited anatomical space.

Compared to these shortcomings of classical biopsies, pCLE offers various advantages:

-decreases the risk of unwanted side effects by performing a non-invasive microscopic analysis of the lesion;

-functional evaluation of the structure (eg dynamic alveoli, real-time elastin fiber, etc.);

-quick diagnosis, by the presence of an anatomopathologist in the bronchoscopy room;

-possibility to enter more difficult areas.

THE IMPORTANCE OF PCLE IN DIGESTIVE PATHOLOGY

It can be used in all parts of the gastrointestinal tract and is often helpful in identifying certain diseases such as: Barret's esophagus, esophageal cancer, pancreatic cystic neoplasms and biliary strictures, ulcerative colitis, celiac disease, polyps, colorectal cancer assessment, inflammatory bowel disease, detection in in vivo of Helicobacter pylori infection [17]. Comparing the results of the two procedures (pCLE and histopathological examination) in the lesions of the gastrointestinal tract, they demonstrated an accuracy of about 86% to 96% [18].

The gold standard for the diagnosis of any disease remains the histopathological examination. However, the percentage of misdiagnosis can be significant due to factors: misinterpretation, inaccuracy of the place of the biopsy or the number of biopsies performed.

The accuracy of this technique in gastric diseases was 94% to 96% for malignant diagnosis, compared to histological biopsies and 88% for premalignant diseases (such as intestinal metaplasia) [19].

In the colon, CLE had an accuracy of 82% when trying to predict the type of histology in the case of polyps when examining *in-vivo*; when used in conjunction with digital chromoendoscopy with narrowband imaging during surgery, its accuracy increased to 94% [20].

In inflammatory bowel disease (IBD), several studies have examined the role of CLE in patient surveillance, analyzing the degree of disease, biopsies, early detection of dysplasia, evaluation of mucosal healing and helping to define treatment stages [21, 22].

New applications have also been studied in the biliary tract for the diagnosis of different subtypes of pancreatic cysts, which have an average accuracy of approximately 85% for neoplastic and non-neoplastic lesions [23, 24]

THE USE OF PCLE IN RESPIRATORY PATHOLOGY

Lung cancer is undeniably the most common malignancy in the world. Globally, lung cancer cases and deaths are increasing. In 2018, GLOBOCAN estimated 2.09 million new cases and 1.76 million deaths, becoming the most common cause of cancer deaths in men and women combined. It is a disease whose diagnosis can be difficult and often at the time of diagnosis is already in a metastatic stage. If it is detected at an early stage, before it is spread, there is a greater chance that it will be treated successfully.

Although screening technology is constantly advancing, research still shows that the death rate from lung cancer has not improved. Most lung cancers are diagnosed in an advanced stage [25]. Its detection is not nowadays a routine practice. The disease can sometimes be detected in the early stages by various tests and analyzes that are performed for completely different reasons.

The most common methods used to detect lung cancer include: lung radiography, chest CT scan, bronchoscopy, sputum cytology.

The diagnosis of lung cancer must include histological confirmation, the degree of tumor spread (staging) but also the analysis of the patient's functional status to decide treatment options [26].

Probe-based laser confocal endomicroscopy is a fairly new technique respiratory medicine. It is an imaging technique that can be used during bronchoscopy and thoracoscopy [27, 28]. The airways, pleura, lung tumors and lymph nodes can be viewed in real time

with a resolution of up to 3.5 μm and a maximum depth of 70 μm, with a field of view of up to 600 nm maximum. Through flexible bronchoscopy, it is possible to visualize in real time, in vivo, the elastin microstructure existing in the walls of the airways, but also of the alveoli. The procedure is performed under moderate sedation. This technique has the advantage of providing high quality images, without major risks, and without significant morbidity. It also allows noninvasive diagnosis. Also, with the help of this technique it is possible to monitor the response to treatment and possibly even the progression of the disease.

However, pCLE has not yet reached its full potential and development is still needed. Although confocal endomicroscopy is about to enter the usual practice in gastroenterology, in pulmonary medicine, it is still limited, there are only studies on small groups of patients [29].

Various studies have proved that pCLE can identify abnormal areas in endobronchial [29-34] and parenchymal malignancies (for both primary and metastatic neoplasm) [35-37]. Filner et al. [34] demonstrated the ability of pCLE to differentiate between normal lesions compared to neoplastic lesions and vice versa in endobronchial lesions.

In their study [38], performed on 32 patients suspected of lung cancer, Fuchs et al compared data from 75,522 images accumulated by pCLE with pathological data obtained from biopsy samples. In this way, they were able to identify different patterns, specific to neoplasms: chaotic and non-overlapping distribution of cells, changes in nucleus - heterogeneous size and fluctuating distances between them. Acriflavin, on the other hand, was not homogeneously absorbed by the neoplastic tissue, leaving areas without absorption. According to these data, the study supports high accuracy for pCLE (91%), neoplastic changes being detected with a sensitivity of 96% and a specificity of 87.1% - because it allowed the differentiation of cellular and subcellular elements and underground imaging. Thus, a clear differentiation was obtained between inflammatory infiltrates, normal mucosa and malignant elements.

Additional and inspiring attempts were made by applying CLE to separate normal mucosa from cancerous tissue, both *in vivo* and *ex vivo* [39, 40], but also alveolar lung microlithiasis [41] and radiation-induced pulmonary fibrosis [42]. CLE has been shown to be useful for accurately highlighting the peripheral lung nodule [43], but also the solitary lung nodules highlighted in all segments of both lungs [44]. Guisier et al. demonstrated the successful application of CLE for apoptosis to tumor xenografts in mice [45].

In her study, Anastasia Sorokina et al [46] presented the collection of 18 lobectomy specimens from 18 different patients. Images of lung cancer samples and microscopy samples prepared from the same pCLE samples were compared. It has been shown that pCLE can detect lung carcinoma in tissues ex vivo from neoplasia areas of lung tissue samples

Sabine Zirlik et al [47] analyzed CLE as a new diagnostic route for differentiating malignant and non-malignant pleural effusions. Thoracentesis was performed in 100 patients with pleural effusions. Cresyl violet and acriflavine were used as contrast agents. CLE was able to identify malignant cells in pleural effusions. Sensitivity was 87% (p: 87%) and 81% (p: 72%) for acriflavin and cresyl violet respectively. In terms of specificity, they obtained an average value of 99% and 92%.

In a study of 91 patients identified with a peripheral lung nodule [48], it was found that although in a fairly high percentage (92%) the pCLE could identify areas of abnormal tissue, it could not discern malign from benign areas. Several authors believe that this occurred because fluorescein does not penetrate so well into the epithelial layer of the bronchi [49,50] and thus it is difficult to stain epithelial cells in the bronchial mucosa [48, 51-54].

Bonhomme et al [55] presented three cases with pulmonary pleural pathology using pCLE (three images of the pleura were presented: normal pleura, non-cellular lung cancer with pleural metastasis and a mesothelioma). pCLE showed a clear differentiation of normal from malignant pleura in these cases.

SAFETY AND COSTS

When taking into account the necessary procedural steps, complications during CLE can happen either when the intravenous fluorescein is administered before performing CLE and during the evaluation of mini-samples from the selected tissue. CLE requires the use of a fluorophore, a dye that is fluorescent when stimulated by the laser beam, allowing the contrast to intensify [56]. However, initial *in vivo* studies that followed pCLE concluded that it was safe and deemed it to be well tolerated by the awake patient, with local anesthesia only and without the requirement of supplemental oxygen administration. In addition, contrary to the significant bleeding episodes that accompanied acinar imaging, pCLE did not cause significant discomfort or adverse effects [57].

pCLE has been proven to have higher performance elements in assessing injuries, possibly reducing the need for repeated procedures and lowering costs.

FUTURE PERSPECTIVES

New studies focusing on resolving relative barriers to the use of CLE are currently needed. The results that have been obtained in recent years demonstrate the

use of CLE in clinical practice, and a first step in this regard could be the treatment of patients with uncertain diagnoses. In addition to improving and resolving many unclear diagnoses, therapeutic decisions and / or follow-up procedures in this type of patient may also be improved [58].

CONCLUSION

Confocal laser endomicroscopy is a procedure that has demonstrated its ability to accurately diagnose and therefore may be helpful in daily practice to increase the ability to diagnose and manage the treatment of patients.

The somewhat prohibitive initial costs to implement the system in some settings still prevent the widespread use of the procedure, along with the fact that it is not implemented in normal countrywide strategies of many states; however, recent regulatory advancements gave way to its introduction into some developed countries. Total or partial reimbursement and its clear indication in several guidelines, along with effective training programs for both performing the procedure and for image interpretation, further pushed the procedure worldwide.

All limitations are thus relative, with easy to implement solutions that can show its use can largely overcome the initial difficulties and thus warrant its widespread implementation.

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Conflicts of interest

The authors declare no conflicts of interests

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O PREZENTARE GENERALĂ A UTILIZĂRII ENDOMICROSCOPIEI CONFOCALE LASER BAZATE PE SONDĂ ÎN PRACTICA MEDICALĂ MODERNĂ

REZUMAT

Endomicroscopia confocală laser este o tehnică relativ nouă utilizată pentru a investiga mucoasa în timpul intervențiilor endoscopice sau laparoscopice. Principiul a fost descoperit în urmă cu câteva decenii; cu toate acestea, doar progresele tehnologice recente au permis o miniaturizare suficientă a componentelor, necesare transpunerii ideii în realitate. Endomicroscopia confocală laser pe bază de miniprobe a fost astfel stabilită ca o modalitate de a obține biopsii optice în timpul procedurilor exploratorii endoscopice standard. Am urmărit să evidențiem importanța procedurii în obținerea unui diagnostic rapid și valid, cu precizie suficientă, rapiditate și economii de costuri pentru a justifica investiția inițială și experiența suplimentară necesară.

Cuvinte cheie: endomicroscopia confocală laser, biopsie optică, tract digestiv, sistem respirator.

CLINICAL APPLICATIONS OF BASOPHIL ACTIVATION TEST IN ALLERGY DIAGNOSIS

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ABSTRACT
Currently, IgE-mediated allergy diagnosis is based mainly on skin prick tests, but these tests are not recommended for patients that had previously experienced a severe allergic reaction to an allergen source. Serum specific IgE quantification has also its flaws. Therefore, i*n vitro* cellular assays, such as the basophil activation test (BAT) are a good diagnosis alternative. Basophil activation test measures the IgE-mediated hypersensitivity to allergens without exposing the patients to a risk of anaphylaxis. BAT is a functional assay that measures the expression of activation markers such as CD63 and CD203c on the surface of basophils. It is widely used in allergy research but recent clinical validations and standardisations made this test available also for routine testing. In this study, we describe the mechanisms behind the basophil activation test and its clinical application in food, respiratory, venom and drug allergy diagnosis.
Keywords: Allergy, Basophil activation test, Allergy diagnosis, CD63, CD203c

INTRODUCTION

IgE-mediated hypersensitivity reactions are usually diagnosed after a careful clinical anamnesis and confirmed by skin prick testing, measurements of serum specific IgE and, when necessary, provocation tests is. Skin testing performed with commercially available extracts is not always possible (e.g. anti-histamines intake) or can be contraindicated (history of life-threatening anaphylaxis). Furthermore, challenge tests cannot be recommended in daily practice due to ethical considerations, practical reasons and variations in sensitivity and specificity [1]. Therefore, clinicians confirm their clinical suspicions after specific IgE quantification. Unfortunately, these assays do not have absolute diagnostic reliability, especially when it comes to pan-allergens with high potential of cross-reactivity. Also, there might be clear discrepancies between sensitization, revealed by serum IgE and the clinical manifestation of that sensitization [1]. Basophil activation test is a good *in vitro* alternative, mimicking an allergic reaction in a test tube. It determines the ability of an allergen to induce basophil activation, measuring the expression of specific markers using flow cytometry. BAT has the advantage of being a functional test, positive results occur only after successful IgE cross-linking, not by monovalent binding as in IgE assays [2]. In this review, we describe the principle of basophil activation test and its clinical applications in diagnosing food, respiratory, venom and drug allergy.

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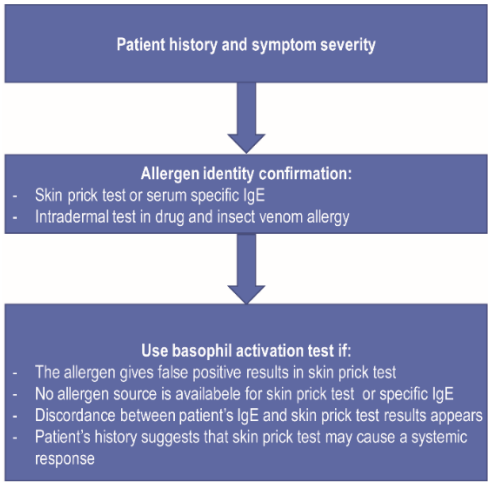

Fig. 1. Diagnostic algorithm for allergic disease (adapted after Hoffmann *et al*, 2015 [3])

BASOPHIL ACTIVATION TEST PRINCIPLES

The main characteristic of allergic effector cells in immediate-type allergy is allergen-specific IgE bound to the high affinity IgE receptor, i.e., FcεRI, from the cell surface. Binding of allergens to surface IgE results in FcεRI cross-linking and elicits the acute phase of the allergic response that causes the sudden release of vasoactive mediators into the tissue and/or circulation. This process is called anaphylactic degranulation (and may induce life-threatening anaphylaxis)[3]. In contrast, in piecemeal degranulation, cells secrete granule contents without exocytosis [4]. Both mast cells and basophils share these key characteristics. Mast cells reside in the tissue and are considered primary allergic effector cells [5]. Basophils are peripheral blood granulocytes, with secretory granules containing histamine and are capable of secreting proteases, cytokines, chemokines, and lipid mediators. Basophils present on their surface unique markers depending upon whether the cells undergo anaphylactic or piecemeal degranulation, which can then be measured by flow cytometry [6]. Basophil activation is a process that starts with intracellular changes (signal transduction from FcεRI by means of phosphorylation of p38MAPK and calcium influx, followed by changes on the cell membrane level (CD203c) with final degranulation of the basophils (CD63) [1](Figure 2).

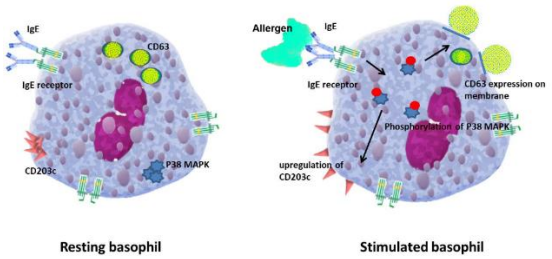

Fig. 2. Comparison between a resting and a stimulated basophil. Cross-linking of membrane-bound IgE from basophil induces mediators secretion and up-regulates the expression of specific activation markers such as CD63 and CD203c (modified after Hausmann *et a*l, 2009 [7]).

Basophil activation test measures an *in vitro* activation of patients' basophils in the test tube. After blood sample preparations (whole-blood assay or leukocyte isolation) and preincubation with interleukin IL-3 (depending on the allergen of interest) or prewarming of blood samples and reagents to 37^0C, the allergen is added, and basophils are incubated for 15 minutes to 1 hour at 37^0C in the water bath (depending on the allergen of interest) (**Stimulation**). Crude allergen extract, purified or recombinant allergens can be used as stimulants. After stimulation, the samples are stained with monoclonal antibodies coupled to fluorochromes (**Staining**). Another step is the lysis of contaminating erythrocytes (**Red blood cell lysis**), followed by **data acquisition** (using a flow cytometer) and **data analysis** (dedicated software) (**Figure 3**).

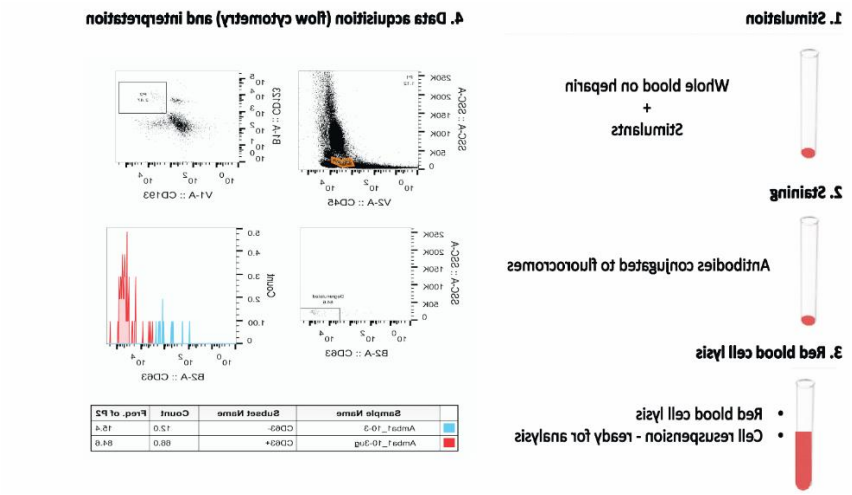

Fig. 3. Basophil activation test includes several steps: stimulation, staining, red blood cell lysis, data acquisition and interpretation

The minimal number of basophils to be analyzed should be 200 per test tube for accurate quantification of basophil activation, but in order to minimize the variation of results caused by equipment measurement error, it is preferred that 600 to 1000 basophils to be evaluated. To select basophils from the whole leukocyte population, several positive and negative selection strategies are possible. CD123, CCR3, CRTH2, IgE can be used as identification markers, while CD63, CD203c, CD107a and b, CD13, CD164, CD69, p38 MAPK, STAT5 represent activation markers (**Table I**) [7].

Table I. Basophil activation test identification and activation markers (modified after Hemmings *et al*, 2018 [2])

	Marker	Cell expression	Description
Identification markers	CCR3	Basophils, mast cells, Th2 lymphocytes	Stable marker for identification [8], [9]
	CD123	Basophils, plasmacytoid dendritic cells	Highly expressed in these cells [10]
	IgE	Basophils, monocytes, dendritic cells	Other cells expressing high-affinity IgE receptor FcεRI [9], [11]
	CRTH2	Basophils, eosinophils, Th2 lymphocytes	Differentiation from eosinophils by side scatter or T lymphocytes by CD3 is reported [12]
Activation markers	CD63	Basophils, mast cells, platelets, macrophages	Widely used activation marker and an accurate marker of anaphylactic degranulation [13]

CD203c	Basophils, mast cells, $CD34^+$ progenitor cells	Upregulation has been reported to be representative of piecemeal degranulation also used as identification marker and expressed in low levels of resting cells [14]
CD107a, CD107b	Basophils, mast cells, T cells and NK cells	CD107a and CD107b was found to be only expressed by activated basophils and its upregulation was similar to CD63.
CD13	Basophils, myeloid cells	CD13 and CD164 has an expression profile comparable to CD203c [15]
CD164	Basophils, $CD34^+$ progenitor cells	
CD69	Basophils, lymphocytes, neutrophils, monocytes, eosinophils	Expressed progressively when stimulated with IL-3; however, found to be weakly expressed to IgE mediated stimulation [16]
p38 MAPK, STAT5	Various cell types	Basophilic phosphorylation of intracellular molecules can alternatively be used to measure basophil activation [17]

Currently, BAT diagnostic kits use CD63 as activation marker (Flow CAST®, Buhlmann Diagnostics Corp), while some kits measure CD203c (Allergenicity Kit, Beckman Coulter).

CD63 is a 53kDa protein from the transmembrane-4 superfamily, known also as lysosomal-associated membrane glycoprotein-3 (LAMP-3). When it comes to a resting basophil, this protein is located on the membrane of intracellular secretory granules. After stimulation by FcεRI, these granules fuse with the plasma membrane and thus, CD63 is expressed on the surface of degranulated basophils [13]. Early studies suggested that this marker is associated with granules containing histamine, suggesting that CD63 up-regulation can be used as an indirect marker of histamine release [13][18]. However, recent studies suggest that histamine release may be the sum of both anaphylactic and piecemeal degranulation, and up-regulation of CD63 may be representative of only anaphylactic degranulation [19].

CD203c is another basophil activation marker present on CD34+ progenitor cells, basophils and mast cells. It is a glycosylated type II transmembrane molecule that belongs to the family of ectonucleotide pyrophosphatase/phosphodiesterase enzymes [20]. CD203c is constitutively expressed in low levels on the basophil surface membrane and is quickly upregulated upon cell activation via allergen (**Figure 2**) or more slowly via IL-3 [21]. This up-regulation differs from CD63 in terms of the inciting stimuli and early signalling events, which suggests that CD203c may be associated with piecemeal degranulation [6].

The quality of basophils obtained is confirmed using for stimulation fMLP, a bacterial peptide. Anti-IgE antibodies can be used as IgE-mediated positive control, while buffer can be used as a negative control. The results of BAT can be displayed as a percentage of basophils expressing the defined activation marker (in the case of CD63) or in terms of mean fluorescence intensity (MFI) (in the case of CD203c), by calculating the stimulation index (SI), represented by the ratio between MFI of the sample and the MFI of the negative control [22].

BAT APPLICATION IN FOOD ALLERGY

When it comes to diagnosing food allergy, oral food challenge (OFC) is the gold standard. However, it requires ingestion of food that can induce an acute allergic reaction and needs to be performed in a supervised environment, capable of treating anaphylaxis. Thus, specialists prefer, when it is possible, to diagnose food allergy based on clinical history, skin prick test and/or serum specific IgE.

These tests have high sensitivity but low specificity. BAT can improve the diagnosis of food allergy proving to be more accurate than IgE sensitization tests with a specificity ranging between 75 and 100% and sensitivity between 77 and 98% [23]. This high specificity may be able to reduce the number of OFC performed. Also, BAT can distinguish between individuals that are clinically allergic to those who are tolerant.

Basophil activation test is also used for monitoring the clinical response to immune-modulatory treatment of food allergy. Studies with immunotherapy for peanut and egg show that the basophil reactivity to those allergens decreased during the treatment [24, 25].

BAT APPLICATION IN RESPIRATORY ALLERGY

Skin prick tests and serum levels of specific IgE, combined with clinical history, were considered sufficient to diagnose respiratory allergies, but allergic reactions to inhaled allergens can be complex due to the diversity of potential sources to which the patients are exposed to and the number of tissues that can be affected. Therefore, patients who report local allergic rhinitis can have negative skin prick tests and undetectable levels of specific IgE, being hard to differentiate between allergic and non-allergic rhinitis. Despite the absence of serum allergen-specific IgE, for these patients, BAT has proved more sensitive and able to diagnose IgE-mediated allergy [26].

Since basophil sensitivity is a reproducible measure [27] it can be used to determine the efficacy of allergen-specific immunotherapy (AIT) to aeroallergens. BAT was used for monitorisation of patients undergoing AIT with birch [28] and timothy [29] and showed reduced allergen sensitivity during the treatment. This reduction may be due to blocking factors competing (specific IgG) for the allergen with the cell-bounded specific IgE [28], [29].

BAT APPLICATION IN VENOM ALLERGY

In the case of Hymenoptera venom allergy diagnosis, basophil activation test report both high specificity (83-100%) and sensitivity (85-100%) [30]–[32]. And even though testing for specific IgE to the major bee and wasp allergens (Api m 1 and Ves v 5) solves many suspect cases, BAT is used in diagnosing unique issues. For example, a small percentage of patients with a clinical history of venom allergy report undetectable specific IgE and negative skin tests. Since sting provocation tests are complicated and have an unethical nature, BAT has proven effective for 80% of these patients [33].

Another issue with the diagnosis of Hymenoptera venom allergy is the double-positive results for bee and wasp venom, approximately 60% of patients exhibit specific IgE to both. This double positivity may be caused by a co-sensitization to both venoms, cross-reactivity due to sequence homology or irrelevant recognition of cross-reactive epitopes [3]. And, in order to prescribe venom immunotherapy (VIT), determination of the sensitizing allergen is crucial [34], especially if the patient had an anaphylactic reaction to only one insect. BAT brings extra information when it comes to detecting the dominant allergen and the real source of venom sensitization [35] and has the lowest rate of double positivity of diagnostic tests for Hymenoptera allergy [36]. In the case of patients double-positive in BAT, the allergen that induces the highest degranulation is considered to be the primary sensitizing allergen [35].

BAT APPLICATION IN DRUG ALLERGY

In the context of an allergic reaction to drugs or related compounds, several studies validate BAT for the diagnosis of an IgE-mediated allergy from neuromuscular blocking agents (NMBAs) [37], beta-lactam antibiotics and clavulanic acid as a b-lactamase inhibitor [38]–[40], iodinated radio-contrast media (RCM) [41].

In case of small molecular weight drugs, called haptens, which are not capable of FcεRI cross-linking themselves (hapten concept [42]) a conjugation to carrier molecules is required. The carriers are usually proteins, for eliciting an immune reaction in susceptible individuals. Moreover, after the drug metabolism, some reactive intermediates may be formed (pro-hapten concept; [43], [44]). Therefore, for the investigation of drug hypersensitivity reactions the use of drug metabolites and hapten-carrier conjugates has been promoted [45]. For example, in the case of propyphenazone (PP) hypersensitivity, basophils reacted in BAT only after stimulation with the drug-carrier conjugate not when they were stimulated with pure PP [46]. Nevertheless, as a consequence of lacking knowledge regarding relevant determinants, metabolic intermediates, their reactive functions, BAT is most frequently performed with solutions of plain drugs [46].

BAT presents some limitations listed in **Table II** alongside with possible solutions. One of these limitations is the stability of blood samples. Using EDTA as anticoagulant means that the sample is stable only for a few hours, so a new method has been developed – passive sensitization, but this method is

applied only in research. The main steps of passive sensitization are peripheral blood mononuclear cells (PBMC) isolation from a healthy donor, with well-characterized basophils; removal of IgE from the receptor with lactic acid (pH 3.9), incubation with patient serum (1 hour, 37^0 C) followed by a regular BAT, as previously described. So the patient's samples are represented by serum, which can be stored for a longer period at 4^0 C [7].

Table II. Limitations of basophil activation test and possible solutions [7], [47]

Limitations	Possible solutions
Reduced basophil reactivity over time	Perform the basophil activation test within max. 24h from blood collection.
Changes in temperature can affect basophil reactivity	A good transportation system that ensures a stable temperature of the sample.
Allergen exposure, chronic inflammation and infection can induce higher basophil reactivity	Avoid basophil activation test after allergen exposure or during infections.
Immunosupressors, including oral corticosteroids, may increase basophil reactivity	Avoid immunosupressors before blood collection for basophil activation test.
Determining optimal allergen concentration	A dose-response curve with patients and healthy (exposed) controls for the exclusion of unspecific stimulation.
Difficulties in establishing the thresholds for positivity	Analysis of allergens in question with confirmed allergic patients and healthy (exposed) controls instead of arbitrarily chosen thresholds.

CONCLUSIONS

Basophil activation test is a functional test that mimics an allergic reaction *in vitro*, proving to be a useful and safe diagnostic method that can be used to evaluate IgE-mediated hypersensitivity reactions. It can be considered a surrogate for provocation test and oral food challenge. Currently, it is applied mostly in research because, in order to be accepted and used in the clinical routine, it needs clinical validation. This means standardised laboratory procedures, standardised allergen concentrations and optimal allergen positivity thresholds. Recent clinical validations and standardisations made this test available also for routine testing of several allergens.

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APLICAȚIILE CLINICE ALE TESTULUI DE ACTIVARE A BAZOFILELOR ÎN DIAGNOSTICUL BOLILOR ALERGICE

REZUMAT
În prezent, diagnosticul alergiei mediate IgE se bazează în principal pe testul cutanat prick, dar această testare nu este recomandată pacienților care au avut anterior o reacție alergică severă la sursa alergenică testată. Cuantificarea IgE seric specific are, de asemenea, neajunsurile sale. Prin urmare, testele celulare *in vitro*, cum ar fi testul de activare a bazofilelor (BAT) sunt o bună alternativă de diagnostic. Testul de activare a bazofilelor măsoară hipersensibilitatea mediată IgE la diferiți alergeni fără a expune pacienții la un risc de anafilaxie. BAT este un test funcțional care măsoară expresia markerilor de activare precum CD63 și CD203c de pe suprafața bazofilelor. Este utilizat pe scară largă în cercetare, dar validările clinice recente și standardizările au făcut disponibil acest test și pentru testarea de rutină. În acest studiu, descriem mecanismele testului de activare a bazofilelor și utilizarea clinică a acestuia în diagnosticul alergiilor alimentare, respiratorii, la venin și la medicamente.
Cuvinte cheie: alergie, test de activare a bazofilelor, diagnostic alergie, CD63, CD203c

ASSESSMENT OF CELL CULTURE MEDIA VARIATION INDUCING CYTOKINE SECRETION IN PBMCS, CD3$^+$, AND CD8$^+$ IMMUNE CELLS

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ABSTRACT
In vivo conditions, the innate immune arm signals shape the activation of naive T cells, however, it is unclear how T cells maintain their function in vitro, in experimental settings, where different culture conditions signals influence the effector T-cell function. In this study of two sets of healthy donors designated here group A (n=10) and group B (n=10), we assessed the cytokine profile of PBMCs, cluster of differentiation 3 (CD3$^+$) cells and cluster of differentiation 8 (CD8$^+$) T cells, which were cultured in different environmental conditions, including Interleukin-2 (IL-2) and/or CD3/CD28 T cell activator, supplemented culture media. The (IL-2), Interleukin-5 (IL-5) and Interferon-γ (IFN-γ) secretion at 24, 48, 72, and 96 hours was determined in a 4.5-hour solid-phase ELISA immunoassay method. The medium containing IL-2 and CD3/CD28 T cell activator was the one that induced increased secretion of IL-2, IL-5, and IFN-γ in all three cell populations, mainly in the CD3$^+$ cells, irrespective of the time frame sampled.
Key words: PBMCs, cytokine, CD4+ T lymphocytes, CD8+ T lymphocytes, ELISA

INTRODUCTION

This study was designed to provide a window in the cytokine profile of peripheral blood mononuclear cells (PBMCs), cluster of differentiation 3 (CD3$^+$) cells, and cluster of differentiation 8 (CD8$^+$) T cells, grown in different environmental conditions and to ascertain which culture condition provided the best quantifiable secretion of Interleukin-2 (IL-2), Interleukin-5 (IL-5), and Interferon-γ (IFN-γ). All three cytokines of interest in this study are involved to a different degree in various aspects of the immune response, from T cell activation to regulatory induction [1-5]. Overall, the vast majority of evidence found in the literature credits IL-2 with an essential part in the primary immune response, a role in immune memory, induction of regulatory T cells, and finally, IL-2 can also be considered a survival factor for T cells via induction of anti-apoptotic proteins [6-7].

In humans, IL-5 acts mainly on eosinophils and mast cells, and activation of B cells via dependent pathways, therefore IL-5 can be viewed as a chemokine bridge between the innate, and the acquired immune arms of the immune system [8-9]. IFN-γ is a soluble cytokine produced by a variety of immune cells like CD4$^+$ helper cells (Th1), CD8$^+$ cytotoxic T-lymphocytes (Tc1), non-cytotoxic innate lymphoid cells (ILC), and predominantly by natural killer (NK), and natural killer T (NKT) cells as part of the innate immunological response (10). Interferon-γ can be viewed as a central node in both the innate and adaptive immune response, with cellular effects like

upregulation of pathogen recognition, inhibition of cellular proliferation, antigen processing and presentation, antiviral, immunomodulation, and leukocyte trafficking [11-12].

Whole blood from two sets of healthy donors designated here group A (n=10) and group B (n=10)

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was used to provide Ficoll-Paque media density gradient isolation of PBMCs, that were characterized via a flow cytometry analysis. Using negative selection, $CD3^+$ and $CD8^+$ T lymphocytes were isolated. Afterwards, a flow cytometry analysis was performed to view the distribution of cell populations. The cells of interest, PBMCs, $CD3^+$ and $CD8^+$ were plated into 24-well plates, and grown using 3 types of environmental conditions, including IL-2 and/or CD3/CD28 T cells activator supplemented culture media.

Secretion of IL-2, IL-5, and IFN-γ was assessed with the 4.5-hour solid-phase enzyme-linked immunosorbent assay (ELISA) immunoassay method on a windowed time frame analysis at 24, 48, 72, and 96 hours [13-19]. All the samples were evaluated in double and when necessary in triple pairs.

MATERIALS AND METHODS

Negative selection of human $CD3^+$ T lymphocytes

The selection of human $CD3^+$ T lymphocytes was made with the EasySep™ Human T Cell Enrichment Kit (STEMCELLS Technologies, Vancouver, Canada) from frozen human PBMCs (two sets of healthy donors group A (n=10) and group B (n=10)). This kit targets non - T cells for removal with Tetrameric Antibody Complexes and dextran-coated magnetic particles recognizing specific cell surface markers. Unwanted cells are labelled with antibodies and magnetic particles and separated without columns using an EasySep™ magnet. The cells of interest ($CD3^+$ T lymphocytes) are simply poured off into a new tube. The cells were resuspended, 1.6 x 10^7 cells in 320 µl buffer (EasySep™ Buffer, STEMCELLS Technologies, Vancouver, Canada) into a polystyrene round-bottom tube and incubated with 16µl of isolation cocktail (EasySep™ Human T Cell Enrichment Cocktail, STEMCELLS Technologies) for 5 minutes at room temperature. The magnetic particles 16µl/sample (EasySep™ D Magnetic Particles, STEMCELLS Technologies) were added to the sample and incubated for 5 minutes at room temperature. The tube was placed into the magnet and incubated for 5 minutes at room temperature, and the enriched cell suspension was poured into a new tube. Following isolation 3 x 10^6 $CD3^+$ T lymphocytes/ml were obtained.

Negative selection of human $CD8^+$ T lymphocytes

The selection of human $CD8^+$ T lymphocytes was made with the EasySep™ Human $CD8^+$ T Cell Enrichment Kit (STEMCELLS Technologies) from frozen human PBMCs (two sets of healthy donors group A (n=10) and group B (n=10)). This kit targets non - $CD8^+$ T cells for removal with Tetrameric Antibody Complexes recognizing specific cell surface markers and glycophorin A, and dextran-coated magnetic particles. Unwanted cells are labelled with antibodies and magnetic particles and separated without columns using an EasySep™ magnet (STEMCELLS Technologies). Following isolation, 3 x 10^6 $CD8^+$ T lymphocytes/ml were obtained. After isolation PBMCs, $CD3^+$ and $CD8^+$ T cells were plated into 24-well plates. All cell types were plated at 5 × 10^5 per well (1 ml/well), with three different types of media: 1. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich); 2. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich) and 30 UI/ml IL-2; 3. RPMI 1640 medium (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 5% human serum (HS, Sigma-Aldrich) and 1% Penicillin – Streptomycin (Sigma-Aldrich), 30 UI/ml IL-2 (R&D Systems, Minneapolis, USA), and CD3/CD28 Dynabeads (Dynabeads™ Human T-Activator CD3/CD28 for T Cell Expansion and Activation, ThermoFisher Scientific, Waltham, MA USA).

Flow cytometry

After using Ficoll-Paque media for density gradient isolation of PBMCs, the resulting cells were aliquoted and stained with Simultest BD Biosciences (San Jose, California, USA) antibodies CD3 FITC/CD8 PE, afterwards following the instructions provided by the manufacturer a two-colour flow cytometric analysis was performed on the BD FACSVerse™ (San Jose, California, USA) flow cytometer. Subsequently, after the negative selection of human $CD3^+$ T lymphocytes from PBMCs, a Multitest BD 4 antibody cocktail (CD3/CD8/CD45/CD4) was used to highlight the distribution of cell populations using the same BD FACSVerse™ platform.

ELISA

The supernatant of all cell types, cultivated in the three different media was collected at distinct time points: 24h, 48h, 72h and 96h. The Quantikine® Human Immunoassay (R&D Systems) was used for assessment of IL-2, IL-5, and IFN-γ secretion of the cells. The procedure followed the instructions provided by the manufacturer. Shortly, the assay employs the quantitative sandwich enzyme immunoassay technique, in which a monoclonal antibody specific for human cytokines is pre-coated onto a 96-well polystyrene microplate; standards and samples are pipetted into the wells in duplicate, and IL-5, IL-2, or

IFN-γ present in the sample is bound by the immobilized antibody. The optical density was determined using Tecan Infinite M200 Pro (Tecan, Männedorf, Switzerland) microplate reader at 450 nm, with wavelength correction at 570 nm, and each cytokine concentration was calculated based on the standard curve and regression equation.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0. (IBM Corp., Armonk , NY).

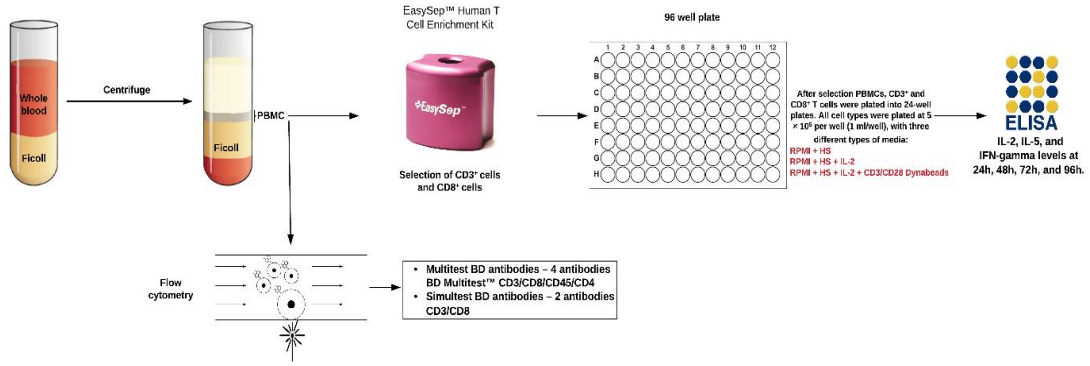
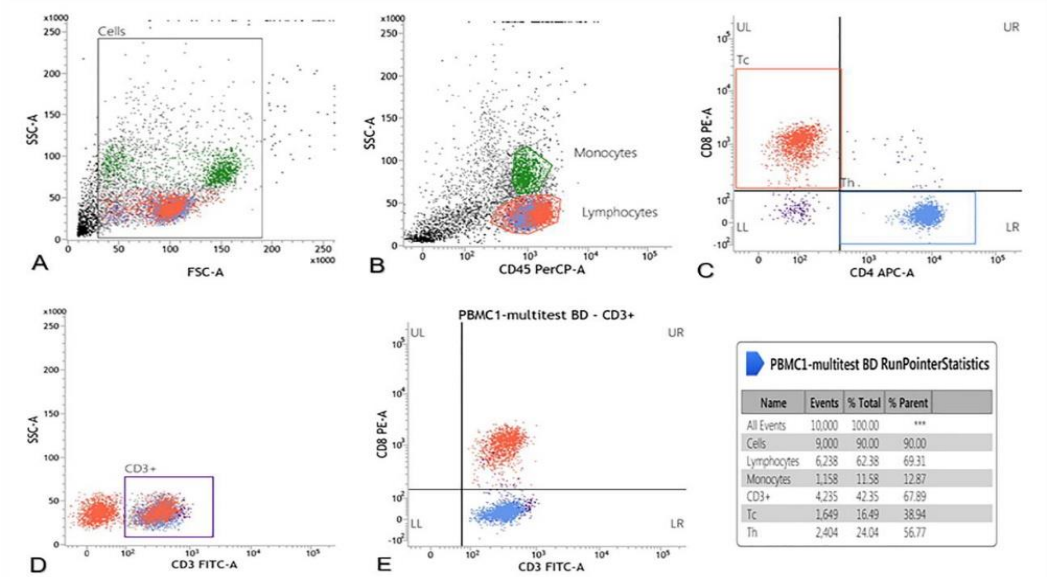


Fig. 1. Graphical abstract of methods used in this study.

RESULTS

Flow cytometry analysis of PBMCs, CD3⁺, and CD8⁺ T lymphocytes

Flow cytometry of PBMCs from both sets of healthy donors, group A (n=10) and group B (n=10) was analyzed with a gating strategy of CD45 (Ly-5), CD3, CD4 with CD8, and CD8 with CD3, with percentage representation of the cell population, both sets of healthy donors showed similar results on the analysis (Fig. 2).



Name	Events	% Total	% Parent
All Events	10,000	100.00	***
Cells	9,000	90.00	90.00
Lymphocytes	6,238	62.38	69.31
Monocytes	1,158	11.58	12.87
CD3+	4,235	42.35	67.89
Tc	1,649	16.49	38.94
Th	2,404	24.04	56.77

Fig. 2. Gating strategy for lymphocytes population. A. Doted plot representing nucleated cells; **B.** Monocyte and lymphocyte population based on CD45 expression marker; **C.** cytotoxic T cells - CTLs ($CD8^+$) and T cells ($CD4^+$) from total T cells; **D.** T cells ($CD3^+$); **E.** $CD8^+$ cells and percentage distribution of cellular populations

.

Following the negative selection of $CD3^+$ and $CD8^+$ T lymphocytes from isolated PBMCs previously quantified and analyzed, a flow cytometry analysis was performed on the isolated $CD3^+$ and $CD8^+$ T lymphocytes, the cell population was gated and percentages were quantified for both sets of healthy donors used in the study (Fig. 3).

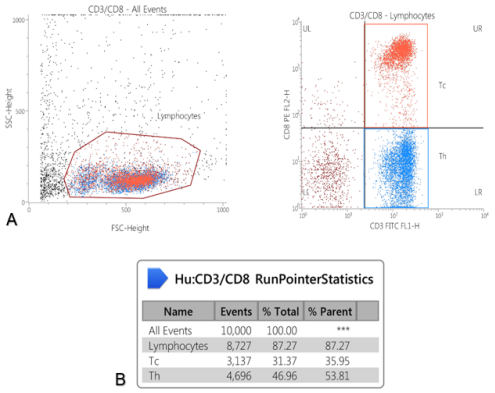


Fig. 3. Flowcytometric analysis of gated selected T cells ($CD3^+$). A. Dot plot of total lymphocyte population and CTLs (CD3+/CD8+); **B.** Percent value of CTLs and T helper cells from the isolated T cells.

CD8+ T cells represented 35.95% of the total CD3+ cells, while the $CD4^+$ cells were 53.81%. We obtained an enrichment of the total number T cells from 67.89% to 87.27% after cultivation with activator and expansion cocktail.

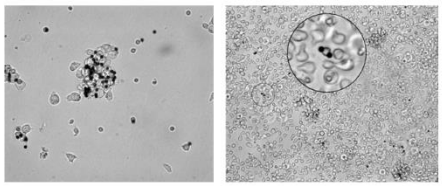


Fig. 4. Cells activated and expanded using CD3/CD28. A. The Dynabeads can be seen coupled with the cells. **B.** Digital magnification of T-cell coupled with CD3/CD28 Dynabeads.

Solid-phase ELISA analysis of IL-2, IL-5, and IFN-γ IL-2

Although IL-2 was added in the culture media from the beginning, the IL-2 concentration determined by ELISA method shows a time-dependent increase, similar to IL-5. The highest concentration value is reached after 96h by the $CD3^+$ cell population, thus confirming synergistic functioning of $CD4^+$ and $CD8^+$ cells when they are together in cell culture conditions. PBMC whole population has a decreased ability to secrete the mitogenic T cell factor. In Figure 5, the differences between cellular populations concerning IL-2 secretion are well represented with statistical significance bars.

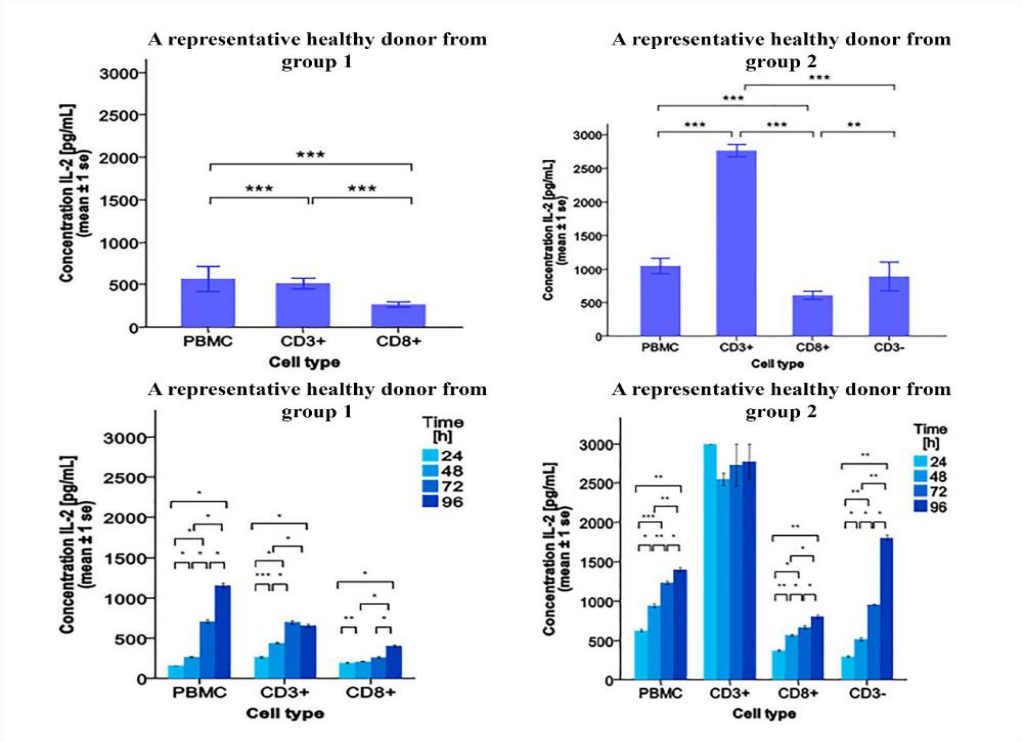


Fig. 5. IL-2 concentration cell types per groups of patients. *p≤0.05, **p≤0.01, ***p≤0.001. Results from posthoc MANOVA GTA Hochberg correction.

IL-5

ELISA assay performed at 24h, 48h, 72h, and 96h revealed that IL-5 concentration increases in a time-dependent manner in all cellular population, for both sets of healthy donors. However, the most important increase was observed for $CD3^+$ T cells, with a significant statistical difference compared with PBMC, $CD8^+$ or $CD3^-$ cellular populations. Interestingly, the $CD3^-$ cellular populations secreted large amounts of IL-5, probably due to monocytes and granulocytes present in the cell culture. ($p \leq 0.001$ between PBMC and $CD3^-$; Fig. 6)

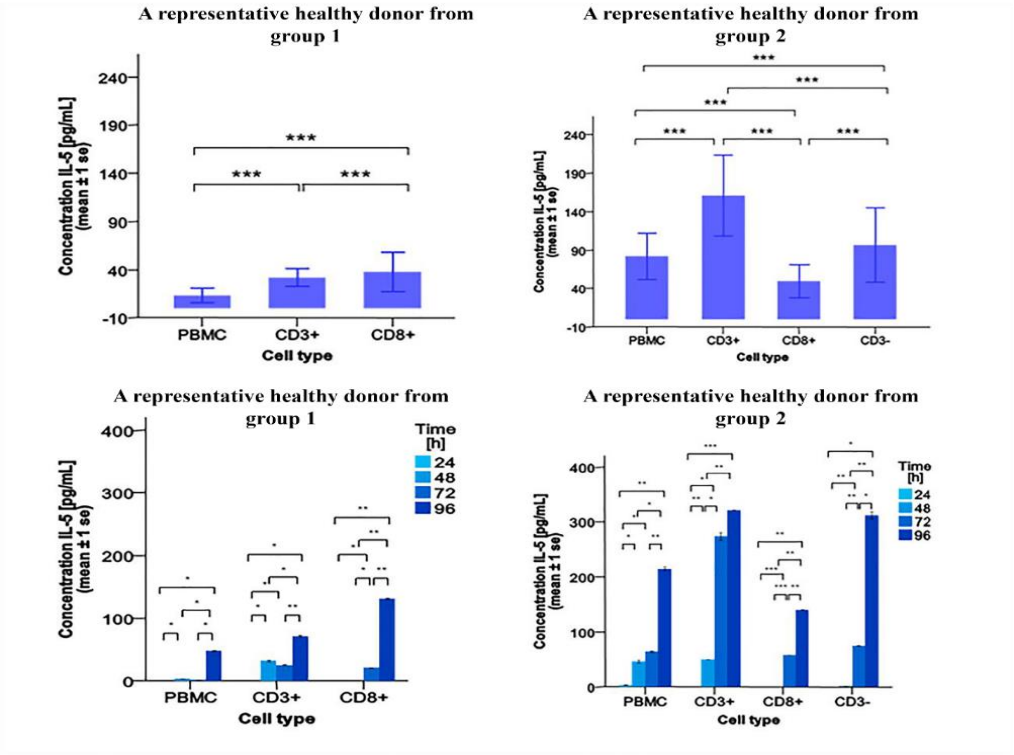


Fig. 6. IL-5 concentration cell types per patient. *p≤0.05, **p≤0.01, ***p≤0.001. Results from posthoc MANOVA GTA Hochberg correction.

INF- γ

INF-γ, although normally secreted also by Tc1 $CD8^+$ cells, it has the maximum concentration in the supernatant of the CD3+ cells, where the contribution is due to Th1 CD4+ cells (Fig. 6). Even though we used the same culture media for all cellular types, supplemented with CD3/CD28 activator and IL-2, it seems that the PBMC population is not able to contribute with higher amounts of functional cytokines compared to $CD3^+$ population of cells. $CD3^+$ cells have the optimum cytokine secretion; further usage of this cell population in further in vitro and in vivo studies of interleukin production is recommended.

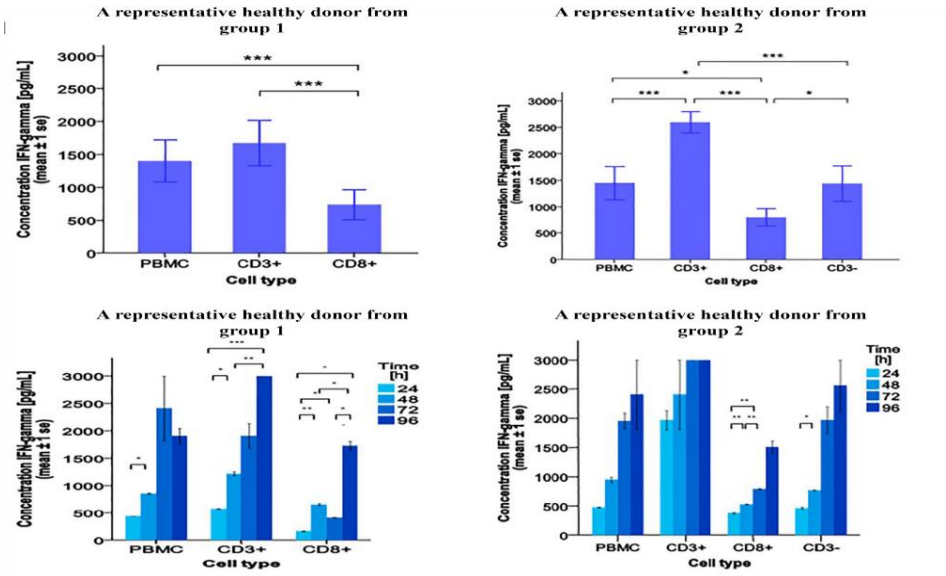


Fig. 7. IFN-gamma concentration cell types per both sets of healthy donors. *p≤0.05, **p≤0.01, ***p≤0.001. Results from posthoc MANOVA GTA Hochberg correction.

DISCUSSION

Scientific data credits IL-2 with a central role in T regulatory (Treg) cell production and homeostasis, with present studies attributing IL-2 a part in T memory recall responses [6]. IL-5's vital role in eosinophil activation and generation is well established, thus, IL-5 expands the innate immune response, and it accentuates the acquired immune response linking the two main branches of the immune system [8]. IFN- γ harmonizes many regulatory cellular programs via its effects on transcriptionally relevant genes in the immune response, specifically those that pertain to macrophage activity [11]. To revenue the best output of cytokines concerning stimulation of production and secretion of cytokines, a well-established stimulatory cocktail is used. In this study, the best outcome, or revenue (quantifiable for cytokines secreted), is in the culture medium supplemented with CD3/CD28 Dynabeads, a stable, well-used activator and expansion kit [20-22].

CONCLUSION

In our present study, there was no observable statistical difference in cytotoxicity in the cell populations in the first and second growth environment, notable IL-2, IL-5, and IFN-γ secretions were registered in the third growth environment supplemented with IL-2, and CD3/CD28 T cells activator. The cell population with the most efficient effector function was CD3+ irrespective of the time frame sampled. Therefore, considering the findings in our study and the evidence in the literature, the best growth environment for the quantitative secretion of IL-2, IL-5, and IFN-γ can be viewed as a cocktail between normal growth media, IL-2, and CD3/CD28 T cells activator supplementation.

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EVALUAREA VARIAȚIEI MEDIULUI DE CULTURĂ ASUPRA SECREȚIEI DE CITOKINE A PBMCS, LIMFOCITE T CD4+ ȘI CD8+

REZUMAT

În condiții *in vivo*, sistemul imun înnăscut modulează activarea limfocitelor T naïve, dar este încă neclară modalitatea prin care limfocitele T reușesc să își mențină funcția in vitro, în condiții experimentale, care influențează funcția limfocitelor T efectoare. În acest studiu, pe două seturi de donatori sănătoși, denumiți ca grup A (n=10) și grup B (n=10), am testat profilul citokinic al PBMCs, limfocitelor T CD3+ și al limfocitelor T CD8+, care au fost cultivate în medii de cultură diferite, care au inclus suplimentarea cu IL-2 și/sau activator al limfocitelor T, CD3/CD28. A fost determinată secreția citokinelor de tip IL-2, IL-5 și -γ (IFN-γ) la 24, 48, 72 și 96 de ore, prin testări assay-uri imune de tip ELISA. Mediul de cultură conținând IL-2 și activator limfocitar CD3/CD28 a fost cel care a dus la secreția crescută de IL-2, IL-5 și IFN-γ în toate cele 3 populații celulare investigate, mai ales la nivelul limfocitelor T CD3+, indiferent de intervalul de timp la care a fost investigată.

Cuvinte cheie: PBMCs, citokine, limfocite T CD4+, limfocite T CD8+, ELISA

THE USE OF SPECIFIC ASSAYS TO QUANTIFY OXIDATIVE STRESS IN MULTIDRUG-RESISTANT BACTERIA

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ABSTRACT
During oxidative stress the balance between the production of reactive oxygen species and antioxidant protective systems is disturbed. Reactive oxygen species (chemical species with an odd electron) include: hydroxyl radical (OH^-), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), while antioxidant protective systems are classified into: enzymatic antioxidants (superoxidismutase, glutathione peroxidase, glutathione S-transferase) and non-enzymatic antioxidants (reduced glutathione, vitamin E, ascorbic acid, selenium, beta-carotene and vitamin A). In the face of microbial invasion, cells defend themselves by releasing reactive oxygen species, but almost all bacteria have evolved and are able to form and use a range of antioxidants to manage the toxic effects of oxidative stress. To understand the defense mechanisms of bacteria, bacterial models were used that managed to characterize their adaptive responses. The development of methods capable of correlating oxidative stress with the bacterial response could contribute to obtaining a perspective on the development of new antibacterial strategies.
Keywords: oxidative stress, reactive oxygen species, enzymatic antioxidants, non-enzymatic antioxidants

INTRODUCTION

Oxidative stress is defined as the situation in which there exists an imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and antioxidant defense [1]. Reactive oxygen species are produced by living organisms as a result of normal cellular metabolism and of environmental factors - such as air pollutants or cigarette smoke. At low to moderate concentrations, they work in physiological cellular processes, however at high concentrations, they are able to damage cellular structures such as carbohydrates, nucleic acids, lipids, proteins, and, in addition, they can alter their functions. Aerobic organisms have developed two systems for controlling reactive oxygen species: the first and most important - the prevention of their formation and the second - their neutralization as soon as they form. Integrated antioxidant systems include enzymatic and non-enzymatic antioxidants that are usually effective in blocking the harmful effects of ROS, but under certain conditions, the balance between oxidants and antioxidants is disturbed in favor of oxidants. This oxidants/antioxidants imbalance is the definition of oxidative stress [2]. Although ROS are cell defense systems against microbial invasion, it has been shown that almost all bacteria have evolved and are able to use an arsenal of adaptive and inducible antioxidants, so that they can manage the toxic effects of ROS [4].

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Bacterial cells have been shown to possess a number of proteins that are able to repair oxidized proteins resulting from ROS attack [3].

REACTIVE OXYGEN SPECIES (ROS) are chemical species that have an odd number of electrons. Being highly reactive molecular fragments, free radicals react with organic molecules, macromolecules and membranes, disrupting intracellular homeostasis and damaging subcellular structures (mitochondria and endoplasmic reticulum) and their decomposition products enter the structure of lipofuscin pigments (also called wear and tear pigments) or of age pigments. They are produced in the cell, either accidentally or during phagocytosis. ROS occur in certain redox reactions in which changes occur, as a result of which the substance often changes its biological function (becomes more hydrosoluble or takes part in another chain of metabolic reactions). ROS intervene as the main amplification factor in the production of "oxidative lesions": peroxides, hydroperoxides, endoperoxides and epoxides, to which a new amplification resulting from the oxidants/antioxidants imbalance is added: through excess of pro-oxidants, doubled by lack of antioxidants. The amplification and accumulation over time of free radicals and oxidative stress (chronic stress), expressed by the imbalance of the ratio of excess pro-oxidants/deficiency of antioxidants, causes the shift of the anabolism/catabolism balance towards anabolic stress (hypo-anabolism)/catabolic stress (hyper-catabolism) with intracellular accumulation of insoluble waste. Oxidative damage can have a devastating effect on the structure and activity of proteins and can even lead to cell death. Amino acids which contain cysteine and methionine sulfur are particularly sensitive to reactive oxygen species and reactive chlorine species, which can damage proteins [3].

Types of ROS produced *in vivo*

1. The hydroxyl radical – $OH^{\cdot}$ is one of the most reactive free radicals, it appears by exposing the body to ionizing radiation. It can react with almost any molecule of the living cell; once formed it destroys everything around it, but it cannot migrate long distances into the cell. Almost all the destructive effects caused by excessive exposure to ionizing radiation are initiated by the attack of the hydroxyl radical on proteins, carbohydrates, DNA, lipids [6].

2. The superoxide anion - $O2^{\cdot -}$ is produced by phagocytes (neutrophils, eosinophils, monocytes, macrophages), as well as other cells such as lymphocytes, fibroblasts, vascular endothelial cells in order to inactivate viruses and bacteria. $O2^{\cdot -}$ is thought to be involved in the transmission of the intracellular signal as well as in the regulation of cell growth. The superoxide anion, as well as hydrogen peroxide, can be generated by autooxidation reactions in which compounds such as catecholamines, tetrahydrofolate react with oxygen to form the superoxide [6]. It will oxidize other compounds initiating reaction chains with the formation of other free radicals. The intimate mechanism of formation involves the addition of an electron to molecular oxygen. This process is mediated by nicotine adenine dinucleotide phosphate [NAD(P)H] oxidase or xanthine oxidase or by a mitochondrial electron transport system. The main site for the production of the superoxide anion is the mitochondria, that is that part of the cell specialized in the production of adenosine triphosphate. Normally, electrons are transferred through the mitochondrial electron transport chain to reduce oxygen to water, but about 1 to 3% of all electrons leak out of the system and produce superoxide. NAD(P)H oxidase is found in polymorphonuclear leukocytes, monocytes and macrophages. During phagocytosis, these cells produce an explosion of superoxide leading to bactericidal activity. Superoxide is converted to hydrogen peroxide by the action of superoxide dismutase (SOD). The superoxide anion is capable of causing a range of damaging effects, including destruction of endothelial cells with increased microvascular permeability, formation of chemoattractants such as leukotriene B4, recruitment of neutrophils for inflammation, lipid peroxidation, destruction of DNA, and formation of the peroxynitrite anion ($ONOO^{-}$). which in its turn can cause a number of changes [5].

3. Hydrogen peroxide (H_2O_2) occurs in natural habitats through reactions between sulfur and oxygen at the oxic/anoxic interfaces, by photochemical reduction of oxygen by chromophores or the redox cycle of pigments. In addition, plants and animals can excrete H_2O_2 to destroy microbes [7]. H_2O_2 can be considered an ideal weapon because it is small and not electrically charged. These characteristics facilitate its passive crossing of the cell membrane at speeds similar to those of water [8]. Once inside the cytoplasm, it disrupts the iron metabolism, virtually destroying the cell. An experimental model using the Escherichia Coli bacterium was used to identify the mechanisms by which H_2O_2 affects cell survival. The results showed that exogenous H_2O_2 damages the cell's DNA and is therefore mutagenic. This effect is known as the Fenton reaction, in which H_2O_2 reacts with intracellular iron [9].

$Fe^{2+} + H_2O_2 \rightarrow [FeO^{2+}] \rightarrow Fe^{3+} + OH^- + HO^{\cdot}$

The explanation why H_2O_2 is so harmful to cells when it interferes with the iron metabolism comes with the discovery that it inactivates two families of iron-containing enzymes: non-redox mononuclear enzymes and dehydrases [10, 11]. Basically, H_2O_2 oxidizes iron from these enzymes and suppresses their activity. It has been observed that only 0.5 μM H_2O_2 is sufficient to destroy the cells in this manner. It has also been observed that following the Fenton reaction, a series of lesions appear in the cell that can cause mutagenesis [10].

ANTIOXIDANT PROTECTIVE SYSTEMS

The increase in the O_2 concentration of the Earth's atmosphere 3 billion years ago forced the primitive forms of life to adapt by creating antioxidant protective systems. These unicellular algae and bacteria not only adapted to the increasing concentration of O_2 but begun to use it as a promoter of the evolution of life and initiator of many essential processes [12]. The body defends itself against excess free radicals with the help of non-enzymatic (natural and synthetic) or enzymatic antioxidants. To increase efficiency, enzymatic and non-enzymatic antioxidants located in various environments - membrane, cytoplasm, extracellular fluid, and in several subcellular fractions - act on the same free radical species [6].

Enzymatic antioxidants

1. Superoxidismutase (SOD) - is found in any aerobic cell, in the mitochondria and cytosol. It converts the superoxide anion to hydrogen peroxide [13]:

$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$

There are several forms of SOD: Fe-SOD in lower organisms and in higher organisms Cu-Zn-SOD in the cytoplasm and Mn-SOD in the mitochondria. Although SOD is found quite abundantly in mammalian cells, diseases have been observed in which the activity of the enzyme is low, either due to the presence of inhibitors, or due to reduced biosynthesis. Decreased SOD has been found in certain foci: inflammation, tumors, cataracts and ischemia of large organs (heart, brain). Attempts have been made to introduce purified SOD in the treatment of diseases, with variable results [14]. Outstanding results were obtained with synthetic SOD, but the high toxicity of this product limits its clinical use.

2. Catalase is found in all aerobic cells, in mitochondria and peroxisomes. It acts on hydrogen peroxide molecules, with a detoxifying effect:

$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$

The large amount of catalase present in red blood cells and liver seems to justify the theory of its involvement in processes where high amounts of H_2O_2 are produced [15].

3. Glutathione peroxidase contains selenium as its active part. The accumulation of the oxidized form of glutathione, formed both through the action of GSH peroxidase or non-enzymatically, seems to be an indicator of oxidative stress at the cellular level. GSH peroxidase also acts as a regulator in prostaglandin biosynthesis by inhibiting lipoxygenase. The importance of GSH peroxidase in prostaglandin biosynthesis is so great that in selenium deficiency endothelial lesions of some vessels and platelet aggregation disorders occur, due to the impairment of prostacyclines biosynthesis [6].

4. Glutathione S-transferase is found intracellularly. It catalyzes the conjugation of glutathione with different compounds under conditions of chemical pollution [6]. A wide variety of isoenzymes of this antioxidant exist, that are ubiquitously expressed in humans. In addition to their catalytic role in conjugating GSH to a variety of harmful components, several isoenzymes reduce hydroperoxides and detoxify the end products resulting from lipid peroxidation.

Non-enzymatic antioxidants

Unlike enzymatic antioxidant systems, which are limited to the four enzymes, non-enzymatic antioxidants comprise a very large group of substances whose list is constantly growing.

1. Reduced glutathione (L-alpha-glutamyl-L-cysteinyl-glycol) (GSH) is the main intracellular non-enzymatic antioxidant. It is a carrier of free -SH groups. Both non-enzymatically and acting as a coenzyme of GSH peroxidase or transferase, GSH is involved in the neutralization of endogenous or environmental harmful substances, of free oxygen radicals generated by acute and chronic intoxications and in reactions with organic compounds and free radicals [6].

2. Vitamin E (α-tocopherol) is recognized as being the most important liposoluble antioxidant, whose basic role is to maintain the integrity of membranes. Vitamin E is a target for free radicals and is an effective inhibitor of the evolution of the oxidative chain [16].

Vitamin E has been shown to regenerate continuously, and this increases its effectiveness in pathological conditions in which cell lysis occurs [17].

3. Ascorbic acid. Discussions on the therapeutic utility of vitamin C have had a great echo with the general public. The main involvements of vitamin C are the anti-infective role based on the modulating effects of ascorbic acid in cellular immunity, the detoxifying role based on accelerating the metabolism of xenobiotics, the antioxidant role – its most controversial since a number of papers list vitamin C among free radicals, while other papers argue in favor of its pro-oxidant role, especially with in vitro experiments [18].

4. Selenium acts not only by GSH peroxidase, but also independently or in association with vitamines [19].

5. Beta-carotenes and vitamin A, while having strong antioxidant effects, are known especially well among anticancer compounds. Numerous works have demonstrated their antioxidant character, peroxidation propagation blocker, and singlet oxygen scavenger [6].

OXYGEN REACTIVE SPECIES IN BACTERIA

Oxidative stress in microbial cells shares many similarities with other cell types, but has its specific characteristics that may differ between prokaryotic and eukaryotic cells [22]. A series of studies aimed at elucidating the mechanism by which bacteria react to ROS attack, used bacterial models (Escherichia Coli and Pseudomonas aeruginosa), thus succeeding in characterizing their adaptive responses, resulting in three conclusions [4, 20]:

- Firstly, each species of ROS triggers its own type of response;
- Secondly, the ROS response is under regulatory control in terms of inducing proteins and enzymes with a role in antioxidant defense;
- Thirdly, the response mechanisms are relatively preserved in most bacterial species. Therefore, it appears that the results obtained using the Escherichia Coli and Pseudomonas aeruginosa models can be extrapolated to visualize the general bacterial responses to ROS. Both bacterial species seem to react in the same way: they achieve antioxidant defense by acting at all possible levels [4]:
  - bacteria are constantly trying to prevent ROS attacks;
  - once the ROS offensive is launched, attempts are made to intercept and block the radicals;
  - if damage has been caused, repairs are made.

Thus, bacterial cells prepare their potential defense by maintaining a basal level of enzymes capable of eliminating ROS such as $O2^-$ and H_2O_2, and thus keeping their concentration within safety limits. The defense against ROS is also performed by antioxidant enzymes, metals, radical scavengers, protective proteins and repair enzymes [21]. Given the recent increase in antibiotic resistance, certain antimicrobial strategies based on the generation of ROS may be considered. To elaborate on the way this could be achieved, we will present a brief review of how antibiotics act on bacteria [23, 24]. It is not yet clear whether this is achieved through a single mechanism or multiple ones, which once triggered will eventually lead to the death of the bacterium. What is certain is the fact is that **the main antibacterial drugs have three targets in bacteria:**

- Bacterial cell wall
  Penicillin, for example, promotes transpeptidation, that is inhibiting the formation of peptidoglycan-type cross-links in the bacterial cell wall. The result is a very fragile cell wall that disintegrates, killing the bacteria.
- Bacterial synthesis of proteins
  Antibiotics can inhibit the synthesis of certain proteins. Tetracycline, for example, can cross the bacterial wall membrane and accumulate in high concentrations in its cytoplasm. It then attaches to the 30S ribosomal subunit and blocks RNA and thus stops the elongation of the protein chain [23, 25].
- Replication and repair of bacterial DNA
  Bacterial chromosomal topology is maintained through the activity of three enzymes: topoisomerase I, topoisomerase IV and DNA gyrase (topoisomerase II). The action of these enzymes is disorganized by synthetic quinolone, an antibiotic that targets DNA-topoisomerase complexes. This action leads to complete inhibition of cell division, with bacteriostatic effect and eventually the death of the bacterium [25].

A study conducted by Kohanski and colleagues in 2007 showed that three of the main classes of bactericidal drugs use a common inactivation mechanism that stimulates the production of lethal doses of hydroxyl radicals by the Fenton reaction [26]. Triggered oxidative stress contributes to antibiotic-mediated cell death. Also in this study it was shown that oxidative stress is involved in acquiring antibiotic

resistance of pathogenic bacteria by altering the antioxidant defense mechanisms of cells. In conclusion, oxidative stress can be considered as a possible mechanism for antibiotics' action, and, on the other hand, as a factor involved in the development of bacterial resistance to antibiotics [27].

PROTECTIVE MECHANISMS OF BACTERIA AGAINST OXIDATIVE STRESS

Bacteria live in a toxic world in which their competitors excrete hydrogen peroxide or redox compounds that generate superoxide. To cope with the ROS attack, bacteria developed a series of defense strategies. The most frequently used study model is with E. Coli and the enzymes involved are SOD in the study of superoxide anion degradation and peroxidases and catalases for the degradation of hydrogen peroxide. Mutants that lack these enzymes also suffer from alterations of enzymes that have iron as a cofactor (catalases, peroxidases and ribonucleotide reductases). Also, following the reaction between intracellular bivalent iron and hydrogen peroxide, hydroxyl radicals result, which in turn will react with all biomolecules. At the level of DNA, these radicals produce lesions that are incompatible with the activity of DNA polymerase and with replication, and if the lesions are major, the end result is cell death.The results of the study published by Imlay JA in 2015 suggest that bacteria protect themselves by activating so-called regulons controlled by a number of transcription factors [11]. A key objective of the current activity is to identify the natural circumstances in which regulons are activated. They perceive peroxide when they oxidize key portions of thiolate or iron, they then induce the formation of sets of proteins that will trigger the defense of vulnerable enzymes. In some non-enteric bacteria, some of the transcription factors control the synthesis and export of redox cycle compounds, while in enteric bacteria the cell defends against the same agents. In addition, some bacteria can induce enzymes that defend the cell against the superoxide produced by these compounds. The results of studies on non-enteric bacteria are promising and seem to provide some clarity in the future on how microbes protect themselves against the attack of ROS.

QUANTIFICATION OF OXIDATIVE STRESS IN PROKARYOTIC SYSTEMS (BACTERIA)

The development of methods capable of correlating oxidative stress with the bacterial response could contribute to obtaining a perspective on the development of new antibacterial strategies. Unfortunately, the ROS's tendency to acquire electrons makes them very reactive, with a short lifespan and therefore very difficult to detect. However, a number of methods useful in this regard do exist.

1. Methods based on chemiluminescence

Several studies have used chemiluminescence to test for the presence of ROS in bacteria. The principle of the method is to bind the dye/enzyme complex (luminophores) to the target reactive oxygen species (ROS) and to emit light, the intensity of which is directly proportional to the ROS. The measurement is made with luminometers, and the values are expressed in relative units of light (RLU) [28] or by flow cytometry [29].

Albesa and colleagues used chemiluminescence to assess the involvement of oxidative stress, especially the role of superoxide in the action of antibiotics against various bacteria, including Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Enterococcus faecalis [30]. Using chemiluminescence, this group of researchers found that various antibiotics can increase the release of superoxide in different strains, but only those antibiotic-sensitive strains respond to oxidative stress. Other studies have shown the role of hydroxyl radicals (OH•) as an essential factor in the response to oxidative stress. A study by Kohanski et al. tracked E. coli treated with norfloxacin, ampicillin and kanamycin and Staphylococcus aureus treated with norfloxacin and chloramphenicol and demonstrated the release of hydroxyl radicals by the Fenton reaction [26]. A specific dye - hydroxyphenyl fluorescein (HPF) was used to evaluate the formation of hydroxyl radicals. Similar results were observed with norfloxacin-treated E. coli after HPF addition. A group of researchers coordinated by Yeom J demonstrated in 2010 that antibiotics could accelerate cell death by promoting the Fenton reaction that leads to an oxidative stress response in ampicillin-treated Pseudomonas aeruginosa [29]. The results of the study show that the antibiotic action is affected by modulating the reduction of nicotinamide-adenine dinucleotide (NADH) levels and iron chelation. In addition to direct or indirect measurements of ROS, the involvement of ROS in cell death and antibiotic resistance can also be assessed through the use of ROS scavengers.

2. Methods which use flow cytometry

This type of method is relatively new in quantifying oxidative stress in antibiotic-treated bacteria. In 2018, a group of researchers from the University of Geneva led by Daniel Manoil set out to evaluate the oxidative stress in bacteria using flow cytometry [31]. They used the DeepRO CellROX® kit which combines an ROS-sensitive fluorophore (CellROX® Deep Red) and a dye for assessing cell viability (SYTOX® Blue) to allow the detection of non-oxidized, oxidized and damaged cells. The bacteria Enterococcus faecalis and Fusobacterium nucleatum were studied and subjected to oxidative stress. An optimal concentration of CellROX® was determined on Enterococcus faecalis and Fusobacterium nucleatum exposed to oxidative stress (H_2O_2). Bacteria were exposed to different concentrations of H_2O_2 and labeled with CellROX® to check if fluorescence increased with oxidative stress. Bacteria exposed to H_2O_2 were also double stained with CellROX® and SYTOX® Blue and analyzed by flow cytometry. Both strains were labeled with CellROX® Deep Red. The fluorescence of CellROX® Deep Red-labeled bacteria increased accordingly with oxidative stress. Flow cytometry analysis of double-stained samples showed subpopulations of bacteria with increased CellROX® signal when stressed and a higher absorption of SYTOX® Blue under higher oxidative stress. The results of this study indicate that CellROX® Deep Red can be applied to measure oxidative stress in E. faecalis and F. Nucleatum and the combination of CellROX® and SYTOX® Blue allowed the discrimination of non-oxidized, oxidized and damaged bacteria.

3. Methods based on the use of free radical scavengers/antioxidants

The principle of this method is based on the addition of antioxidants (thiourea; 2,2-bipyridyl; glutathione; ascorbic acid) in bacterial cultures exposed to antibiotics that cause oxidative stress that protects cells from the harmful effects of ROS and thus increases the survival rate of bacteria. Goswami and colleagues investigated the involvement of ROS in the action of Ciprofloxacin on Escherichia coli [32]. Ciprofloxacin is an important and frequently used member of the fluoroquinolones family of drugs. Its mechanism of action is based on the inhibition of the activities of DNA topoisomerase II and DNA topoisomerase IV respectively, eventually leading to the death of bacterial cells. In addition, an increase in ROS in bacterial cells in response to the action of ciprofloxacin has been demonstrated. These researchers investigated the role of ROS in the antibacterial action of ciprofloxacin by studying the effects of various antioxidant compounds on the susceptibility of Escherichia coli to ciprofloxacin. Among the antioxidants tested, glutathione and ascorbic acid provided substantial protection against ciprofloxacin. The involvement of the superoxide anion (O_2) and hydrogen peroxide (H_2O_2) in the antibacterial action of ciprofloxacin was analyzed using superoxide dismutase, catalase and alkyl hydroperoxide reductase. The results of this study suggest that O_2 and H_2O_2 may be involved in the antibacterial action of ciprofloxacin, that ROS species may have a similar role in the antibacterial action of all fluoroquinolones, and that glutathione-mediated protection is not a general phenomenon but one that is specific to fluoroquinolones.

4. Methods based on the use of mutant strains

Investigating the processes related to oxidative stress and the role of ROS in bacteria exposed to antibiotics provides valuable information on the mechanism of cell death mediated by bactericidal antibiotics and the involvement of ROS in this process. Another strategy for evaluating the release of ROS in antibiotic-treated bacteria is the use of mutant strains that have a modified antioxidant defense mechanism. The antioxidant defensive system of bacteria includes specific antioxidant enzymes, including superoxide dismutase (SOD), catalase and peroxidase. By manipulating E. coli strains, a knockout strain can be generated to create a state of artificial imbalance that allows the selective study of oxidant/antioxidant mechanisms.

The principle of this determination method is based on:

1. use of antibiotic-sensitive strains compared to resistant or knockout strains (bacteria whose oxidative stress defense genes, in particular those responsible for SOD, catalase and peroxidase, have been inactivated through genetic manipulation)
2. highlighting certain genes that are expressed in response to oxidative stress

5. Proteomic investigations

Are based on the use of the following methods:

- Mass spectrometry - ESI (electrospray ionization) and MALDI (ionization by laser desorption assisted by a solid matrix) – identification of proteins/isoforms affected by oxidative stress and their grouping according to the function they perform

- Gel electrophoresis - identification of proteins/isoforms affected by oxidative stress, based on molecular weight.
- PCR - identification and subsequent expression of DNA sequences responsible for the occurrence of OS.

A study published by Huang CH and colleagues in 2011, aimed at proteomic analysis of up-regulated proteins of Helicobacter pylori under oxidative stress led to very interesting results [33]. The development of gastric cancer is known to be associated with chronic inflammation that occurs as a consequence of Helicobacter pylori infection. In such inflammations, the oxidative stress induced by reactive oxygen species in vivo can exert bidirectional effects on both hosts and Helicobacter pylori. In this study, ROS-induced oxidative stress was mimicked in coculture of gastric epithelial cells with Helicobacter pylori treated with hydrogen peroxide (H_2O_2). To investigate the effect of H_2O_2 on the Helicobacter pylori proteome, two-dimensional polyacrylamide gel electrophoresis was performed, followed by liquid chromatography with mass spectrometry and bioinformatics analysis of the database. The presence of overexpressed proteins was found: protein A associated with cytotoxin (CagA), vacuolating cytotoxin (VacA), adhesion protein (AlpA), two antioxidant enzymes - alkylhydroperoxide reductase (AhpC) and catalase (KatA), plus a serine protease (HtrA), aconite hydrate and fumarate reductase. In addition, up-regulation of virulence factors and antioxidant proteins in several strains of Helicobacter pylori isolated from patients have been observed. Furthermore, the rate of Helicobacter pylori infection was found to decrease and proliferation increase after exposure to H_2O_2. It has also been observed that gastric epithelial cells can be protected from oxidative H_2O_2 damage in the presence of Helicobacter pylori. In conclusion, this study supports the assumption that reactive oxygen species containing H_2O_2 may induce up-regulation of Helicobacter pylori virulence factors and antioxidant enzymes and may explain the inflammation that contributes to the development of gastric cancer due to Helicobacter pylori infection.

6. Electrochemical sensors

They allow the real-time quantification of oxidative stress in complex biological systems. Disadvantages: difficulties in calibration and sometimes operation, in complex biological systems, due to the interference between the free radicals formed and their instability.

CONCLUSION

The development of methods capable of in-depth analysis of oxidative stress, and of correlating oxidative stress with the bacterial response could contribute to obtaining a perspective on the development of new antibacterial strategies.

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UTILIZAREA DE ASSAY-URI SPECIFICE PENTRU CUANTIFICAREA STRESULUI OXIDATIV LA BACTERIILE MULTIREZISTENTE

REZUMAT

În cursul stresului oxidativ echilibrul dintre producția de specii reactive ale oxigenului și sistemele protectoare antioxidante este tulburat. Dintre speciile reactive ale oxigenului (specii chimice care posedă un electron impar) fac parte: radicalul hidroxil ($OH^{\cdot}$), anionul superoxid ($O_2^{\cdot -}$), peroxidul de hidrogen (H_2O_2), în timp ce sistemele protectoare antioxidante se clasifică în: antioxidanţi enzimatici (superoxiddismutaza, glutation peroxidaza, glutation S-transferaza) și antioxidanţii neenzimatici (glutationul redus, vitamina E, acidul ascorbic, seleniul, beta-carotenii şi vitamina A). În fața invaziei microbiene, celulele se apără eliberând specii reactive ale oxigenului, dar aproape toate bacteriile au evoluat și sunt capabile să formeze și să utilizeze o serie de antioxidanți, pentru a gestiona efectele toxice ale stresului oxidativ. Pentru a înțelege mecanismele de apărare ale bacteriilor s-au utilizat modele bacteriene care au reușit să caracterizeze răspunsurile adaptative ale acestora. Dezvoltarea metodelor capabile să coreleze stresul oxidativ cu răspunsul bacterian ar putea contribui la obținerea unei perspective asupra dezvoltării de noi strategii antibacteriene.

Cuvinte cheie: stres oxidativ, specii reactive ale oxigenului, antioxidanți enzimatici, antioxidanți neenzimatici

IMMUNOCAP ISAC MULTIPLEX MICROARRAY IGE TESTING – A TOOL FOR COMPONENT-RESOLVED DIAGNOSIS IN ALLERGY

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ABSTRACT

Introduction: Allergy has become a major health concern worldwide, affecting more than 20% of the population in the industrialized world. Allergy symptoms negatively impact quality of life of the sensitized individuals, increase in severity over time and with multiple allergen sensitizations. Component-resolved diagnosis can provide insights into patients' sensitization profiles, identifying primary sensitizers, cross-reactive components and risk to develop more severe symptoms in the future.

Aim: To identify IgE repertoires of polysensitized patients and to test whether multiplex ImmunoCAP ISAC assay can help to differentiate between genuine sensitization and cross-reactivity.

Methods: Three ragweed-allergic patients presenting sensitizations to at least two other allergen sources have been included in the study. Serum from these individuals was tested in ImmunoCAP ISAC containing 112 allergen components derived from 50 allergen sources according to manufacturer's instructions.

Results: All patients showed reactivity to the major allergen from ragweed Amb a 1 and had reactivity towards species-specific components from timothy grass. The main cross-reactive components towards which two patients showed low to moderate response were profilins, while one patient also had IgE towards tropomyosins. Highest IgE levels towards these cross-reactive components were for those contained in the same source as species-specific components.

Conclusions: ISAC multiplex testing helped to identify sensitization to an allergen source by reactivity towards the species-specific allergens, while also identifying reactivity towards cross-reactive allergens found in multiple sources. Additional allergens could improve the identification of primary sensitizers. For proper allergy diagnosis and treatment, patient history and exposure would also need to be evaluated by the allergist to determine the appropriate immunotherapy for the patient.

Keywords: allergy, IgE, polysensitized, cross-reactivity, ISAC multiplex

INTRODUCTION

Atopy is defined as the disposition to develop IgE antibodies against otherwise harmless external agents, which by cross-linking result in the release of inflammatory mediators like histamines or leukotrienes by the effector cells [1,2]. In the industrialized world, allergic disease occurs in around 20% of the population and appears to be increasing [3, 4]. The most common symptoms of allergic diseases are rhinitis and conjunctivitis [5]. Rhino-conjunctivitis reduces the quality of life and implies increased health costs for sensitized individuals. Additionally, rhino-conjunctivitis can develop into more severe forms of allergic disease, like asthma [6], while rhinitis and

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asthma is more prevalent among patients sensitized to more than one allergen source [7]. Patients with multiple sensitizations and morbidities also have more persistent and more severe asthma symptoms [6]. Most frequent sensitizations are towards house dust mites (HDM) and pollen sources like timothy grass and short ragweed, while most severe allergic manifestation of allergy, anaphylaxis, may be caused by sensitization to allergens in food, insect venoms, and drugs [3], [8].

Allergen sources contain multiple proteins with sensitizing potential, some of which are considered species-specific major allergens and used as markers for sensitization while others are known panallergens (proteins with functional and structural homology belonging to the same protein family) (Fig.1). Proteins which are known as panallergens belong to the profilin, tropomyosin, polcalcin, nonspecific Lipid Transfer Protein (nsLTP) and Pathogenesis-Related protein (PR) families [9]. Allergy onset is triggered by sensitization towards the major and some minor allergens, while sensitization towards panallergens occurs after high exposure to allergen source and is sometimes an indicator of disease severity [9, 10]. When IgE antibodies are raised against a specific antigen, but are able to recognize sequences from other antigens based on their primary or tertiary structure, cross-reactivity occurs [11, 12]. Allergens with an amino acid identity above 70% are often considered to trigger reactions of cross-reactivity, while allergens with identities below 50% rarely cross-react [13]. Sequence homology is not always a prerequisite for cross-reactivity, since these reactions were also found between non-related proteins, i.e. soy Gly m 6 and cow milk Bos d 6 [12]. These reactions can be clinically relevant when both sources can trigger symptoms or they can be irrelevant to the patient [11]. Patients sensitized to certain pollens have often reported symptoms after ingestion of certain foods, known as pollen-food syndrome (PFS) e.g. birch pollen allergic patients, sensitized to the major allergen Bet v 1 report symptoms after ingesting apple, due to the homology between the two PR-10 proteins Bet v 1 and Mal d 1. Birch immunotherapy was also found to reduce the symptoms towards apple [14]. Similarly, patients with sensitization to plant food allergen Pru p 3, nsLTP from peach, also had rhinitis symptoms in response to mugwort nsLTP Art v 3 [15]. Another common PFS is ragweed-melon-banana which is probably induced by primary sensitization with reactivity towards nsLTP or profilin from ragweed [16]. Due to its predicted dispersal and increased allergenicity, ragweed has become a major health problem in Europe and worldwide [17]. Therefore it is important to investigate potential cross-reactive allergen sources among ragweed allergic patients to determine primary sensitization and select the appropriate immunotherapy [18].

Diagnosis of type I allergy requires the determination of allergen-specific IgE (sIgE) in the blood of patients or induction of immediate symptoms towards allergen in provocation test (nasal, skin, oral or bronchial provocation test) [19, 20]. Skin prick test (SPT) using allergen extracts were preferred by physicians and patients, since they were a fast, cost-efficient and easy way to diagnose allergy [21]. However, disadvantages include the lack of standardization of allergenic components when obtaining allergen extracts as well as the presence of cross-reactive carbohydrate determinants and panallergens in natural extract which could give false positive results [22]. To identify the specific sensitization profile of patients, production of recombinant allergens has proven to be an invaluable tool in the component resolved diagnosis of allergy and precision medicine [19, 23]. Two different techniques are most frequently employed in component-resolved diagnosis: singleplex ImmunoCAP and multiplex ImmunoCAP Immuno Solid-phase Allergen Chip (ISAC). An important advantage of ImmunoCAP testing is obtaining quantified allergen-sIgE levels against an allergen coated on a cellulose matrix without interference from IgG. On the other hand, ISAC provides an overview of IgE sensitization patterns towards 112 allergens from 50 allergen sources coated on polymer-coated glass slide using only small quantities of serum. A drawback for ISAC being that it gives you only semiquantitative data at a relatively high cost per assay [24, 25]. In ImmunoCAP the bound IgE antibody is measured with an enzyme labelled anti-IgE antibody, where the fluorescence intensity is proportional to the amount of bound IgE. The principle behind the multiplex assay is similar, in ISAC the allergen-bound IgE is measured using fluorescently labelled anti-human IgE which is detected using a laser scanner. Both component-resolved assays can provide valuable information regarding genuine sensitization by the use of marker (species-specific) allergens or enabling to identify cross-reactivities by sensitization to panallergens. This assay provides also the opportunity to identify IgE reactivity profiles at risk of developing sever symptoms, e.g. anaphylaxis [26]. The ISAC assay has recently been evaluated by different operators in 23 sites worldwide, showing high repeatability across sites and variability estimated at 25.5% [27].

Aim of this study is to illustrate the effectiveness of component-resolved diagnosis in deciphering primary sensitization in ragweed allergic patients presenting multiple sensitizations in SPT. The patients

chosen for this assay present multiple sensitizations towards at least three types of pollen. Therefore, the aim was to test whether ISAC multiplex assay can help to differentiate between genuine sensitization and cross-reactivity in patients presenting multiple sensitizations.

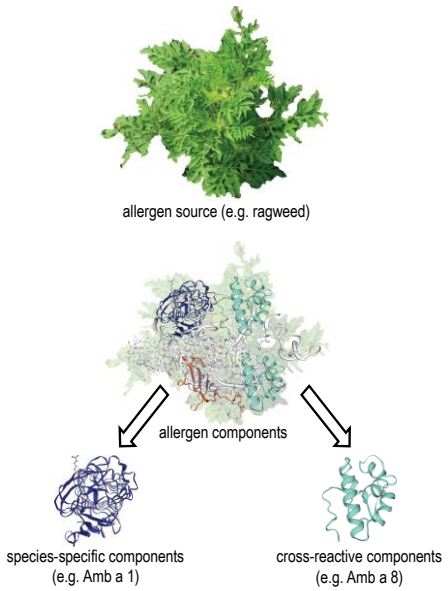

Fig. 1. Component-resolved diagnosis. Allergen sources contain multiple allergenic components, which can be either species-specific or cross-reactive.

MATERIALS AND METHODS

Patients sera

Three ragweed-allergic patients were selected for inclusion in this study after examination by an allergist at the Allergology Unit in Clinics, Timisoara, Romania. Inclusion criteria were reactivity towards ragweed pollen extract (SPT or blood IgE) as well as reactivity towards at least two other allergen sources (pollen from trees, grasses, other weeds, house dust mites, fungi, furry animals, latex or food) and symptoms of allergic rhinitis and conjunctivitis during allergy season. The participants in this study signed an informed consent to donate blood voluntarily. Sera samples were stored at -80°C until use. The study was approved by the local ethics committee of County Emergency Clinical Hospital „Pius Brînzeu" Timisoara (Ethical approval number 102, 10.01.2017).

Determination of IgE profiles using ISAC 112i

For this study four samples were measured: the calibrator sample (CTR03), provided by the manufacturer and three sera from polysensitized patients. IgE sensitisation to 112 allergen components was determined in triplicate by a semi-quantitative allergen microarray assay using the ImmunoCAP ISAC 112i (ThermoFisher, Uppsala, Sweden) platform following the manufacturer's guidelines (Fig. 2). CTR03 is composed of known amounts of humanized chimeric monoclonal antibodies against 16 molecular components (Amb a 1, Art v 1, Art v 3, Bet v 1, Can f 1, Can f 2, Can f 5, Der f 1, Der p 1, Fel d 1, Gal d 1, Gal d 2, Mal d 1, Pla a 3, Phl p 5, Pru p 1, Pru p 3). The antibodies against these allergenic components are found in the calibrator in a range of 4 different concentrations expressed in ISAC Standardized Units (ISU-E) values for sIgE (1, 4, 15 and 50). The calibration curve is plotted from the 4 points of fluorescence intensity which correspond to the different ISU (Fig. 3 A, B).

Fig. 2. ImmunoCAP ISAC array layout. Layout shows positions and names of 112 allergen components (MIA software)

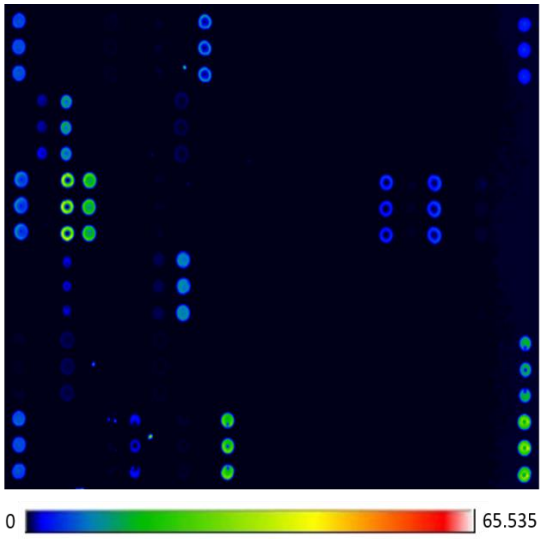

Fig. 3A. ImmunoCAP ISAC image scan of the calibrator sample (CTR03). Allergen components are arrayed in vertical triplicates. Image is shown in false colour display mode to better visualize the fluorescence output. Linear scale of false colour display is shown below the image.

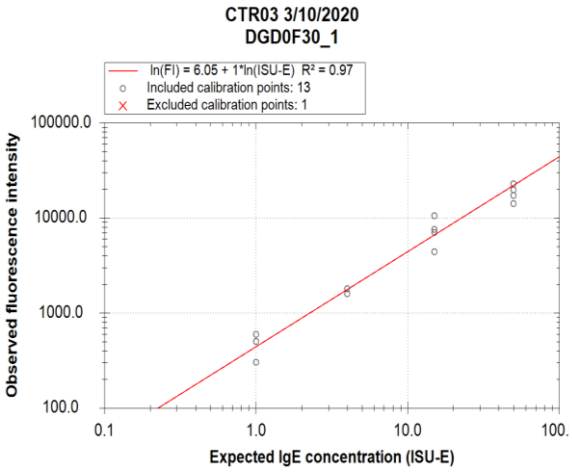

Fig. 3B. ISAC calibration curve used for this assay analysis (CTR03)

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The microarray was incubated with 30 µL of patient serum for 120 minutes at room temperature. Slides were then washed and 30 µL fluorescence-labelled human IgE antibody was added on each microarray. After 30 min incubation in the dark humidity chamber, unbound antibodies were removed by washing and the fluorescence intensity was measured by microarray scanner (LuxScan 10K/A, CapitalBio, Beijing, China) with the following parameters: laser power (LP = 60) and photo-multiplier tube (PMT = 600). Results were interpreted using Phadia Microarray Image Analyzer software (MIA, ThermoFisher). The sIgE values expressed semiquantitatively as ISU-E units are assigned to one of the following four categories: Undetectable (<0.3 ISU-E), Low (0.3-0.9 ISU-E), Moderate/High (1-14.9 ISU-E), Very high (≥15 ISU-E). Phadia Xplain add-on (ThermoFisher Uppsala, Sweden) was used for data interpretation.

RESULTS

All patients had a positive response in skin prick test/ blood IgE test towards weed pollen and grass pollen, while patients 2 and 3 were also reactive towards tree pollen (Table I.). Patient 1 also had positive results towards house dust mites (HDM), fungi, furry animals and food, while patient 2 was positive towards latex extract and HDM (Table I).

Table I. Patients characterization and sensitization to different allergen sources. Patients sensitization towards extracts as evaluated at the allergist clinic. (+ positive, - negative, NA not available)

Pat.	Age	Sex	Allergen sources							
			Tree	Grass	Weed	House Dust Mite	Fungi	Furry animals	Latex	Food
1	39	M	-	+	+	+	+	+	NA	+
2	63	M	+	+	+	+	-	-	+	-
3	16	F	+	+	+	NA	NA	NA	NA	NA

Component resolved analysis revealed that all three ragweed-allergic patients (patient 1, 2 and 3) were positive (ISU-E≥0.3 ISU-E) for major ragweed component Amb a 1 with measured values between 8.8-20 ISU-E, corresponding to moderate – very high response (Fig. 4 A-F). Patient 2 reacted to major component from mugwort Art v 1 with a value of 3 ISU-E (Fig. 4 D). Patients 1 and 2 also reacted with profilin Mer a 1 from annual mercury with values of 0.5 and 8.7 ISU-E respectively (Fig. 4 B, D).

All three patients with allergy to grass pollen reacted to one or more species-specific components from timothy grass pollen as follows: patients 1 and 3 reacted to Phl p 1 with values of 2.3 and 71 ISU-E, to Phl p 2 with values of 0.9 and 17 ISU-E, patients 2 and 3 reacted to Phl p 4 with values of 1.6 and 2.3 ISU-E, patient 3 reacted to Phl p 5 and Phl p 6 with values of 25 and 5.2 ISU-E. Patient 2 also reacted to cross-reactive profilin Phl p 12 with a value of 1.7 ISU-E (Fig. 4 D). Cyn d 1 resulted in a moderate IgE response in patient 2 (2 ISU-E) and a very high response in patient 3 (36 ISU-E) (Fig. 4 C-F).

Among the patients sensitised to tree pollen, patient 3 showed reactivity to major allergen component from birch Bet v 1 with a value of 1.1 ISU-E, whereas patients 1 and 2 showed reactivity to profilin Bet v 2 with values of 0.4 and 7.6 ISU-E (Fig. 4 A-F). Patient 3 reacted slightly to major component from Japanese cedar Cry j 1 with value of 0.9 ISU-E.

Patient 2 showed a positive response to allergens from house dust mites (HDM) and mould (Fig. 4 D). Species-specific allergens Der f 2 and Der p 2 resulted in moderate response with values of 3,8 and 4,6 ISU-E, while the cross-reactive component tropomyosin, Der p 10, also had a moderate value of 1.7 ISU-E. Patient 2 also reacted to tropomyosins from three other animal sources: anisakis Ani s 3, cockroach Bla g 7 and shrimp Pen m 1 with low to moderate values of 0.8, 0.9 and 1.2, respectively (Fig. 4 D). Patient showed low reactivity to the enolase from mould Alt a 6 with a value of 0.6 ISU-E.

Other reactivities in ISAC were to profilin from latex Hev b 8 for patients 1 and 2 with values of 0.4 and 8.4 ISU-E and to food allergen from hazelnut Cor a 1.0401 for patient 3 with a value of 0.4 ISU-E. Also, patient 2 and 3 showed reactivity to CCD (cross-reactive carbohydrate determinant) MUXF3 with low levels of 0.7 and 0.9, respectively (Fig. 4 D, F).

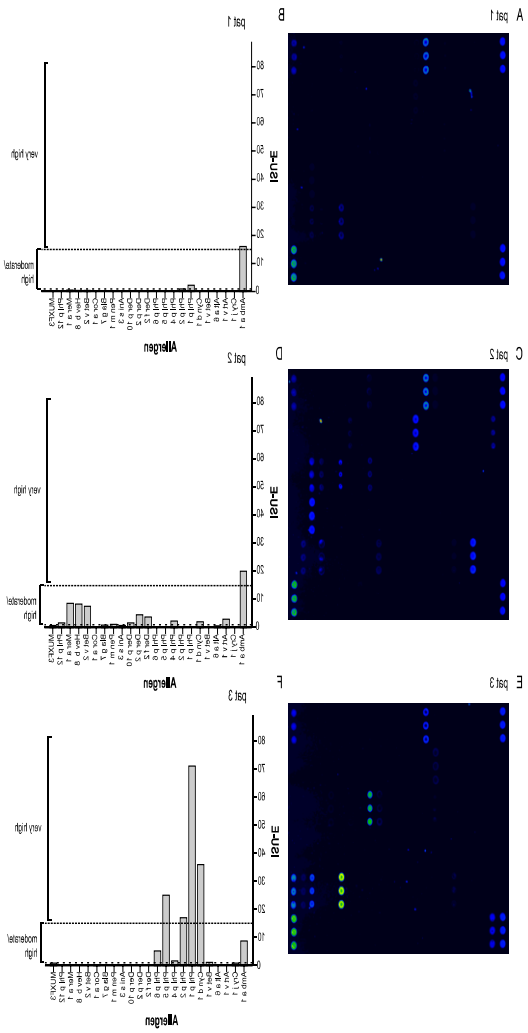

Fig. 4. ISAC IgE measurements for the three patients. ISAC readings and average ISAC Standardized Units (ISU-E) results for patient 1 (A, B), patient 2 (C, D) and patient 3 (E, F).

DISCUSSION

ImmunoCAP ISAC Molecular Allergy Test has proven to be a highly sensitive and specific method of diagnosis in allergy, showing a strong correlation with other methods as SPT and sIgE tests [27, 28]. Studies found that in Europe individuals are exposed to overlapping pollen seasons of a variety of allergenic plants [29]. Ragweed, mugwort, birch and timothy grass are only a few of the most common pollen sources which can be found in Western Romania [30, 31]. Previous studies conducted in Timisoara, Romania, showed that ragweed allergy has the

highest prevalence in both monosensitized and in polysensitized patients and ragweed pollen is considered to be the most significant outdoor allergen [32]. Therefore, three patients with ragweed allergy in addition to at least two other sensitizations were chosen to investigate allergen-specific IgE repertoire to 112 components from 50 allergen sources.

The sensitisation rate for the three patients included in our study was higher for pollen-derived components than for non-pollen derived components. All three ragweed-allergic patients showed reactivity to the species-specific component Amb a 1 from ragweed with high IgE levels, as expected, since older studies show that Amb a 1 is a highly allergenic molecule recognised by more than 90% of the ragweed-allergic patients and high IgE titres are found in patient sera [33]. Although other major and minor allergens are known from ragweed pollen to be clinically relevant contributing to the clinical picture of ragweed allergy, in this case, the patients are genuinely sensitised to ragweed pollen. In addition, all patients also reacted to at least one component from timothy grass.

Patient 1 showed specific IgE to major allergens Phl p 1 and Phl p 2 which indicate genuine sensitisation to timothy grass [34]. Low IgE reactivity to the profilins from annual mercury Mer a 1, latex Hev b 8 and birch Bet v 2, can indicate cross-reactivity due to the high sequence homology of these proteins. Studies found that Bet v 2 was an indicator of cross-reactivity with profilins from other pollen sources, plant-based foods and latex, similar to Mer a 1 which cross-reacted with other pollen profilins and Hev b 8 which was found to cross-react with other pollen profilins rather than to indicate genuine latex sensitization [34, 35]. All this data suggests that this patient may be sensitised to other profilins which are currently not spotted on the chip. The reactivity to the beforementioned profilins and not Phl p 12 (from timothy grass) could be attributed to sensitization towards profilin Amb a 8, a minor allergen from the ragweed pollen with an IgE frequency of 20-35 % in ragweed-allergic patients and a sequence identity of over 65% with other known allergenic profilins [36]. However, profilin sensitization was indicated to have little clinical relevance in allergic diseases [37].

Patient 2 appeared to be sensitised to Bermuda grass and timothy grass due to the positive response to natural components Cyn d 1 and Phl 4, respectively. This patient also had low IgE level to CCD. Proteins purified from extract can contain CCD components and IgE reactivity to Bermuda grass Cyn d 1, timothy grass Phl p 4, cypress Cup a 1, Japanese cedar Cry j 1 and plane Pla a 2 could be partially based on cross-reactivity to these CCDs [38, 39]. Although more than 20% of the allergic patients were found to have IgE that binds to CCDs, the clinical relevance of these molecules has yet to be proven [39]. Both Cyn d 1 and Phl p 4 are considered species-specific allergens and in this case, patient 2 is probably sensitised to both sources with only a small part of the IgE to Cyn d 1 and Phl p 4 being attributed to CCDs [34]. Art v 1 is the main specific mugwort component as at least 95% of the mugwort allergic patients react to this allergen and IgE to this marker shows sensitisation to mugwort pollen [34]. However, in ragweed pollen (towards which the patient is sensitized according to the high reactivity to Amb a 1) there is a minor allergen (defensin Amb a 4) 1 which is not included on the chip, showing 50% sequence identity with Art v 1 [36]. Pablos et al. found that between 52 and 74% of patients which reacted to Art v 1 (depending on region) also responded with a similar intensity towards the homologs in ragweed and *Parthenium* [40].

Patient 2 showed low reactivity to Alternaria enolase Alt a 6 minor allergen indicating probably weak sensitisation to mould [41]. Specific IgE towards marker allergens Der f 2 and Der p 2 indicated genuine sensitisation to house dust mites (HDM). Der p 2 and Der f 2 can cross-react. This patient also showed low-moderate reactivity to tropomyosins Ani s 3, Bla g 7 and Pen m 1, which could be due to cross-reactivity to Der p 10 from house dust mites towards which the patients is sensitized and which had slightly higher IgE level [34]. In order to determine the primary sensitizer in case of cross-reactivity, the highest IgE values for a protein from that family has been suggested as indicator of primary sensitization, while lower IgE values may be to cross-reactive proteins [38].

This patient also showed IgE reactivity to cross-reactive components from birch Bet v 2, latex Hev b 8, annual mercury Mer a 1 and timothy grass Phl p 12 indicating cross-reactivity between these profilins [34]. Based on the IgE levels towards these components, it is likely that patient 2 was primarily sensitized to annual mercury and this led to cross-reactivity with other profilins. On the other hand, given that patient 2 appeared to be sensitized to timothy grass, Phl p 12 could also be considered as primary sensitizer, although this could not explain why the IgE value to Phl p 12 was four or five times lower than to Bet v 2, Hev b 8 and Mer a 1. Another possibility could be that patient 2 was primarily sensitized to profilin from other allergen sources known to cross-react and towards which that patient showed high IgE reactivity, but which are unfortunately not available on the chip for testing (e.g. ragweed Amb a 8 or mugwort Art v 4).

Patient 3 showed a high reactivity towards the species-specific components from grass pollen, five mainly species-specific components of timothy grass

(Phl p 1, Phl p 2, Phl p 4, Phl p 5, Phl p 6) indicating genuine sensitization to timothy grass [42, 43]. High IgE values to Bermuda grass Cyn d 1 could also indicate a genuine sensitization to Bermuda grass, since Cyn d 1 is considered representative marker allergen for this species [34]. However, we also have to consider that this reactivity may be due to cross-reactivity to timothy grass Phl p 1. Previous studies have found a high degree of allergenic similarity or cross-reactivity between Bermuda grass pollen and timothy grass pollen and IgE to Cyn d 1 may cross-react to Phl p 1 [38, 44, 45].

Patient 3 showed a weak sensitization to tree pollen. IgE reactivity to Bet v 1 component indicates sensitization to birch [46]. IgE reactivity to hazelnut Cor a 1.0401 is associated with local allergic reactions in patients with pollen-food-syndrome caused by sensitization to birch (e.g. Bet v 1) or related trees [34]. In this case, sensitization to Cor a 1.0401 could be due to cross-reactivity to birch Bet v 1 belonging to the same protein family (PR-10 protein). Part of the IgE reactivity to components Cyn d 1 and Phl p 4 for this patient could be due to IgE against CCD, as mentioned before. Since the IgE values to Japanese cedar Cry j 1 and CCD are identical, it is possible that patient 3 was not primarily sensitized to Cry j 1 and the positive response could be entirely due to the CCD-specific IgE [34].

CONCLUSION

ISAC multiplex assay has proven to be an important tool to determine the IgE profiles of the polysensitized patients included in our study. After data interpretation, patients included in this study were sensitized to fewer allergen sources according to chip than was previously determined by extract testing. Therefore, ISAC measurements could help to explain certain reactivities obtained when testing the allergen extracts due to the cross-reactive allergen components or carbohydrate determinants within those allergen sources. Additional allergens on the chip could provide more information regarding primary sensitizer among cross-reactive allergens, especially when considering the exposure of the patients to the allergens. This information is very useful for precision medicine and personalized treatment in allergy. However, for the clinical diagnosis and selection of allergen immunotherapy, patient and symptom history as well as allergen exposure will have to be evaluated by the allergist to establish the appropriate treatment for each patient.

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TESTAREA DE ANTICORPI IGE CU MULTIPLEX IMMUNOCAP ISAC MICROARRAY – O METODĂ DE DIAGNOSTIC MOLECULAR ÎN ALERGII

REZUMAT

Introducere: Alergiile au devenit o problemă majoră de sănătate în toată lumea, afectând mai mult de 20% din populația din lumea civilizată. Simptomele alergiei reduc calitatea vieții pacientului și manifestarea lor devine tot mai severă pe măsura creșterii ratei sensibilizării la alte alergene. Diagnosticul molecular oferă o înțelegere mai amplă a profilului de sensibilizare al pacientului, ajută la identificarea sursei sensibilizării primare, a componentelor alergenice care pot da reactivitate încrucișată și la evaluarea riscului de a dezvolta simptome mai severe pe viitor.
Scop: Studiul de față propune identificarea repertoriului de anticorpi IgE al pacienților polisensibilizați prin utilizarea tehnicii ImmunoCAP ISAC pentru a face diferența între sensibilizarea primară și reactivitatea încrucișată.
Metode: Trei pacienți alergici la polen de ambrozia care au prezentat și simptome la cel puțin alte două surse alergenice au fost incluși în acest studiu. Serul de la acești indivizi a fost testat în ImmunoCAP ISAC la 112 componente din 50 de surse alergenice conform instrucțiunilor producătorului.
Rezultate: Toți pacienții au prezentat reactivitate la alergenul major din ambrozia și la anumite alergene din timoftică. Principalele alergene cu potențial de reactivitate încrucișată la care pacienții au prezentat un răspuns slab sau moderat au fost profilinele; un singur pacient a prezentat reactivitate la tropomiozine. În cazul pacienților care au răspuns la mai multe alergene omolage, răspunsul IgE cel mai crescut a fost pentru sursa la care pacientul s-a sensibilizat inițial la alergene majore.
Concluzii: Testarea cu ajutorul tehnologiei ISAC a ajutat la identificarea sursei de sensibilizare prin determinarea reactivității față de alergenele specifice unei surse, dar și la identificarea alergenelor care dau reactivitate încrucișată. Adăugarea suplimentară pe cip a altor alergene minore ar putea contribui la identificarea sursei alergenice primare în cazul reacțiilor de reactivitate încrucișată. În vederea stabilirii unui diagnostic corect al alergiei, istoricul pacientului și expunerea la diferite surse alergenice trebuie evaluată de medicul alergolog în cazul administrării imunoterapiei adecvate pentru fiecare pacient.
Cuvinte cheie: alergie, IgE, polisensibilizare, reactivitate încrucișată, ISAC multiplex

POST TONSILLECTOMY PAIN IN ADULTS – A COHORT STUDY

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ABSTRACT
Tonsillectomy is one of the most common procedures performed annually. Pain is reported in all patients in the postoperative period and persists for a various extent of time. Numerous patients address themselves to the emergency department after a tonsillectomy for pain relief. The aim of the study is to compare the effect of Ibuprofen vs Paracetamol vs Tramadol in the post-operative recovery after tonsillectomy and their link to postoperative bleeding.
Keywords: tonsillectomy, AINS, acetaminophen, tramadol, bleeding

INTRODUCTION

Tonsillectomy is one of the most frequently performed surgical procedure in the ENT specialty. In the United States, more than half a million of the pediatric population undergo this surgical procedure each year [1]. This procedure is usually performed in case of recurrent tonsillitis or complications of tonsillitis (peritonsillar abscesses or deep neck infections), for the treatment of OSAS or in case of tonsillar malignancy [2,3].

One of the most postoperative complaints of the patients after the intervention is pain. The intensity of pain depends on the surgical technique and the medical postoperative pain relief treatment. The pain after a surgical procedure is due to the surgical damage to the mucosa and the irritation of the nerve fibers in the peritonsillar space [4]. Various analgesic protocols have been used to reduce the post tonsillectomy pain but significant gaps remain in the literature as a result of the lack of evidence-based criteria for the ideal treatment of pain and the link between some antalgic treatments and postoperatory bleeding.

Acetaminophen (paracetamol) is commonly used for surgical pain as well as for analgesic and antipyretic properties [5]. The potential for hepatic injury exists and has been found in some young patients [6].

Ibuprofen is a NSAID well known for his great analgesic effect but also for the increased risk of bleeding. Ibuprofen acts by inhibiting the cyclooxygenase enzymes resulting in a thromboxane inhibition (an important component of the coagulation process) [7].

Opioids are widely used in postoperative, treating the acute pain. However, they are well known for their many side effects such as vomiting, nausea, sedation [8]. The use of opioids in the pediatric population is under discussion and research because they can produce severe respiratory distress and in some cases death by the rapid conversion of codeine to morphine via the CYP2D6 pathway [9].

The aim of our study was to compare the efficacy of the Acetaminophen vs Ibuprofen vs Tramadol for the treatment of post-tonsillectomy pain in adults and the incidence of complications such as post-

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tonsillectomy bleeding.

MATERIAL AND METHODS

Three groups were compared regarding the pain severity and the post-tonsillectomy occurrence hemorrhage. The groups were sorted according to gender and age with a similar distribution in the cohorts. The aims were defined as readmission for pain control, readmission for post tonsillectomy bleeding and surgical hemostasis.

The indication for tonsillectomy was repetitive tonsillitis. The cold dissection technique of all the tonsillectomies was performed by the same surgeon for the entire duration of the study. The hemostasis was realized by compression and bipolar cauterization. The postoperative control was made by the same team of surgeons and resident doctors. None of the patients received preoperative or intraoperative antibiotics.

The patients were appointed every 3 days for a medical visit and they filled a questionnaire for the evaluation of the pain (a scale from 1 to 10). No patient missed their appointment. The questionnaire filled by the patients included also items about the difficulty of swallowing (for liquids, for solids, for both), and experience of nausea and other possible side-effects of the treatment.

The patients with a history of coagulopathy, renal or liver diseases, peptic ulcer disease, and intolerance to NSAIDs, asthma or allergies to the substances used in this research had been excluded from this study. The patient with acute infections had been also rejected from the inclusion of this study.

To every age cohort group was appointed a similar number of patients treated with a different pain relief drug (Table I).

Age	Acetaminofen	Ibuprofen	Tramadol	Total
31-40 years old	2	2	2	6
41-50 years old	2	2	2	6
51-60 years old	2	2	2	6
Total	6	6	6	**18**

Our standard treatment for post tonsillectomy analgesia has been: Acetaminofen 1 g every 6 h for a maximum of 4 grams per day, Ibuprofen 400 mg every 12 h for a maximum of 800 mg per day, and Tramadol 5 mg every 6 h for a maximum of 200 mg per day. All the treatments were prescribed for 12 days. The monitoring of the patients was over a period of 21 days, with a medical appointment every 3 days.

Our goals were to assess the surgical hemostasis readmission because of a post tonsillectomy bleeding and readmission for pain control. In our opinion, the surgical hemostasis is a more reliable indicator of bleeding events because some patients might report a bleeding without the evidence by the surgeon. The medical team remained the same through the duration of the study in order to have the same objective criteria's.

The incidence of the tonsillectomy hemorrhage was 16% in the Tramadol group vs 0% in the Ibuprofen group and 33.3% in the Acetaminophen group. The incidence of re-intervention for post-tonsillectomy bleeding was 16.67%.

Some studies indicated that the risk of post tonsillectomy bleeding is increasing with age. Our study is in agreement with observation because 2 out of the 6 patients with an age between 51-60 years old were readmitted in the hospital during the 7-10 postoperative days for reintervention. Both of them were under Acetaminophen treatment [10].

One of the 6 patients treated with Tramadol presented with postooperatory bleeding, he was included in the 41-50 years age group.

One patient treated with Ibuprofen was readmitted for post tonsillectomy pain despite the ibuprofen treatment. He was in the 31-40 years cohort group of patients.

Pain intensified in our patients during the 5th to 7th day. In this period, one of the patients (under Ibuprofen treatment) was readmitted because he presented dehydration as a consequence of the intensification of the pain (10 /10 in the questionnaire at day 6). The Tramadol group of patients had the best pain control (the highest grade was 5/10). The Acetaminophen proved to reduce pain after tonsillectomy (the highest grade was 7/10).

DISCUSSION

Post tonsillectomy pain is a significant morbidity among patients undergoing this intervention making the pain control and the risk of bleeding the most common concern among surgeons. Poor analgesia may cause dysphagia, dehydration and leads patients to emergency medical visits and readmissions [11].

Acetaminofen is widely used after tonsillectomy due to their analgesic effect comparable with the opioid but without the gastro-intestinal side-effects [12].

The Ibuprofen, a NSAID well known medication, has the ability to reduce pain and inflammation without the side effects of the opioid treatment. Yet, the use of ibuprofen after tonsillectomy remains controversial due to the increased bleeding time. A study by Krishna

et al. shows that the bleeding time in a patient with normal coagulation pathway remains normal under the use of the NSAID medication. This study is supported by many trials in the existing literature that assessed that the use of Ibuprofen is not influencing the post tonsillectomy bleeding [13,14]. Our study supports this theory. Only one of the 6 patients treated with Ibuprofen was readmitted in the hospital for the pain control (and not for a bleeding as we expected).

Opioid pain control has been used for the management of pain with reports of many side-effects and also fatalities in children as a result of genetically altered metabolism of codeine-morphine in the pediatric population [15].

Our research compared by a randomized trial the efficacy of Paracetamol vs Ibuprofen vs Tramadol for the treatment of post tonsillectomy pain in adults over a 21 days period. Our results indicated that the best pain relief was achieved by Tramadol. However, all the patients included in our study described the pain after tonsillectomy to be less intense than the pain they felt each time during the acute tonsillitis.

Our study has several limitations. It is based on a small population number and thus it lacks of statistical power. Further, a larger study has to be made.

CONCLUSION

The pain management of post tonsillectomy pain is challenging and needs to find a balance between the efficacy controlling of the pain and the possible side effects. Further larger studies are required to find the best adequate treatment with the less side effects.

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DUREREA POST-AMIGDALECTOMIE LA ADULŢI – STUDIU COHORTĂ

REZUMAT
Amigdalectomia este una dintre procedurile comune efectuate cel mai frecvent anual. Durerea este raportată la toți pacienții în perioada post-operatorie și persistă ca simptom pe intervale variabile de timp. Numeroși pacienți se adresează Serviciului de Urgență după amigdalectomie pentru a primi medicație antialgică. Scopul acestui studiu a fost de a compara efectul Ibuprofenului vs. Paracetamol vs. Tramadol în perioada post-operatorie de recuperare după amigdalectomie și legătura dintre aceste medicamente și sângerarea post-operatorie
Cuvinte cheie: tonsilectomie, AINS, acetaminophen, tramadol, sângerare

INTERACTIVE PEN DIGITIZER TO FOSTER TEACHING

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ABSTRACT

Background: Blended learning with chalk-board and PowerPoint is the most preferred method of teaching in Indian Medical College. With evolving technology, new digital learning aids are devised to spawn a better teaching method. Medical teachers keep them updated with newer teaching technologies and tools for better engagement of students in lecture. One such newer teaching aid with advanced technology is "interactive pen digitizer".

Objectives: To compare the two different teaching tools- "traditional chalkboard" versus "interactive pen digitizer" from the student's perspective

Methods: A prospective, cross-sectional, questionnaire-based study was carried out among first-year Indian MBBS students.

Results: A total of 49 students participated in the study, 33 (67%) participants were males, with a median age of 19.47 years and 16 (33%) participants were females with a median age of 19.39 yrs. 92% of Students preferred "digital pen digitizer as compared to traditional chalk and board.

Conclusions: Students preferred Pen digitizer over a traditional chalkboard for being more fascinating, more class-engaging, better summarization, memorization, and focused teaching with fewer distracters.

Keywords: chalkboard, pen digitizer, digital chalkboard, tablet, medical students.

INTRODUCTION

Teaching and learning are active and continuous processes. Since ancient times, the most common and preferred large group teaching method is lectures. A large group method to teach medical physiology is mainly didactics lectures. A good lecture is the most efficient way to assimilate and impart a large volume of information from multiple sources in a limited time. So planning the lecture is a crucial step. It should deliver the content stepwise, logically, utilize the time wisely, and focus on the important points. A blend of PowerPoint presentations added with chalkboard serves this basic motto. Many studies are conducted to compare these two teaching aids and a blend of both teaching aids is preferred by medical teachers and students [1-4].

With evolving, technology newer teaching modalities are introduced. New digital learning aids are devised for better teaching-learning. To improve the quality of teaching, medical teachers are progressively using the latest teaching-learning media and tools. One such newer teaching aid with advanced technology is "interactive pen digitizer". An interactive pen digitizer consists of a digital pen with a writing pad which can be connected to the laptop. The size of the writing pad is 12 inches which are light and easy to hold in one hand while writing. Handwritten notes and diagrams can be drawn with a digital pen and can be displayed on a laptop screen by using software like "Paint"/ "whiteboard. With pen digitizer, multiple colors and different tools like pencil, brush or shapes, etc. can be used. The laptop screen can be projected with the help of a projector. The matter written on the writing pad is displayed on the projector in real-time.

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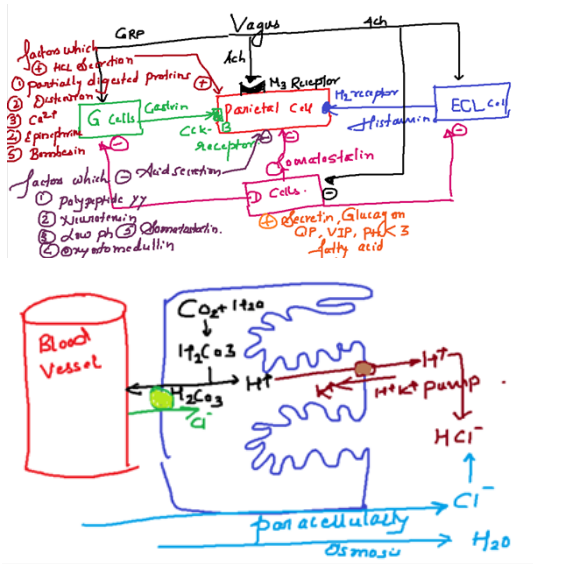
Thus pen digitizer is the digital form of Chalk and board with some added advantage. The impact of Tablet PC [5,6] (similar to pen digitizer) in teaching and learning has been investigated in many disciplines such as engineering [7-9] mathematics [10] science [11,12] and education [13,14].



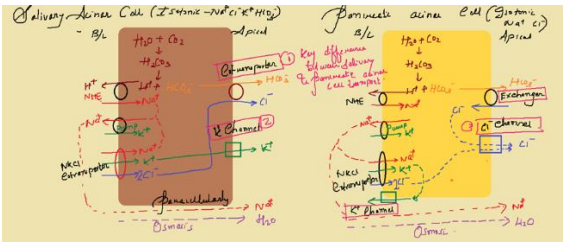


Fig. 1. Shows the screenshot of GIT Physiology taught by using the "digital pen digitizer/Digital Chalk and Board"

There are fewer studies in the medical field [5, 15] and no studies in Indian Medical Colleges comparing the traditional "chalk and board" versus "interactive pen digitizer". To determine medical student's perception comparing the traditional chalkboard with the pen digitizer, a prospective, cross-sectional, questionnaire-based study was carried out among Indian MBBS students.

METHOD AND MATERIAL

The prospective, cross-sectional study was conducted on 1st-year medical students. Written consent was taken from the students who were willing to participate in the study. A total of 49 (n = 49) 1st-year medical students participated in the study.

Renal physiology was taught to the students using the traditional chalkboard method. The gastrointestinal physiology was taught to the students using pen digitizer by the same faculty. After completion of renal and gastrointestinal physiology, a predesigned and pretested 16 item questionnaire consisting of five sections was given to the students. The internal consistency of questionnaire was 0.8. The answers were analyzed and expressed in percentages.

RESULT

Out of 50 students in the batch, 49 students participated in the study, 33 (67%) participants were males, with a median age of 19.47 yrs and 16 (33%) participants were females with a median age of 19.39 yrs.

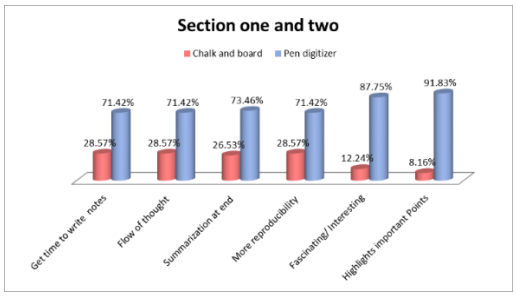


Fig. 2. Showing percentages of students preferring the teaching aids for various questions in section one and two

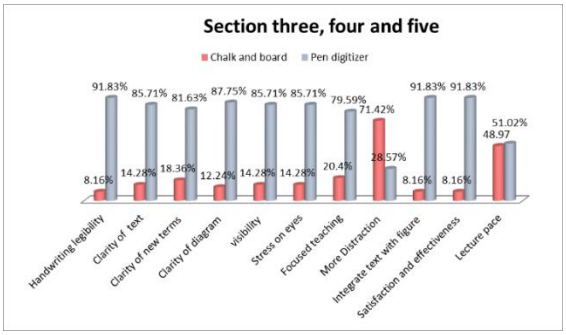


Fig. 3. Showing percentages of students preferring

the teaching aids for various question in section three four and five

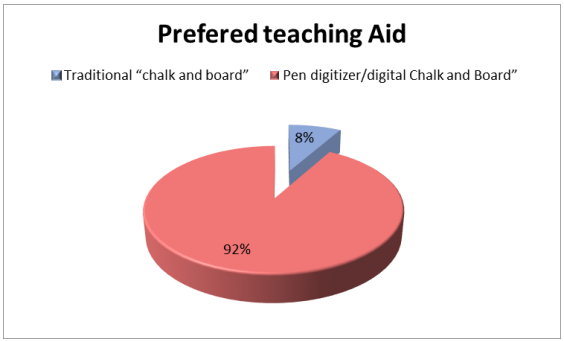


Fig. 4. Showing percentages of students preferring the teaching aids

92% Students preferred digital pen digitizer as compared to traditional chalk and board.

DISCUSSION

Section one asked the students to assess their learning capability with both tools. In this study, we observe that students were able to perceive the step-by-step sequential development of the concept better when the topic was taught with pen digitizer. This allows student's sufficient time to takes notes. These findings are supported by studies of Frolik et.al (2004) and Pomales-Garcia. et. al. (2007) and Stickel (2008) [16-19]. In pen digitizer, a good balance between writing and listening is achieved. Screenshots and screen recording of the class taught by pen digitizer can be saved and used to summarize the topic at the end of the class. These notes can be sent or emailed to the students which can be utilized by students to revise and memorize the topic [16, 20].

Section two addressed the use of multiple colors for teaching with both teaching tools. Limited color options are available with chalk and board. Pen digitizer provided the ability to deliver the traditional lecture using digital ink [21, 22]. Use of multiple colors and tools helped to highlights important points and increased the focus teaching [20, 23-25].

Section three addressed the visibility and clarity of the board. The legibility of handwriting, clarity of text, and diagram were better with the use of pen digitizer. Students were able to see the handwritten notes by pen digitizer on the projector screen from any corner in the classroom. This has decreased the stress on the eyes. These findings are supported by the studies done by Derting, *et. al*. (2008), and Walker *et. al*. [26,27]. In open-ended questions students reported difficulties in seeing the entire blackboard due to glare on some corners or reading notes written on the blackboard, distracted them from learning.

Section four asked the question about the student's concentration and distracters. The partial field of the blackboard is blocked by the teacher standing in front of the board while writing notes on the blackboard. It blocks the flow of thought and decreases the speed of notes taking by the students. It acts as distracters and decreased focus learning.

Teacher-student's eye contact facilitates the students to concentrate on the lecture. When the teacher faces students while teaching, it allows the teacher to track and estimate the student's comprehension and understanding [28]. But while writing on the blackboard the teacher has to face the blackboard. Thus the teacher cannot maintain eye contact with the students. The students concentrate in the class slash down and the attention span drives down in the class. Pen digitizer benefits teachers to face the students while teaching. The potential technological difficulties which can act as distracters using pen digitizer were documented in other studies. These are power cut, computer freezing, slow computer speed, discharged batteries of the stylus, etc. [20]. These difficulties were not encountered in our classes hence were not reported by our students.

Section five about the student perceptions for overall satisfaction and effectiveness between two methods, nearly all students agreed that the pace of the lecture was appropriate regardless of the method used and pen digitizer was the preferred teaching aid.

Students reported greater engagement and greater satisfaction with Pen digitizer as compared to traditional chalk and board. Pen digitizer is a promising tool and does not require a specific teacher training and preparation. It can switch between other applications during lectures and prevents messy chalk and dusty fumes from dry erase markers.

Hong Wang had reports that the use of tablet PCs with a digital pen or stylus has enhanced students' learning experiences through learner-interface interaction, learner-content interaction, learner - instructor interaction, and learner-learner interaction [29].

Although our study has limitations of small sample size and was conducted in single-institution, it provided initial evidence that the Pen digitizer enhances the effectiveness of didactic lectures. This teaching aid is preferred by the students. We recommend the medical teachers should utilize this technology for efficient lectures and better student's engagement in the class. In the breakneck changing world, newer educational technology can be an efficient tool for teaching and learning. It can be a component of a comprehensive system for lifelong

education. Further studies with larger sample size and multi-centric studies are required to independently corroborate our data.

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FOLOSIREA STILOULUI DIGITAL INTERACTIV IN PEDAGOGIE

REZUMAT

Învățarea combinată cu tablă și PowerPoint este metoda preferată de predare în Indian Medical College. Cu tehnologia mereu în evoluție, sunt concepute noi mijloace de învățare digitale pentru a genera o metodă de predare mai bună. Cadrele medicale sunt la curent cu noile tehnologii și instrumente de predare pentru o mai bună implicare a elevilor în cursuri. Un astfel de ajutor didactic mai nou, cu tehnologie avansată, este stiloul interactiv digital.

Obiective: comparatia intre două instrumente didactice diferite, o tablă tradițională si stiloul interactiv digital, din perspectiva elevului.

Metode: Un studiu prospectiv, bazat pe chestionar, a fost realizat în rândul studenților din India din anul întâi MBBS.

Rezultate: Un total de 49 de studenți au participat la studiu, 33 (67%) participanți au fost bărbați, cu o vârstă medie de 19,47 ani și 16 (33%) participanți au fost femei cu o vârstă medie de 19,39 ani. 92% dintre studenți au preferat stiloul interactiv digital cu stilou în comparație cu creta tradițională și tablă.

Concluzii: Studenții au preferat stiloul interactiv digital unei table tradiționalã, fiind mai atractiv și permițând o sintetizare mai bună a cunoștințelor, precum și memorizare mai buna și predare mai concentrată, cu mai puține distracții.

Cuvinte cheie: tablă, stilou digital, tablă digitală, tableta, studenți la medicină.

EVALUATION OF THE POSSIBILITY OF POSTPARTUM HEMORRHAGE IN PATIENTS WITH PLACENTAL STRUCTURE ABNORMALITIES

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Abstract

Obstetric hemorrhage is associated with an increased risk of severe maternal morbidity and mortality. Postpartum hemorrhage is the most common form of obstetric hemorrhage, and worldwide, a woman dies from massive postpartum hemorrhage about every 4 minutes.

In addition, after hemorrhage, many women experience severe morbidity, such as multiple organ failure, complications of multiple blood transfusions, peripartum hysterectomy, and unintentional pelvic organ damage, loss of fertility, and psychological sequelae, including posttraumatic stress disorder.

In this context, I consider useful a study to analyze the risk factors that were correlated with severe postpartum hemorrhage in probably the most common placental pathology associated with obstetric hemorrhagic syndromes, namely, placenta praevia, trying to establish a model for predicting postpartum severe hemorrhage., derived from clinical and imaging results, to guide obstetricians to accurately assess severe bleeding and to prepare treatment strategies for patients with severe postpartum hemorrhage.

Keywords: obstetric hemorrhage, postpartum hemorrhage, placenta praevia

INTRODUCTION

Anticipating massive postpartum hemorrhage, prompt recognition of the cause and the establishment of correct and appropriate measures to control bleeding [1,2] and replace lost blood volume and restore oxygen transport capacity (normal hemoglobin values) and correct the "washing phenomenon" / " washout phenomenon" that leads to coagulopathies will help save lives [3,4].

The obstetric shock index can help avoid underestimating blood loss and the use of tranexamic acid, oxytocics but also peripartum hysterectomy, performed in a timely manner, if and where appropriate, will help save lives [5,6,7].

Obstetric hemorrhage may occur before or after birth, but over 80% of cases occur postpartum [8,9,10]. Globally, massive obstetric hemorrhages, resulting from the failure of normal obstetric, surgical and / or systemic hemostasis, are responsible for at least 25% of the several hundred thousand maternal deaths each year [11,12].

Most women will not have identifiable risk factors [13,14,15]. However, the primary prevention of postpartum hemorrhage begins with an assessment of identifiable risk factors [16,17,18].

In this context, I consider useful a study to analyze the risk factors that were correlated with severe postpartum hemorrhage in probably the most common placental pathology associated with obstetric hemorrhagic syndromes, namely placenta praevia, trying to establish a model for predicting severe hemorrhage. postpartum, derived from clinical and imaging results, to guide obstetricians to accurately assess severe bleeding and to prepare treatment strategies for patients with severe postpartum hemorrhage [19,20].

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AIM AND OBJECTIVES

The impact on pregnancy in cases of placenta praevia is most likely reflected in both mother and fetus, each of which can frequently suffer severe complications due to it, especially increasing the risk of obstetric hemorrhage[5]. In addition, after hemorrhage, many women experience severe morbidity, such as multiple organ failure, complications of multiple blood transfusions, peripartum hysterectomy, and unintentional pelvic organ damage, loss of fertility, and psychological sequelae, including posttraumatic stress disorder [9].

In this research we set several specific objectives, which would allow the achievement of the initial proposed goal and the subsequent elaboration of conclusions derived from the results obtained from the study, with a high degree of objectivity and representativeness. Having as origin the desideratum evoked previously, we proposed that in this study to carry out in a first stage a clinical study, in order to investigate the existence of correlations regarding the influence of insertion and placental structure on pregnancy in general and on obstetrical hemorrhagic syndrome.

At a later stage of the research we considered it an objective to study the impact of clinical situations associated with obstetric hemorrhage, by careful analysis and correlation of these data with ultrasonography study and macroscopic morphological study of placenta. Finally, I proposed a comprehensive and integrated approach to these objectives, in order to generate a broad perspective on maternal-fetal management in the context of obstetric hemorrhagic syndrome associated with placenta praevia, in order to generate the main objective of this study, namely the generation of a model to be able to predict severe postpartum hemorrhage, a model derived from clinical and imaging information.

MATERIAL AND METHODS

We conducted our research study, performed both retrospectively and prospectively, between 2014 and 2019, on a study group consisting of 82 selected pregnant patients diagnosed with placenta praevia. The cases included in the study groups were selected from the case studies of the Obstetrics-Gynecology Department of the "Caritas" Hospital Roșiori de Vede, the private practice of the Obstetrics-Gynecology office "Dr. Caravețeanu Dragoș Cristian" and private practice of 2 other Obstetrics-Gynecology offices in Teleorman County. This study was conducted entirely in accordance with existing regulations in the field of scientific research, provided in both European and Romanian legislation but also according to the rules of the University of Medicine and Pharmacy of Craiova and by approving the Ethics Commission of the Senate of the University of Pharmacy from Craiova.

Blood loss was assessed using a combination of gauze weighing methods and blood clot collection, based on the postpartum hemorrhage management and prevention protocol issued by the Obstetrics and Gynecology Associations of Europe and Romania, was measured at the beginning of the skin incision for cesarean section or at the beginning of perioperative vaginal bleeding for cesarean section or at the beginning of parturition for vaginal birth and blood loss over 1,000 ml was defined as severe postpartum hemorrhage and was the only clinical observation in this study. The diagnosis for placenta praevia was made after 28 weeks of pregnancy using ultrasonography. Clinical suspicion of placenta praevia was maintained in the following cases: pregnant patient with vaginal bleeding at a minimum time after 24 weeks of amenorrhea, unprovoked associated with the absence of painful uterine contractions, with a normal uterine tone present, with oo dystocic presentation, with fetal heartbeats present and normal. In order to better outline the diagnosis, we associated the following associated conditions: intrauterine growth retardation; the presence of maternal risk factors, including a history of placenta praevia; premature rupture of membranes.

Based on the guidelines and recommendations published by the profile associations, we collected clinical information regarding postpartum hemorrhage, noting the following aspects: age; residence; level of education; assisted reproduction; pregnancy history; number of tasks; hours of birth; periods of miscarriage; history of vaginal birth; history of cesarean section; history of uterine surgery; history of surgical complications; history of premature births; history of placenta praevia; current information about the task; body mass index before pregnancy; number of pregnancy tests; the presence of uterine fibroids; the presence of anemia; the presence of thrombocytopenia; diagnosis of hypertension; amniotic fluid index at the last ultrasound; history of vaginal bleeding during pregnancy; bleeding volume; possible treatment with magnesium sulfate; fetal cephalic presentation; gestational weeks at termination of pregnancy; surgical anesthesia; vaginal blood loss; blood transfusion.

In developing the model for predicting the possibility of obstetric hemorrhage and given the case of the existence of medical units in disadvantaged areas, which do not have adequate ultrasonography equipment or in the case of massive hemorrhage, which do not allow the patient to move immediately to

a clinic benefit from such a facility, the formulation of the diagnosis of placenta praevia was also used based on the specialized clinical examination, obstetrical and especially the vaginal one.

Based on the guidelines and recommendations published by the profile associations, we collected information about the placenta related to postpartum hemorrhage, including the type of placenta: lower inserted placenta, the lowest location of the placenta at 2 cm from the internal cervical orifice; marginal placenta: the placenta that reaches the internal cervical orifice; partial placenta: part of the placenta that covers the internal cervical orifice; complete placenta: placenta that completely covers the internal cervical orifice); place of the placenta (the main body of the placenta that covers the anterior wall of the uterus, defined as the anterior wall of the placenta and the one that covers the posterior or lateral wall of the uterus defined as the posterior wall of the placenta); echo of the uterine placenta junction (spongy placenta; appearance of placental fissure: 4 or more regions with reduced echo, with a diameter greater than 5 mm in the placenta); the placenta covering the uterine-bladder junction (the uterine serous line is clear and continuous, the uterine serous line is discontinuous or not completely visible); abundance of blood vessels at the junction of the uterine placenta assessed under Color Doppler control.

Depth of the placenta invading the wall of the uterine muscle: based on the richness of vascularity at the echo of the junction of the uterine placenta, the junction of the uterine bladder covered by the placenta and the junction of the uterine placenta. The correlation was preliminarily assessed based on abnormal implantation, placental adhesion, placental implantation and placental penetration.

Statistical processing of results and comparative data analysis was performed using the following softwares: Microsoft Excel version 2007, Anova One Way, respectively SPSS version 22.0. Sublots analyze was performed by statistical indicators: weight, median, standard deviation. Testing the consistency of the samples was performed using the independent Student t-test, bilateral, and testing the combination of parameters by using the Chi square test of association. Statistical differences of the test were representative when the p value was <0.05 at a confidence interval of 95%.

RESULTS

Our results shows that from the total of 82 patients enrolled in the study, a number of 6 had a vaginal birth, while a number of 76 pregnant women included in the study group, gave birth by cesarean section. Analyzing the data that characterize the main characteristics of the study group, it is observed a predominance of patients from urban areas, namely 61 from the study group, the mean age was 31 years, the duration of gestation was in most patients included in the study under 39 weeks (over 90%) and only a number of 8 patients had over 39 weeks of pregnancy.

We assessed collateral pathology, identifying 6 patients with high blood pressure during pregnancy, 14 patients with preeclampsia, 1 patient presented high values of fasting blood glucose and subsequently, by consultation in the outpatient clinic, was diagnosed with gestational diabetes. Nevertheless we identified obesity in a number of 13 patients, the presence of abortion in the antecedents (13 patients), 4 patients from the studied group (representing 4.88% of the total) reported the loss of a pregnancy, while 1 patient from the studied group (representing 1.22% of the total) evoked the loss of two previous pregnancies. From the personal pathological antecedents, it was also highlighted that 2 patients from the studied group (representing 2.44% of the total) had venous thrombosis.

On the other hand, out of a total of 82 patients enrolled in the study, a percentage of 7.31% from the study group had a vaginal birth, while the rest gave birth by cesarean section (all of these patients were initially diagnosed with the lower placenta inserted).

The most important findings was reporting postpartum hemorrhages All the 82 patients included in our group presented postpartum hemorrhages, including a number of 21 patients, representing a percentage of 26% having severe hemorrhages, over 1000 ml, while a number of 61 patients, representing a percentage of the studied group of 74% had minor bleeding.

Regarding the age of pregnant patients included in the study group, in the case of patients with severe postpartum hemorrhage, the mean age was, according to statistics, about 32 years, with extremes of 23 years and 41 years, respectively, in while the age of pregnant patients included in the study group who had minor postpartum hemorrhage, the mean age was approximately 29 years, with extremes of 19 years and 38 years, respectively. There is a difference of about 3 years in favor of patients who had severe bleeding, the difference being maintained even in patients who had the extreme ages of the group.

Also, a number of 59 patients, of all those who had minor postpartum hemorrhage (61 patients) had a gestation period of less than 38 weeks of pregnancy,

while in the case of pregnant patients included in the study group who had severe postpartum hemorrhage, 90.48% of the total had a gestation period of less than 38 weeks of pregnancy.
We report a slight difference of about 6 percent in favor of patients with minor bleeding, but without a strong statistical significance and a higher percentage in favor of patients born by caesarean section and had major postpartum hemorrhage.
In the statistical calculations we also took into account a whole series of other clinical indices in the prediction model of postpartum hemorrhages, among which history of premature birth 2 patients with minor hemorrhage, and a patient with severe hemorrhage, history of placenta praevia, one patient with severe hemorrhage, the presence of uterine fibroids one patient with severe hemorrhage.
We also observed a higher percentage in favor of patients born by caesarean section and had major postpartum hemorrhage, both to patients with natural birth and major postpartum hemorrhage, and to both subcategories (natural birth and cesarean section) within patients with minor bleeding studied. According to clinical evaluations, a number of 32 patients with minor hemorrhage, representing a percentage of 52.46%, had anemia in pregnancy. Regarding the patients with severe hemorrhage, included in the studied group, anemia during pregnancy was diagnosed in a number of 11 patients, but representing a percentage of 52.38%. Of all the patients included in the study group, a number of 9 patients had a history of bleeding in the last trimester of pregnancy, of which 6 patients, representing a percentage of 9.84% included in the subgroup with minor hemorrhage, while 3 patients, representing a percentage of 14.29% included in the subgroup with severe hemorrhage, having a statistically significant higher percentage.
The primary factor we reported in this study was, as it results from the formulation of the working hypothesis and the establishment of batches, the volume of postpartum hemorrhage, which was calculated according to the algorithm presented in the chapter Material and methods. From the evaluations of obstetricians and doctors of anesthesia and intensive care, the following were observed: the patients with minor hemorrhage included in the studied group presented an average value of blood loss of 647 ml with extremes between 367 ml and 927 ml respectively; the patients with severe hemorrhage included in the studied group presented an average value of blood loss of 2145 ml with extremes between 1346 ml and 3332 ml.
From the point of view of the type of placenta, evaluated by ultrasonography and according to the classifications taken in the clack, the patients considered in the study group presented: lower inserted placenta - 17 patients with minor hemorrhage, one patient with severe hemorrhage; marginal praevia placenta: 4 patients with minor hemorrhage, one patient with severe hemorrhage; partial praevia placenta - one patient for each, minor and severe hemorrhage; central placenta praevia - 39 patients with minor hemorrhage, respectively 18 patients with severe hemorrhage.
Thus, patients with minor hemorrhages presented a distribution of the placenta type as follows: 27.87% - lower inserted placenta, 6.56% - marginal praevia placenta, 1.64% - partial praevia placenta, 63.93% - praevia placenta central. Also, the patients with severe hemorrhages presented a slightly different distribution, but keeping the hierarchy of the placenta type as follows: 4.76% - lower inserted placenta, 4.76% - marginal praevia placenta, 4.76% - partial praevia placenta and the vast majority 85.72% - central placenta praevia.

DISCUSSION

Logical stepwise regression analysis of risk factors indicated that age, duration of birth, history of vaginal birth or cesarean section, weeks of pregnancy, depth of implantation of the placenta invading the uterine muscle wall, and blood transfusion during birth were risk factors, independent for severe postpartum hemorrhage.
We found that blood transfusions during birth were an intraoperative variable and should be predetermined before birth when variables are included to determine the pattern of bleeding. Therefore, it should be omitted when establishing the model. The logical regression prediction model was used to predict severe postpartum hemorrhage after random sampling using the R language, setting the discriminant limit value of the model, with a probability between 0 and 1 (0.3). In other words, after entering each variable in the model equation, if the calculated probability is greater than 0.3, it denotes a higher occurrence of severe postpartum hemorrhage, while a value lower than 0.3 denotes a lower probability.
The equation of the P prediction model of the logistic risk was as follows:
P = Log (F / 1 - F), where F (hemorrhage factor) = −6.9 + 0.075 x V (age) + 1.5 x P (first birth = 1, multiplied by the number of pregnancies) - 3 .5 x N (vaginal birth: 1 for yes and 0 for no) + 1.7 x N (duration of pregnancy - 0 for <38 weeks, 1 for ≥38 weeks) + 1.5 x D (relationship between placenta and wall uterine muscle: 0 for normal, 1 for placental adhesion, 2 for placental implantation, 3 for placental penetration) + 0.2 x A (miscarriage = 1, no = 0).

For example, a 30-year-old pregnant woman (V = 30), who gave birth once (P = 1), vaginal birth 0 (V = 0), gestational weeks at termination of pregnancy 37 (N = 0), normal in the placenta (P = 0), miscarriage once (A = 1) an F value of: F = −6.9 + 0.075 × 30 + 1.5 × 1 - 3.5 × 0 + 1.7 × 0 + 1.5 × 0 + 0.2 x 1 = −2.95, F was introduced into the equation logic to obtain P = 0.0434 <0.3, indicating a lower chance of severe postpartum hemorrhage.

To verify the effectiveness of the logistic risk prediction model equation, 15 patients were randomly selected from the total (n = 82) to determine the verification rate. The results showed that the sensitivity was = 0.73 (negative prediction accuracy rate), the specificity was = 0.9 (positive prediction accuracy rate), and the total accuracy rate was 0.8.

CONCLUSION

The established model we generated predicted severe postpartum hemorrhage in patients with placenta praevia based on independent risk factors for severe postpartum hemorrhage and included clinical and ultrasonography data. The sensitivity and specificity of the model was 73% and 90%, respectively.

Obstetricians could effectively use this model to determine the risk of severe postpartum hemorrhage in patients with placenta praevia.

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EVALUAREA POSIBILITĂȚII DE HEMORAGIE POST-PARTUM LA PACIENȚII CU ANOMALII DE STRUCTURĂ PLACENTARĂ

Rezumat
Hemoragia obstetricală este asociată cu un risc crescut de morbiditate și mortalitate maternă severă. Hemoragia postpartum este cea mai frecventă formă de hemoragie obstetrică și, la nivel mondial, o femeie moare din cauza unei hemoragii masive postpartum aproximativ la fiecare 4 minute.
În plus, după hemoragii, multe femei suferă de morbiditate severă, cum ar fi insuficiența multiplă a organelor, complicațiile transfuzionale multiple, histerectomie totală și afectarea involuntară a organelor pelvine, pierderea fertilității și sechelele psihologice, inclusiv tulburarea de stres posttraumatic.
În acest context, considerăm util un studiu pentru a analiza factorii de risc care au fost corelați cu hemoragia severă postpartum în probabil cea mai frecventă patologie placentară asociată cu sindroamele hemoragice obstetricale și anume placenta praevia, încercând să stabilim un model de previziune a hemoragiei severe postpartum, derivat din rezultatele clinice și imagistice, pentru a ghida obstetricienii să evalueze cu precizie sângerările severe și să pregătească strategii de tratament pentru pacientele cu hemoragie severă postpartum.
Cuvinte cheie: hemoragie obstetricală, hemoragie postpartum, placenta praevia

DEVELOPMENT OF A MINIATURIZED METHOD FOR ESTIMATING ARISTOLOHIC ACID I (AAI) IN ENVIRONMENTAL SAMPLES

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ABSTRACT
Aristolochic acid I (AAI) is known as the principal poisonous nitrophenanthrenic compound belonging to the big group of aristolochic acids (AAs), naturally produced by the *Aristolochia* and *Asarum* genus.This phytotoxin is considered the main causative factor involved in Balkan endemic nephropathy (BEN), a deadly environmentally-induced chronic kidney disease present in particular Balkan locations, including few villages from Romania. In this article, we highlighted various types of extraction techniques used for AAI detection and quantification in different environmental samples, developed for specific chromatography methods. In order to extend the scope of our previous studies regarding the occurrence and distribution of AAI contamination in "soil-plant-environment" systems, we proposed a novel sensitive method of miniaturized extraction techniques: in-tube solid-phase microextraction (IT-SPME) coupled with capillary liquid cromatography (Cap-LC). Chromatographic separation and quantification of AAI were carried out under isocratic conditions, using a particulate LC column (Zorbax SB-C18) and DAD connected to the capillary column (TRB-35) by means of a switching valve. Linearity ranged between 10–100 ng/ml, achieving a limit of detection (LOD) of 8.12 ng/mL. The proposed method is cost effective to determine AAI in environmental samples, has good analytical characteristics and allows the identification and quantification of AAI in appropriate concentrations to control environmental quality. The characterization of samples can be achieved in less than 10 minutes.
Keywords: Aristolochic acid I; Balkan endemic nephropathy; Environmental samples; Extraction; Liquid chromatography

1. INTRODUCTION

Data collected over the past sixty years indicate that certain rural villagers from the Balkan countries along the Danube River (Romania, Serbia, Bulgaria, Bosnia and Croatia) are affected by a particular renal disease known as Balkan endemic nephropathy (BEN). The most distinguishing features of BEN are: its endemic nature, affecting residents from certain rural farming villages in the Balkan area, long-incubation period and a familial, but not inherited, pattern of disease; occurrence in adults but not children; and a close association with carcinomas of the upper urinary tract (UTUC) [1-7].

BEN is a unique type of gradual chronic renal disease, with arguable etiology, but throughout the time several factors have been speculated: Pliocene lignite, heavy metals, fungal toxins and polycyclic aromatic hydrocarbons (PAHs) without any concluding results [1-5]. In 1969 Ivic was the first to propose another etiologic factor, aristolochic acids (AAs), coming out from the widespread toxic *Aristolochia* plants and therefore entering in contact with the food around and contaminating

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it [8]. The nephrotoxicity of the AAs became clear in the 1990s, in Belgium, when over 100 young consumed slimming herbal preparations made from *Aristolochia* species and accused symptoms of renal disease [9,10]. This rapidly progressive kidney disease was originally named Chinese herb nephropathy (CHN) and later renamed aristolochic acid nephropathy (AAN), having similar clinical and morphological parameters. [11].

Aristolochic acids (AAs) are a group of nitrophenanthrene carboxylic acids, naturally found in the *Aristolochia* species, classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC), therefore some countries banned the commercialization of AA-containing herbal medicines [12].

In the past ten years, different groups of researchers further proposed and consolidated the hypothesis that BEN is an environmentally induced disease by demonstrating the toxicity of AAs and their occurrence in food and the environment [13-15]. Environmental samples such as: soils, common vegetables and drinking water were analyzed and confirmed the existence of AAs in rural food crops around the endemic BEN areas or where *Aristolochia clematitis* grows [16-23]. As a result AAs-containing weed comingling with the food crops around (cucumber, maize, grain, wheat) [16,17] was found to be responsible of environmental exposure AAs and chronic dietary poisoning [14-23].

Due to the high toxicity, the quantification of AAs has an obvious significance for the prevention of adverse events through early detection. In recent years, a number of methodologies have been developed for the detection and quantification of AAs extracts [24].

Although many articles have been published on this topic, the separation efficacy needs to be further improved, especially for the analysis of real samples. Most of the proposed methods are used to quantify AAs in herbal remedies, which contain relatively high concentrations of AAs, but they may not be sufficiently sensitive to detect low levels of AAs in the complex matrices of food and environmental samples [25].

BEN represents a public health problem, so there is an increasing interest to develop a simple and sensitive detection method for emerging pollutants like aristolochic acids (AAs) that are affecting sporadic populations in the Balkan area. *Aristolochia clematitis* plants are considered the main cause of BEN, so controlling the growth of this weed, together with prevention and remediation methods would necessary to fight this deadly disease [26].

Nowadays there is a general trend in analytical methods towards simplification and miniaturization of the sample preparation process, which reduce the sample handling and analysis time, as well as the consumption of large volumes of solvents and the generation of wastes. In this sense, in-valve in-tube solid phase microextraction (IT-SPME) has emerged as a reliable technique for the online enrichment of analytes from environmental samples [27-29]. Therefore, in this study we are proposing a new minuaturized extraction technique in order to achieve good separation and sensitivity for the determination of AAI in environmental samples.

2. MATERIALS AND METHODS

2.1 Reagents and materials

All chemicals and reagents were of analytical quality and were used without further purification.The standard AAI was purchaised from Sigma-Aldrich (St. Louis, Missouri,USA). The standard solution of AAI was prepared in methanol at the concentration of 70 mg/mL. Working solutions of AAI were obtained by dilution of the stock solutions with water. All solutions were stored in amber glass bottles at 4◦C. Methanol, acetonitrile and formic acid were purchased from Merck (Darmstadt, Germany). High purity water was prepared using a Milli-Q water purification system (Millipore, Milford, MA, USA).

2.2 Instrumental analysis & Chromatographic conditions

The capillary chromatographic system consisted of a LC isocratic capillary pump (Agilent) and a UV diode array detector (Agilent 1260, Palo Alto, CA, USA) with six-port injection valveequipped with a GC capillary column (TeknokromaTRB-35) which acted as the injection loop. The detector was linked to a data system (Agilent, HPLC ChemStation) for data acquisition and calculation. The Zorbax SB-C18 analytical column (150 mm x 0.5 mm i. d., 5 um) purchased from Agilent was used for the separation of the analyte (AAI). The column was eluted with a isocratic binary solvent system consisting of acetonitrile:water (50:50) plus 0.1% formic acid, at a flow rate of 20 uL/min. The excitation and emission wavelengths of the UV detector were set at 315 nm.

2.3 Extraction techniques used for aristolochic acid I (AAI)

2.3.1 Solvent extraction

In order to efficiently extract AAI, several factors have to be considered in this process, starting with the sample preparation. A pre-cleaning step of the samples before the extraction process would be essential to reduce interactions with other compounds [30-32]. The extraction method should be non-selective in order to avoid destroying the structure of the chemical compounds. From all extraction methods, solvent extraction is the most widely used and methanol is most commonly reported to be used in AAs extractions [33-35]. The yield of AA extraction depends on the type of solvents, the volume of the solvent, the extraction time and the temperature. Among the solvents with variable polarities, the highest yields of AAs were obtained with 70% methanol in

aqueous medium. Extraction conditions were optimized by investigating the efficiency of different extraction methods (ultrasound versus reflux). Sonication with methanol, a method often reported in the literature, has proven to be simple and effective [31-36].

The analysis and quantification of AAs can be done by developing a method that takes into account the properties of AAs but also the matrix, which can be a raw extract, medicinal preparation, herbal supplement or other environmental sample. Therefore, over the past years, several extraction methods have been proposed [37]. In our previous studies, we employed different chromatography methods (HPLC, LC-MS and GC-MS) in order to analyze environmental samples (soils and vegetables)

For the case of HPLC and UHPLC-MS analysis, we employed a simple extraction method adapted after Trujillo *et al*. [36] where we mixed 5 grams of sample with 3 mL extraction solvent, which consists of: 80% methanol and 20% aqueous solution with 10% formic acid. The solid-liquid mixture was centrifuged at 4000 rpm for 10 minutes and then the supernatant was collected and filtered through a 0.45 µm polyethersulfone (PES) filter before injection into HPLC and UHPLC-MS [19].

Another extraction technique used for GC-MS analysis involves 1:1 ratio of weight of sample:volume of methanol. After combining the two components, we obtained a mixture that was heated to 350 Watts in a microwave oven in two rounds of 30 seconds. In order to separate the extract, the samples were centrifuged at 2000 rpm for 10 min, then the supernatant was collected and evaporated to a continuous stream of nitrogen. The samples were resuspended in 20 µL methanol and vortexed to obtain a homogeneous content and then injected into the GC-MS [38].

2.3.2 IT-SPME extraction

IT-SPME (*In-Tube Solid Phase Microextraction*) extraction technique was carried out using a capillary column of the type TRB- GC 35 (Teknokroma, Barcelona, Spain) with the length ranging from 40 to 70 cm, covered with 35% diphenyl and 65% polydimethylsiloxane (3 µm coating and 97 µm thickness) connected in a six-port injection valve, replacing the injection loop. Capillary connections to the valve were facilitated by the use of 2.5 cm sleeve of 1/16 in polyether ether ketone (PEEK) tubing and ferrules (Teknokroma, Barcelona, Spain).

Following preliminary studies, the volume of processed samples was optimized. For this aim, we tested volumes between 25-1500 µL. In the optimized procedure, aliquots of 500 µL standards and samples were injected manually using a high-precision syringe (Hamilton, Bonaduz, Switzerland) a volume of 500 µL into the extraction column of the IT-SPME device. Finally, the injection valve was rotated manually, so the analyzes were transferred directly to the LC system through the capillary analytical column, so that extraction, preconcentration, separation and detection are performed in a single step, using dynamic desorption. The mobile phase is passed through the capillary column and the desorbed analytes are sent to the injection valves of the chromatographic system, following the normal circuit of the Cap-LC system [27].

3. RESULTS AND DISCUSSION

3.1 Method calibration and validation

The standard AAI solution was prepared in methanol at a concentration of 70 µg/mL. AAI working solutions were obtained by diluting the stock solutions with distilled water. All solutions were stored in amber glass vials at 4°C.

Detection and quantification of AAI was possible for all extracted samples, which were analyzed by Cap-LC method with UV detection, a calibration curve with serial dilutions was performed daily, in the range of concentrations 10 - 100 ng/mL. The AAI quantification was based on several key parameters obtained from the calibration curve. Calibration curves for AAI were linear in the concentration test range and were generated by graphically representing the area of AAI peaks relative to AAI concentrations of working standards (Figure 1). The detection limit (LOD) was calculated at 8.12 ng/mL, obtaining a signal-to-noise (SN) ratio of 3:1 and a quantification limit (LOQ) of 24.61 ng/mL. The accuracy and precision of the method was less than 15% and the correlation coefficient R^2 was higher than 0.99.

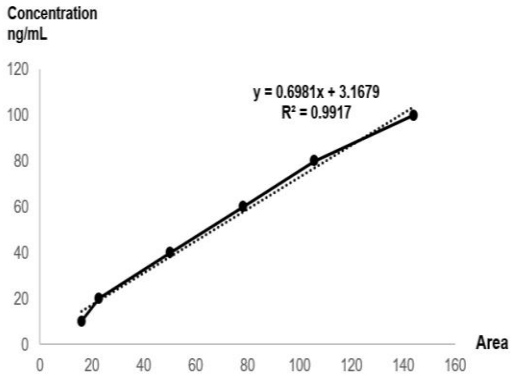

Fig. 1. Calibration curve for AAI with the equation of the line (y) and the regression coefficient (R^2)

3.2 Analytical performance of the proposed method by IT-SPME coupled with Cap-LC

After several attempts to find the best combination of mobile phases, taking into account the properties of AAI, we concluded that an acetonitrile-water mixture (50% - 50%) in the isocratic mode and a pH adjustment using 0.1% formic acid gave the best results in terms of separation of the analyte and peak intensities from the chromatograms obtained. The flow rate of the tested mobile phases ranged between 5 and 20 µL/minute, and the flow rate of 20 µL/minute proved to be suitable for a good intensity and resolution of the chromatographic peak. To choose the optimal value for analyte detection we used the Zorbax C18 column specific for hydrophobic compounds. Another analytical parameter studied, the injection volume, we started with injections with a minimum volume of 25 µL, then we gradually increased to 50 µL, 100 µL, 500 µL to large volumes of 1 mL to 1.5 mL. Although the highest intensity is directly proportional to the highest volume, in analytical chemistry there must be a compromise when it comes to quantities, volumes and intensities, because even if we apparently get a very good sensitivity. Therefore, the optimal result was reached at a volume of 500 mL, as can be seen from the graph in Figure 2. Making these settings for optimization, AAI was eluted before 10 minutes.

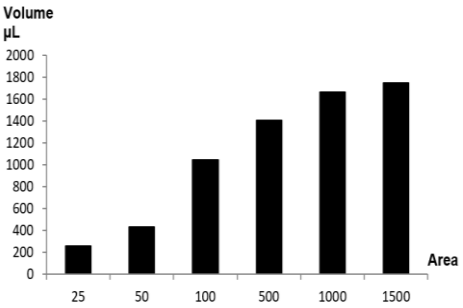

Fig. 2. Bar plots for the volume of injection and the corresponding area

The IT-SPME coupled with Cap-LC system has many advantages in terms of analytical performance, being suitable for environmental samples, such as the samples in the present study. Using dynamic desorption, the analyte (AAI) is extracted and preconcentrated inside the capillary column covered on the inside with a thin film of extractive phase and then the analyte is transferred to the analytical column using the mobile phase by switching the valve position, thus separating and detection of the compound. In our experiments, we replaced the stainless steel injection loop of a conventional injection valve with a GC capillary column covered with a phase of 35% dimethylpolysiloxane 65% diphenylpolysiloxane, considered suitable for AAI extraction, being a hydrophobic compound.

The final optimal conditions for the detection and quantification of AAI standards were: acetonitrile-water mixture (with 0.1% formic acid) in a proportion of 50%:50%, injection volume 500 µL, flow rate of 20 µL/minute. The addition of only 0.1% formic acid as a pH modifier significantly increased AAI peaks. The following image (Figure 3) shows overlapping chromatograms of the 100 ng/mL AAI standard solution, injected in the same concentration several times, and immediately after each injection, a *clean-up injection* was performed directly into the capillary tube with volumes of water between 50 and 100 µL. This procedure is well known and is applied to increase the sensitivity and resolution of the analyte peaks. Optimal results were obtained by injecting 50 µL water immediately after injecting the standard or sample, observing a proportionality in terms of peak intensity but also retention time (RT = 6.8 minutes).

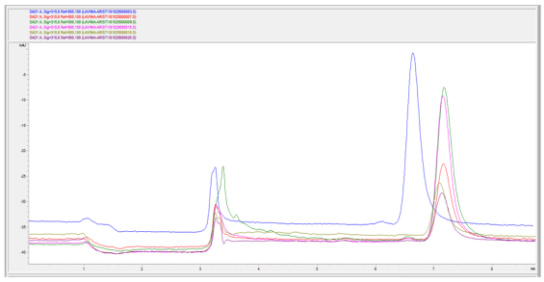

Fig. 3. Chromatograms obtained from the same concentration of 100 ng/mL AAI standard solution with different volumes (0, 50, 100 µL) of clean-up injections with water

3.3 Comparison of the new method with previously reported methods

Current approaches for AAs separation detection and quantification include chromatographic methods, mainly liquid chromatography (LC) coupled with ultraviolet (UV), fluorescence (FLD) detection or mass spectrometry (MS) [16-23]. Widely, HPLC is considered the routine method

for detecting AAs and specialized articles identified a detection limit (LOD) at the nanogram levels [18,19,22,23]. Most HPLC methods focus on AAI and/or AAII (aristolochic acid II) analysis in various samples, from the simplest to the most complex. Throughout the process of optimization of analytical methods, which involves the composition and percentage of mobile phases, pH and analytical columns, the separation and quantification of AAs is increasingly accurate [18-23,39]. A better analytical sensitivity for the detection of AAI and AAII in extracts was obtained with a liquid chromatograph coupled with tandem mass spectrometry (LC-MS/MS), using an ion trap MS (MS-IT), but the best characteristics Ionizations were obtained using atmospheric pressure chemical ionization (APCI) and by including ammonium ions in the mobile phase. Detection limits for AAs are also influenced by interferences with the sample matrix [40-42].

The toxic contaminants, AAs, have multiple sources of entry into the human body: soil - plant - water - food - air, therefore they have been declared extremely toxic compounds by IARC [12]. To support these claims, new improvements in chromatographic methods in terms of selectivity and sensitivity have been able to detect AAs at low concentrations in all these environmental samples, which could help to understand the entry of AAs into the human body.

Studies started by Ivic [8] and continued by Hranjec et al. [40] demonstrated that *Aristolochia clematitis* seeds have an increased content of AAs and deduced that these phytotoxins have an imperative role in causing BEN. Afterwards, many research groups have followed the same direction and developed modern chromatographic methods to identify and detect AAs in various environmental samples: extracts from Chinese plants [36,44], soils [18-22,39], groundwater [23] and plants cultivable in crop fields: wheat and corn [18,22,45]. The detection of AAs was studied in vegetables grown in laboratory conditions: peas and cucumbers [16] and lettuce, spring onions and tomatoes [17]. In recent years, several extraction methods have been proposed using large volumes of solvents.

For the same comparative purpose, we designed the table below (Table I), which contains a summary of modern analytical methods used to extract, detect and quantify AAs from various samples or matrices, but not only from the Balkan countries but from around the world. The genus *Aristolochia* includes up to 600 different species, present mainly in Asia, and in Romania the characteristic species is *Aristolochia clematitis* . Due to the ubiquity of this genus of plants, comes the similarity between the three types of kidney disease with almost identical pathology: BEN, AAN and CHN. Table 1 focuses on one of the most important analytical parameters, LOD (limit of detection), which is the minimum concentration at which the signal can be detected for a specific analyte. The detection techniques generally used are chromatographic of various types. The matrices or samples from which the extraction is made are largely environmental samples or biological samples. LODs vary from 0.02 ng/mL to 0.03 ug/mL. Good sensitivity of 0.02 ng/mL was reached by adding Zn/H+ to induce nitroreduction, so AAs convert into their respective aristolactams (AL), which dramatically enhances their analytical sensitivity [17-21,33-36,44-53].

Table I. Table comparing various modern methods used for estimating AAI in different types of samples; Principal characteristics of analytical methods and parameters.

Sample/ Matrix	Extraction method	Detection technique	LOD	Reference
Environmental samples (soils/vegetables)	**IT-SPME**	**Cap-LC**	**8.12 ng/mL**	**Current method**
Chinese herb soup	MSPE (Solid phase magnetic extraction)	HPLC	0.05 µg/mL	[46]
Soils from Serbia (Endemic area)	Solvent extraction (methanol: water: acetic acid = 70:25:5)	LC-MS / MS (Zn / H) Transformation AAI -> AL I (Aristolactam I)	ALI: 62.5 ng/kg	[20]

Wheat grains, corn grains and soil (Endemic area)	Extraction solvent (methanol: water:acetic acid = 70:25:5)	LC-MS / MS (Zn / H) AAI -> AL I	AL I: 0.02 ng/mL	[21]
Plants, medicinal plants	MPE (Multiphase Extraction) - Methanol: Water = 60:40	HPLC	2.4 µg/g	[47]
Soil and SOM (soil organic matter)	Extraction solvent (80% m ethanol 20% aqueous mixture (90% water 10% formic acid))	HPLC, MS	100 ng/mL	[19]
Medicinal plants, weight loss supplements	Extraction solvent (m ethanol)	SIA (Sequential Injection Analysis) and CL (Chemiluminescence)	3 ng/mL	[48]
Medicinal herbs	Extraction solvent (70% m ethanol)	SPE (Solid Phase Extraction) and HPLC	0.03 µg/mL	[49]
Wheat grains, corn grains, soils	The extraction solvent (methanol: water: acetic acid = 70:25:5)	HPLC-FLD and UPLC - MS / MS	3.3 ng/g	[18]
Lettuce leaves grown in soil contaminated with AAs (laboratory conditions)	Extraction solvent (methanol: water: acetic acid, 70:25:5)	HPLC-FLD	0.73 ng/mL	[50]
Chinese herbal medicines	Extraction solvent (methanol)	LC-FLD	0.271 ng/mL	[51]
Herbal food supplements	QuEChERS solid phase extraction method (deionized water 2% formic acid, acetonitrile, buffer salts)	UHPLC-MS	1 ng/mL	[52]
Herbal products, biological samples (rat serum)	Extraction solvent (m ethanol)	UHPLC-MS / MS	0.14 ng/mL	[53]
Biological samples (rat urine and plasma)	Extraction solvent (Methanol: Water: Acetic acid = 70: 25: 5)	HPLC-FLD	0.09 ng (urine) 0.07 ng (plasma)	[154]
Medicinal herbs	Extraction solvent (methanol: water: acetic acid = 70: 25: 5)	LC-FLD	0.39 ng / mL	[44]

4. CONCLUSION

In this work, IT-SPME coupled to Cap-LC with DAD detection has been proposed to the determination of AAI in environmental samples such as soils and vegetables. First, the experimental conditions were optimized in order to achieve the proper selectivity and sensitivity. Under the optimal conditions, the described method offers good accuracy and reproducibility, lineal answer ($R^2 > 0.99$) and precision (RSD < 15%) are reached. Finally, high sensitivity is achieved by achieving lower detection limit can be reached with the processing of large sample volumes. Some alternative strategies such as increasing the in-tube capillary length or the size of the loop of the valve can be applied particularly if lower detection limits want to be reached. It joins the advantages of both systems allowing high speed time analysis since separation is achieved in less than 10 minutes.

The proposed method is cost effective to determine AAI in environmental samples, has good analytical characteristics and allows the identification and quantification of AAI in appropriate concentrations to control environmental quality. Moreover, the considerable reduction on the amounts of sample and chemicals in reference to other procedures proposed in the literature. Compared with previous studies done by other research groups, this method is a good alternative.

Although many papers have been published in recent years, the efficiency of separation needs to be further improved, especially for the analysis of real evidence. Most of the proposed methods are used to quantify AAs in herbal mixtures, which contain relatively high concentrations of AAs, but may not be sensitive enough to detect low levels of AAs in complex matrices of environmental samples. There is a general trend in analytical methods towards simplification and miniaturization of the sample preparation process, which reduces the time of handling and analysis of samples, as well as the consumption of large volumes of solvents and waste generation.

This study on chromatographic methods for complex environmental analysis has a major impact on the health of certain vulnerable or exposed people, but also on the chemistry of the environment. Further studies would be useful to clarify the role of soil contamination with other phytotoxins or environmental factors involved in the etiology of BEN.

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DEZVOLTAREA UNEI METODE MINIATURIZATE PENTRU ESTIMAREA ACIDULUI ARISTOLOHIC I (AAI) ÎN PROBE DE MEDIU

REZUMAT

Acidul Aristolochic I (AAI) este cunoscut ca principalul compus nitrofenantrenic otrăvitor aparținând grupului mare de acizi aristolochici (AA), produs în mod natural de genul *Aristolochia* și *Asarum*. Această fitotoxină este considerată principalul factor cauzal implicat în nefropatia endemică balcanică (BEN), o boală cronică renală cronică indusă de mediu, prezentă în anumite zone balcanice, inclusiv în câteva sate din România. În acest articol, am evidențiat diferite tipuri de tehnici de extracție utilizate pentru detecția și cuantificarea AAI în diferite probe de mediu, dezvoltate pentru metode specifice de cromatografie. Pentru a extinde sfera studiilor noastre anterioare privind apariția și distribuția contaminării cu AAI în sistemele „sol-plantă-mediu", am propus o nouă metodă sensibilă a tehnicilor de extracție miniaturizate: microextracția în fază solidă în tub (IT-SPME) cuplat cu cromatografie lichidă capilară (Cap-LC). Separarea cromatografică și cuantificarea AAI au fost efectuate în condiții izocratice, folosind o coloană LC sub formă de particule (Zorbax SB-C18) și DAD conectate la coloana capilară (TRB-35) prin intermediul unei supape de comutare. Liniaritatea a variat între 10–100 ng/mL, atingând o limită de detecție (LOD) de 8.12 ng/mL.

Metoda propusă este economică pentru determinarea AAI în probele de mediu, are caracteristici analitice bune și permite identificarea și cuantificarea AAI în concentrații adecvate pentru a controla calitatea mediului. Caracterizarea probelor poate fi realizată în mai puțin de 10 minute.

Cuvinte cheie: acid Aristolochic I; Nefropatie endemică balcanică; Eșantioane de mediu; Extracţie; Cromatografie lichidă