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A winter song in the black forest

Sanda-Maria Copotoiu*
Editor-in-Chief

At the beginning of a new year, the editors decided to publish an article dedicated to the emergency rescue system in Romania. It is basically not only a brief recall of the history of the airborne medical system in Romania, but also an update on the assets and the performances of the medicalized helicopters. Twenty-eight years following the establishment of the SMURD in Romania, and fifteen years from the birth of the helicopter fleet in Romania, airborne crews experienced the leapfrog from debutants to experienced, consolidated medical teams.

I remember that as a lecturer at the first Pan European Emergency Medicine Conference in Budapest in 1992, I was in the process of arranging the meager slides I had to illustrate the teaching of Emergency medicine in ROMANIA and the means of transportation at the time. Besides me stood an Italian lady doctor taking pride in manipulating some 50 colored slides picturing among others, the Italian helicopter fleet. I asked her to lend me some in order to resuscitate my lecture, but she did not concur. I am sure she could not imagine it was a joke, a self-persiflation, and decided to completely ignore me. I had courage enough to step up and support the beginnings of what developed subsequently in the contemporary national emergency system. Ten years later, the Romanians benefitted from the first fleet composed of nine helicopters and two airplanes.

The article authored by Sebastian Tranca et al., hosted in this issue of the AMM, is dedicated to the description of the emergency air rescue system in our country. I will not go into details for the article is worth it to be read, but I cannot forget the helicopter's crew who crashed in Cojocna, near Cluj in 2003, in a mission that transformed a charismatic medical team in heroes. Helicopters used to perform salvage missions even before, and I remember a stunned child of six petrified in front of a cherry red helicopter parked besides the SMURD building in Tg.Mures. It was in 2000 that the pilot of the chopper invited the bewitched little boy to stay by his side in the fantastic shiny flying machine. Due to the boy's bewilderment and to the understanding of the pilot, this child is a today in his final year of medical studies to graduate at the Faculty of Medicine in Tg.Mures. Sadly enough, the magnanimous pilot became a hero of the airborne fleet.

An image of an helicopter over a difficult to reach area reminded me how appropriate seem to be Nichita's Stanescu lyrics:

"And no one passes-
only the white suns revolve in quiet worship.
and the thought spreads in circles
ringing the trees
in twos
in fours."
Winter song by Nichita Stanescu

The impressive work of the air fleet led to the transportation and thus the rescue of some of the victims of the catastrophic accident in Bucharest, at the Colectiv club. Burned victims who survived and we who followed the news are aware of the implication of the emergency teams involved.

And again I think Stanescu's lyrics may be most appropriate to tell these stories:

"Black snow was falling. The tree line
shone when I turned to see-
I had wondered long and silent,
Alone, trailing memory behind me.

And it seemed the stars, fixed as they were,
ground their teeth, a stiffened nexus,
an infernal machine, tolling
the halted hours of consciousness."
Black Forrest by Nichita Stanescu

Thus I think it is about time to acknowledge the efforts made to build up a rescue system that is both efficient and affective.

This is why at the beginning of the New Year 2018, I wish you a better year, more realistically dedicated to those in need. I also would like to thank my colleagues of the editorial board who constantly struggled to improve the performances of the published authors and to attract valuable materials to be published.

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REVIEW

Emergency Air Rescue System in Romania

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The helicopter, as a means of transport, has facilitated a significant decrease in intervention time at the site of request, increasing the chances of survival of the critical patient. Since 2003, SMURD has managed to form a fleet composed of nine helicopters and two airplanes. From an operational and strategic point of view, the SMURD intervention unit, set up seven Aeromedical Operational Bases (A.O.B.) equipped with helicopters and materials necessary for their operation. There is a dynamic increase in the number of air rescue missions in Romania, with most missions being carried out by the air rescue bases in Târgu Mureș and Bucharest. Specialty literature has clearly demonstrated the positive impact on the survival of critical patients assisted by airborne crews, so it is necessary for the Romanian air rescue system to grow up. It is necessary to increase the number of air bases, purchase new helicopters and to continue the training programs of both pilots and medical personnel.

Keywords: emergency medical service, helicopter, air rescue, retrieval medicine, critical care transport

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Helicopter emergency medical service (HEMS) has become a significant component of prehospital treatment of traumatized patients in many countries [1,2]. Since its introduction into the civilian rescue system, the effects of HEMS towards time and cost efficiency has been discussed controversially [1,3–5]. In this context, some potential disadvantages of HEMS (e.g. high financial burden [6], availability of HEMS due to weather conditions) have been reported [1,3,7]. However, HEMS also seems to provide several presumable advantages compared to ground emergency medical services (GEMS). Firstly, HEMS is expected to facilitate rapid and wide ranged transport due to increased transportation velocity [8]. Secondly, HEMS medical crew members are supposed to be more experienced in trauma management improving preclinical treatment of traumatized patients [8-10].

SMURD (Mobile Emergency Service, Reanimation and Extrication Service) is a public intervention unit, integrated, without jurisdiction, of great strategic importance for Romania [11].

Air rescue is an aircraft equipped in accordance with the legal provisions in force for emergency aero-medical interventions to rescue critically ill patients requiring rapid and high-level intervention. A critical patient is defined as having unstable vital functions or with conditions that may have irreversible complications requiring special investigation, intervention and / or special care provided by a complex, multidisciplinary team in a general or specialized intensive care clinic or department.

Airborne transfer is used when a suitable transfer cannot be secured by land, or if the transfer time on the terrestrial route is longer than the condition of the patient permits, causing worsening or irreversible complications. The traumatized or non-traumatized patient, unstable or with a high potential for worsening during transport, requiring inter-hospital transfer to a specialized facility, should benefit from an optimal transport mode to ensure safe and timely delivery.

The use of aviation for humanitarian purposes was formally implemented following the Geneva Red Cross Convention in 1925. In Romania this was introduced in the Decree of Organization and Operation of the Central Civil Aviation Service of 1929, according to which, civilian aviation tasks also included air and medical transport.

Thus, in 1935, on the territory of Romania a donation from the Bucharest City Hall to the civil aviation, consisting of a medical plane, was the first structure of this kind. This aircraft was exclusively used for aero-medical missions in Romania and was the first of its kind.

The transportation of the wounded was carried out during the war by the squadron, proved that the suitably equipped aircraft is one of the most effective mean of rescuing human lives. These aspects lead to the development of sanitary aviation in Romania, therefore on 14th of November 1946 the first Aviasan sanitary aviation unit in the country was established. This aviation unit was directly subordinated to the Ministry of Health of that period.

In September of 1990, an Emergency System named SMUR was established in Targu-Mures and later after collaboration with the Fire Brigade (operational part of the service for the decommissioning) became the current SMURD which is a mobile emergency, rescue and extrication unit. Over time, beside the usual missions performed with specialized ambulances, this organization saw the need and importance of using the air transport for shortening the reaction and transport time of patients in need of medical intervention, so it started to rent aircrafts from private operators. These aircrafts were helicopters or planes

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of various types, belonging to the Ministry of the Interior, the Ministry of National Defense or to the Utility Aviation from the Ministry of Transportation. These aircrafts were also used to transport critical patients from Targu-Mures to other medical centers in Bucharest or abroad. In such situations, the medical crew consisted of two medical professionals, one of whom was a physician with experience in the field of emergency medicine or intensive care.

Since 1999, SMURD has permanently rented an IAR-316B (Alouette III) helicopter from the Utility Aviation of the the Ministry of Transportation [11]. The helicopter was based at the Targu-Mures County Clinical Hospital and was equipped with the necessary equipment for resuscitation and accident interventions. The medical staff on board had an emergency physician or an anesthesia and intensive care specialist. This helicopter carried out approximately 20 to 30 flying hours a month, initially serving Mures County and later extending its services to neighboring counties.

In 2003, the current SMURD form of air-medical interventions was born, a strong organization that exceeded the SMAR project by far. Through the initiative of SMURD-Mures, directed by Dr. Raed Arafat, in 2003 the foundations were laid for a national helicopter emergency medical assistance system.

A major factor that determined the increase of the efficiency of SMURD operations, was the development of the air-lift department. The helicopter, as a mean of transport, has contributed to a significant decrease in reaction time at the place of request, increasing the chances of survival.

The year of 2003 represents the start of the SMURD cooperation with the Special Aviation Unit of the Ministry of Interior. The context in which this collaboration began was, first and foremost, linked to the major shortcomings represented by the need for SMURD to hire helicopters from private operators. It all culminated in the first recorded aviation accident for SMURD. In 2003, a IAR-316B type helicopter rented from Transgaz (a private company from Medias) crashed in Cojocna, Cluj County with 4 people on board. On impact, all personnel (pilot, co-pilot and SMURD medical crew consisting of a physician and nurse) were fatally injured. The Romanian helicopter used for this mission was an old model used by Aviasan in the 1970s. The cause of the tragedy was unfavorable meteorological conditions (fog, night, ice) and human factor (spatial disorientation).

During the same year, financed from the state budget, SMURD succeeded in acquiring the first Eurocopter EC-135 helicopter. Later that year, a second helicopter , the same model, was purchased by the Government. In order to keep the operating costs at a minimum, taking into account all the favorable factors presented, the Special Aviation Unit of the Ministry of the Internal Affairs was asigned to operate both helicopters and all other future aircrafts of SMURD.

Since 2003, SMURD has managed to form a fleet of nine helicopters and two airplanes. From an operational point of view the SMURD organization established seven operational bases equipped with helicopters and equipment necessary for their operation (the eighth Aeromedical Operational Base is to be opened in Jibou, Salaj County).

Since 2008, the Special Aviation Unit has been transformed into the General Inspectorate of Aviation (G.I.o.A.) of the Ministry of Internal Affairs. G.I.o.A. is the air operator that performs independent SMURD missions.

The Aeromedical Detachment from the G.I.o.A. is responsible for the operation of SMURD aircrafts. This department of the G.I.o.A. runs all the Aeromedical Operational Bases in the country.

The fleet of the Aeromedical Detachment is composed of ten aircrafts with military registrations and one with civil registration. From these eleven SMURD aircrafts, eight are EC-135, one is H 135 and two airplanes: a Piper PA 42 (turboprop) - Cheyenne and a Cessna Citation V Turbo-Jet (Figure1).

From the operational point of view, since 2014 the G.I.o.A. is a part of a joint organization called the Emergency Situations Department (D.S.U. in Romanian abbreviation / E.S.D.). The Emergency Situations Department is subordinated both to the Ministry of Internal Affairs and to the Ministry of Health.

The Aeromedical Detachment, component of the G.I.o.A, has subordinated operational flight structures specifically designed for intervention, search-rescue and sanitary transport called Aeromedical Operational Bases (P.O.B.).

Aeromedical Operation Bases and aircraft serving these structures are:

- **Bucharest** Aeromedical Operational Base Base, equipped with a helicopter type EC-135 (registration: 334) and two airplanes. A Piper PA-42 Cheyenne (registered: 1121) and a Cessna Citation V type Turbo-Jet (registered YR-SMD).

- The Aeromedical Operational Point **Targu-Mures**, which has a helicopter type EC-135 (registration 340). It operates from the Targu-Mures Aeromedical Operational Base platform located within the County Emergency Clinical Hospital in Targu-Mures. The aircraft is parked in the SMURD hangar located in the same location.

- The **Iasi** Aeromedical Operational Base, which has a helicopter type EC-135 (registration ⁻342). It operates from a platform within Iasi International Airport, located in the Iasi Special Aviation Unit. The aircraft is hangered in the Iasi Aeroclub facility.

- The **Arad** Aeromedical Operational Base, which has a helicopter type EC-135 (registration 341). It operates within the Arad International Airport. It is parked in the hangar of Arad Aeroclub.

- The **Craiova** Aeromedical Operational Base , which has a helicopter type EC-135 (registration 344). It operates from the SMURD platform at Craiova International Air-

Fig. 1. Aircrafts of the SMURD organization, at left is the Airbushelicopters H-135 helicopter, at the top-right is the Piper PA-42 Cheyenne, and the Cessna Citation V is at bottom-right.

port. The parking of the aircraft is made in the SMURD hangar located in the same location.

- The **Constanta** Aeromedical Operational Base, which has a helicopter type H 135 (registration 349). It operates from the platform of a trading company near Constanta County Emergency Clinical Hospital. The parking is in the special hangar on the outskirts of Constanta.

- **Galaţi** Aeromedical Operational Base, which has a helicopter type EC-135 (registration 346). It operates from the platform of the Border Police in Galati. The aircraft is parked at the Pegas Aeroclub in Galati. The SMURD hangar is going to be built until the end of the year of 2017.

- The **Jibou** Aeromedical Operational Base will be equipped with the registered 345 EC-135 helicopter, which will operate from the platform of the Jibou Fire Unit (Salaj County). At this location a hangar and spaces for the proper conduct of the activity were built .This Aeromedical Operational Base is not yet operational.

The distribution of the aircraft on the territorial structures (P.O.A. and S.S.A.V.) can be seen on the figure 2, each being represented by the G.I.o.A on the map of Romania.

The SMURD helicopters are equipped according to Order no. 1.092 of September 7, 2006, regarding the determination of competence and attributions of intervention teams on different levels in the pre-hospital phase, issued by the Ministry of Health. These are equipped with medical devices that provide critical patients with a high performance mechanical ventilator, difficult intubation tools, defibrillator - external cardiac stimulator, rapid infusion systems, trauma immobilization devices, suction, thoracic drain kits, central vascular catheterization kits, cricotiroidotomy kits, specialized medication, blood gas and acid-base balance analyzer, amputation kit, burn kit and an incubator for neonatal transport missions (Figure 3).

The missions carried out by the SMURD organization within the Aeromedical Department of the G.I.o.A. are divided into three categories: primary missions, secondary missions and special missions.

- Primary missions: are the missions carried out in the event of serious accidents in which a person urgently needs medical assistance due to injuries suffered or due to the circumstances. The methodological norms of the Government Emergency Ordinance no. 126/2003 regarding the operations define as primary missions the following:

(a) serious road accidents and other accidents in which one or more people are at vital risk due to the injuries suffered or the circumstances in which they occurred;

b) critically ill patients in small or rural settlements, in inaccessible localities or places, and in places lacking qualified emergency medical assistance for the case in question; (Figure 4). Rapid procedures are preferred to sustained on-scene treatment, particularly when surrounding conditions are hostile. HEMS emergency physicians attempt to keep on-site intervals short, treatment and monitoring to minimize delay in rescue [12]. HEMS should be used more often in case of trauma in order to guarantee the proven benefit for multiple traumatised patients [13]. The combination of the transfer by HEMS and treatment in a level I trauma center has a significantly positive effect on the survival rate of the patient, especially in patients with traumatic brain injury (TBI)[14].

c) critically ill, injured, intoxicated or other acute illnesses transported by ambulances or other means of transport to sanitary units or hospitals that cannot provide the necessary stabilization and emergency care for the respective cases, with the need for emergency transfer to specialized medical centers.

(d) patients suffering from acute conditions with a high potential for aggravation in the absence of other appropriate means of intervention nearby or likely to succumb faster than the helicopter;

e) intervention in support of the emergency and first-aid crews of the public or private emergency medical care

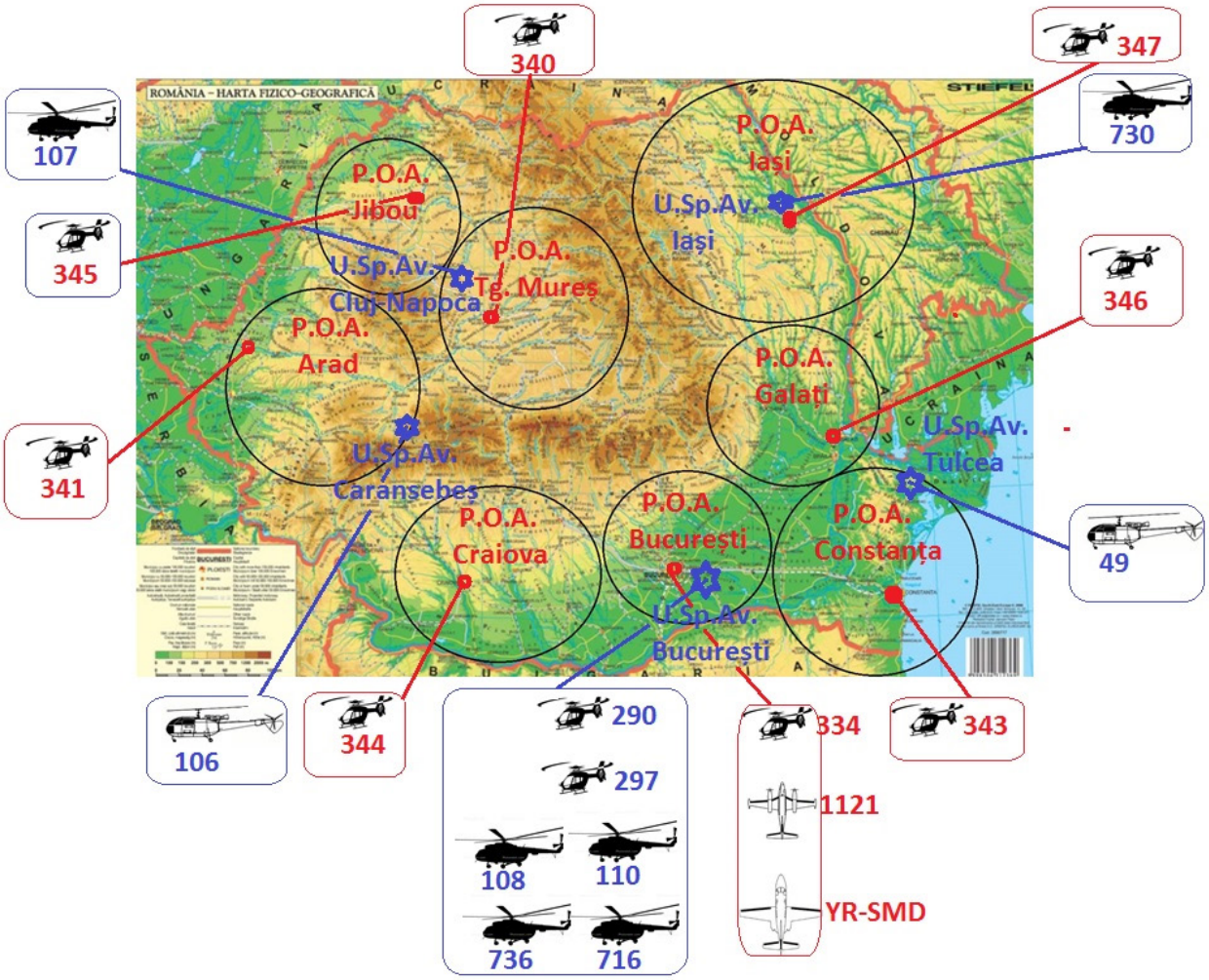

Fig. 2. Distribution of the operational structures of the G.I.o.A. on the map of Romania

services or other authorized public services upon their request (Figure 5)

Secondary missions: are the transfer missions of patients from health care units that cannot provide the appropriate investigation or medical care, requiring an emergency transfer to a specialized health facility. Currently large, and increasing, numbers of critically ill patients require transfer between critical care units [15]. Inter-unit transfer poses significant risks to critically ill patients, particularly those requiring multiple organ support. Documentation of the patient's condition, the investigations and their results, the medication administered, with the specification of the doses and hours of administration, the specialized consultations, etc. are copied and sent to the hospital receiving the patient.

Special missions are considered: Transport missions of transplant, organs for transplant and search and rescue mission.

The way of requesting the intervention of a SMURD aircraft is made exclusively by calling the 112 emergency service.

The medical crew of the helicopter includes 2-3 members, of which at least one physician. Only the following categories of doctors may serve on the helicopter:

a) Emergency medicine specialists, certified for helicopter activity;

b) Specialists in anesthesia and intensive care accredited / certified for helicopter intervention;

c) Resident doctors in emergency medicine or anesthesia and intensive care, 3rd, 4th and 5th year, with the approval from the director of the helicopter medical institution and from the chief officer of the Aeromedical Operation Base and only after accreditation or attestation for helicopter intervention .

Following the tragic fire that took place on the night of 30/31 October 2015 at COLECTIV Night Club, which resulted in the death of 63 people and more than 150 injured, the Aeromedical Detachment aircraft performed 7 missions to transport seriously injured patients to hospitals abroad. Thus, between 7 and 30 November 2015, 7 flight missions were carried out, 2 by PYPE Cheyenne Pa42 in Vienna and Graz (Austria) and 5 by Cessna Citation V in Zurich (Switzerland), Vienna (2 missions) and Graz (Austria) and Le Bourget - Paris (France).

During 2015, the implementation of the cross-border project "Improving the Response Capacity of the Mobile Emergency Service for Resuscitation and Extrication (SMURD) through the joint integration of systems

Fig. 3. The inside of a SMURD helicopter

Fig. 4. Helicopter intervention in hard-to-reach areas

Fig. 5. Support provided by the SMURD helicopter to medical crews in the field

for effective monitoring and disaster mitigation has been completed, with regard to the population within the borders of Romania, Ukraine and the Republic of Moldova "(Joint Operational Program Romania-Ukraine-Republic of Moldova). As a result, helicopters serving Iasi and Galati A.O.B. have performed so far several missions on the territory of the Republic of Moldova.

Continuous crew training is a priority. The training courses conducted in Romania by the Swiss Air Salvage Service specialists REGA, within the framework of the Swiss-Romanian Cooperation Program of Preparation, had three main components:

• NVIS - Night Vision Imaging System - Romanian flight instructors have strengthened their acquired knowledge so as to become trainers for other pilots. The training was supervised by REGA flight instructors.

• The use of the winch - Romanian pilots and doctors had the opportunity to develop new skills in case of special situations that can be encountered during the interventions and how these interventions are influenced by weather conditions, visibility or different obstacles.

• Simulator training - Pilots participated in standard and emergency procedures on the Swiss Air Force Base in Emmen.

As a result of the analysis of Table 1, there is a dynamic increase in the number of air missions in Romania, most of them being carried out by the air rescue bases from Târgu Mureş and Bucharest. When analyzing the data, we also need to take into account the following:

-The SMURD Galaţi Airbase became operational from 20th of June 2014;

-From the 15th of December 2014 until 31st of July 2015 the SMURD Airbase in Constanţa was inoperative;

-From the 2nd of June 2016 until 17th of January 2017, the Iasi SMURD Airbase was not operational;

It is also desirable to arrange landing grounds - heliports in the vicinity of hospitals. At this very moment , few hospital units have their own landing space (Figure 6).

Helicopter Emergency Medical Services (HEMS) are used worldwide in order to provide potentially life-saving pre-hospital medical support to trauma patients at the accident scene. The literature has clearly demonstrated the positive impact on the survival of critical patients assisted by aircrew, so it is necessary to further develop the air res-

Table 1. Number of aeromedical interventions

Location	2013	2014	2015	2016
Târgu Mureș	361	656	760	746
București	514	534	753	719
Iași	335	415	561	276
Arad	248	401	436	451
Craiova	286	317	550	539
Constanța	107	286	142	378
Galați	-	104	340	551
Total primary and secondary missions	1851	2713	3542	3660

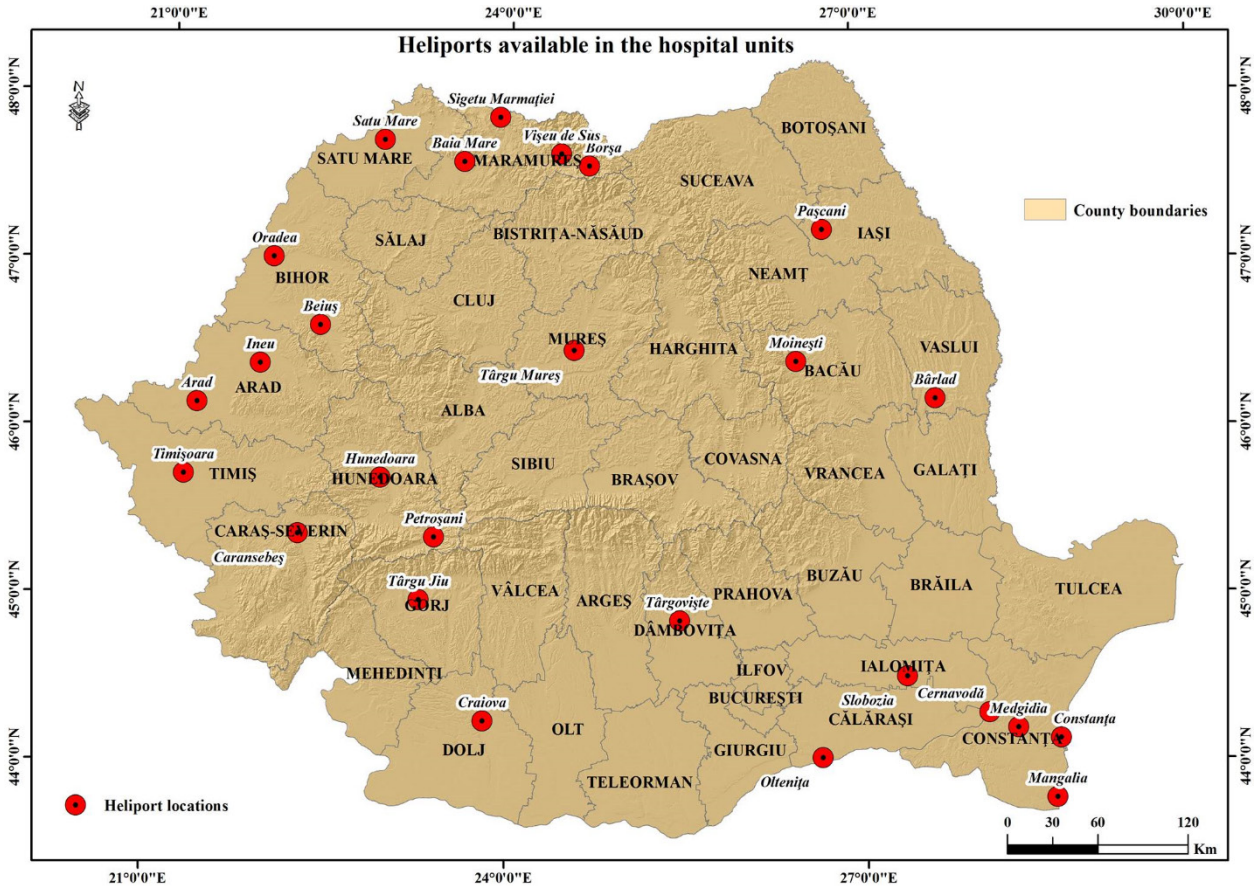

Fig. 6. Cities with medical units that own land helipads

cue system in Romania in the future [16]. It is necessary to increase the number of air bases, increasing the level of training of both pilots and the medical team. It would also be desirable to equip the helicopter with the possibility of blood transfusions in the event of severe haemorrhagic shocks. In the future, it is also desirable to implement the endovascular balloon occlusion of the aorta (REBOA).

Conflicts of interest

None

References

1. Andruszkow H, Lefering R, Frink M , et al. - Survival benefit of helicopter emergency medical services compared to ground emergency medical services in traumatized patients. Crit Care. 2013;17:R124.
2. Sturm JA, Pape HC, Dienstknecht T - Trauma care in Germany: an inclusive system. Clin Orthop Relat Res. 2013;471:2912–2923.
3. Andruszkow H, Frink M, Zeckey C, Krettek C, Hildebrand F, Mommsen P - Merits and capabilities of helicopter emergency medical service (HEMS) in traumatized patients. Technol Health Care. 2012;20:435–444.
4. Galvagno Jr SM, Haut ER, Zafar SN et al. - Association between helicopter vs ground emergency medical services and survival for adults with major trauma. JAMA. 2012;307:1602–1610.
5. Galvagno Jr SM, Thomas S, Stephens C et al.- Helicopter emergency medical services for adults with major trauma. Cochrane Database Syst Rev. 2013;3:CD009228.
6. Taylor C, Jan S, Curtis K, et al. - The cost-effectiveness of physician staffed Helicopter Emergency Medical Service (HEMS) transport to a major trauma centre in NSW, Australia. Injury. 2012;43:1843–1849.
7. Westhoff J, Hildebrand F, Grotz M, Richter M, Pape HC, Krettek C. - Trauma care in Germany. Injury. 2003;34:674–683.
8. Butler DP, Anwar I, Willett K. - Is it the H or the EMS in HEMS that has an impact on trauma patient mortality? A systematic review of the evidence. Emerg Med J. 2010;27:692–701.
9. Biewener A, Aschenbrenner U, Rammelt S, Grass R, Zwipp H. - Impact of helicopter transport and hospital level on mortality of polytrauma patients. J Trauma. 2004;56:94–98.
10. Andruszkow H, Hildebrand F, Lefering R Et al. - Ten years of helicopter emergency medical services in Germany: Do we still need the helicopter rescue in multiple traumatised patients? Injury. 2014;45:S53-S58.
11. Arhiva Istoric [Internet]. SMURD. [cited 2018Jan8]. Available from: http://smurd.ro/category/despre/istoric/
12. Taubenböck S, Lederer W, Kaufmann M, et al. - HEMS in alpine rescue for pediatric emergencies. Wilderness & environmental medicine. 2016;27:409-414.
13. Andruszkow H, Lefering R, Frink M, et al. - Survival benefit of helicopter emergency medical services compared to ground emergency medical services in traumatized patients. Critical Care 2013;17:R124.
14. Schweigkofler U, Reimertz C, Lefering R, Hoffmann R. - Importance of air ambulances for the care of the severely injured. Der Unfallchirurg. 2015;118:240-244.
15. Droogh JM, Smit M, Absalom AR, Ligtenberg JJ, Zijlstra JG. Transferring the critically ill patient: are we there yet? Critical Care. 2015;19:62.
16. Ringburg AN, Thomas SH, Steyerberg EW, van Lieshout EM, Patka P, Schipper IB.- Lives saved by helicopter emergency medical services: an overview of literature. Air medical journal. 2009;28:298-302.

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REVIEW

Clinical Conditions and Predictive Markers of Non-Dipper Profile in Hypertensive Patients

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Hypertension remains one of the primary causes of premature cardiovascular mortality representing a major independent risk factor.
The importance of ambulatory blood pressure monitoring in clinical evaluation of hypertensive patients, beyond diagnosis, is the identification of circadian dipping/non-dipping profile. The non-dipper pattern in hypertensive and normotensive patients is associated with significant target organ damage and worse outcomes, as an increased cardiovascular risk condition. Non-dipping pattern has been found to be associated with specific clinical conditions. Obesity, diabetes mellitus, metabolic syndrome, obstructive sleep apnea syndrome, chronic kidney disease, autonomic and baroreflex dysfunctions, salt sensitivity, hormonal changes, gender and age were extensively studied. Research efforts are focused on recognizing and exploring predictive markers of abnormal blood pressure circadian pattern. Previous studies acknowledge that red cell distribution width, mean platelet volume, fibrinogen level, C-reactive protein, serum uric acid and gamma-glutamyltransferase, are independently significant and positive associated to non-dipping pattern. Moreover, research on new biomarkers are conducted: Chitinase 3-Like-Protein 1, atrial and B-type natriuretic peptide, brain-derived neurotrophic factor, chemerin, sphingomyelin and the G972R polymorphism of the insulin receptor substrate-1 gene. This review summarizes the current knowledge of different clinical conditions and biomarkers associated with the non-dipper profile in hypertensive patients.

Keywords: systemic hypertension, non-dipper profile, cardiovascular risk, clinical conditions, predictive markers

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Introduction

Hypertension was identified as a main preventable cause of morbidity and premature mortality worldwide, 31.1% adults suffering from this condition. Hypertension prevalence displays a significant difference between high-income and low-and middle-income countries. [1]

Different approaches to treatment, awareness and control along with additional increase of life expectancy, grant the emerging incidence of hypertension. [2]

In 2012 hypertension prevalence in Romanian population was 40.4% as reported by SEPHAR II survey. 85% patients were taking antihypertensive treatment and adequate control was obtained in a quarter. [3]

SEPHAR III survey (2016) results revealed an ascending prevalence, with 19.1% newly diagnosed hypertensive patients out of 45.1% total prevalence. [4]

Ambulatory blood pressure monitoring (ABPM) is the recommended standard method in accurate diagnosis of true high blood pressure (BP), systolic and diastolic values along with all particular aspects of the circadian BP variation. [5,6]

The dipper profile is defined by at least 10% decline, but not more than 20% in systolic and/or diastolic BP value during the night-time, compared to daytime. The term non-dipper refers to patients whose blood pressure does not exhibit these variations. [6] Non-dipper hypertensive patients manifest an increased mortality risk, cardiac and extracardiac morbidity. [7,8]

Possible underlying mechanisms of non-dipper profile were explored in a multitude of studies conducted over the years, identifying clinical conditions linked to disturbances of 24-h systolic and/or diastolic BP variations. [9]

Complex mechanisms originating in endocrine, renal, neural, and vascular areas are involved in the pathogenesis of arterial hypertension and its circadian variability. Poor sleep quality and the absence of physical exercise during the day, are likely affecting the abnormal night-to-day BP ratio. (Table I)

Significant target organ damage (TOD) such as left ventricular hypertrophy, stroke, changes in carotid wall thickness and atherosclerotic plaques, along with renal and ocular damage were recognized in non-dipper hypertensive patients. [7,8,21,22]

Non-dipper profile is not a static marker of the cardiovascular status. Hypertension pattern identification and therapeutic interventions can improve patient prognosis. [23-25]

This review summarizes the current knowledge of different clinical conditions and biomarkers associated with the non-dipper profile in hypertensive patients.

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Table I. Conditions associated with non-dipping pattern

Endocrine system disorders	Hypo/Hyperthyroidism [9,10] Primary hyperparathyroidism [11] Aldosteronism [9] Cushing syndrome [9] Pheochromocytoma [10] Hypopituitarism [12] Impaired nocturnal melatonin secretion [13] Acromegaly [14]
Renal dysfunction	Chronic kidney disease [10] Renal transplantation [15] Unilateral nephrectomy [16]
Autonomic nervous system dysfunction	Autonomic failure Orthostatic hypotension [17, 18]
Sleep disturbances	Obstructive sleep apnea [10] Stress disorders [19] Impaired sleep quality [20]
Other [10]	Obesity Diabetes mellitus Metabolic syndrome Salt sensitivity Age, gender

Autonomic nervous system

Neural mechanisms are believed to play an important role in non-dipper hypertension profile.

Increased autonomic nervous system activity is involved in triggering and maintaining high BP. Different techniques were used to measure sympathetic nervous system (SNS) activity: electrophysiological studies and measurement of norepinephrine neurotransmitter values in plasma and urine samples. [26]

Dauphinot and colab. report that patients with impaired autonomic nervous system activity present an insufficient decrease in nocturnal BP. [27] Non-dipper hypertensive patients have low circulating norepinephrine and higher peripheral vascular resistance compared to dippers. [28]. Grassi et al. provide evidence that in arterial hypertension is a close correlation between the sympathetic activation degree and the magnitude of nocturnal decrease in systolic or diastolic BP. [10,29]

Orthostatic hypotension

Orthostatic hypotension is a relatively common and multifactorial disorder, often secondary to autonomic dysfunction. BP variation related to posture was associated to non-dipper pattern in female gender taking at least 2 antihypertensive drugs. Orthostatic hypotension could be a marker of non-dipper profile. [30]

Alquadan et al. demonstrated that postprandial hypotension and cardiac rhythm variability computed on ABPM are potent predictors of autonomic dysfunction in routine clinical practice. [31] Frequently, patients with orthostatic hypotension may develop non-dipper hypertension profile, which is a treatment challenge for physicians. [18]

Obesity

Compared to normal weight, obesity is responsible for five times increase in hypertension incidence. [32] In the complex obesity-associated hypertension process, activation of renin-angiotensin-aldosterone system (RAAS), endothelial and adipose tissue dysfunction and the sympathetic nervous system (SNS) stimulation, represents the underlying mechanisms. [33-35]

Through pathological activation of RAAS, in visceral adipocytes renin-angiotensin-aldosterone metabolites involved in BP regulation are released: angiotensinogen, renin and renin receptor, angiotensin-converting enzyme, angiotensin I type and angiotensin II receptors. Angiotensinogen via angiotensin II induce hypertension by systemic vasoconstriction, sodium and fluid balance, and stimulates aldosterone secretion. [33]

The role of metabolic dysregulation in obesity-associated hypertension implies the malfunction of connection between microvascular and perivascular adipose tissue inflammation to adipokine and neuropeptides synthesis. Leptin, resistin, adiponectin, visfatin, TNF- α, IL-6, MCP-1 and IL-1 further disturb the sympathetic activity and influence the tight link with insulin resistance. Both mechanisms are responsible in hypertension development. [33,36,37]

Different studies observed that basic mechanisms of obesity-associated hypertension are very much alike to mechanisms determinants of non-dipping pattern of hypertension. [10,38-40]

Ayukusuma et al. in a small study investigated the serum level of interleukin-6 (IL-6), as a key mediator of mechanism between hypertension and inflammation. Performed on forty-eight hypertensive patients, study concluded that IL-6 serum level did not differ among dipper and non-dipper hypertensive patients. [41]

Diabetes mellitus and metabolic syndrome

Decline of physiological mechanisms in hypertensive patients with diabetes mellitus or metabolic syndrome (MS), are considered to be also responsible for the non-dipper hypertension pattern: insulin resistance (anti-diuretic action of insulin), exaggerated response to internal vasoconstrictors, surge of SNS activity, damage endothelium-vasodilatation dependant and results of thickening in vascular smooth muscle. [10]

Duggal and colab. showed that 46% of hypertensive patients with type 2 diabetes had a non-dipper profile. This profile is associated to higher prevalence of microalbuminuria and advanced age. Authors emphasize the importance of BP profile identification and early specific treatment in particular patients, stratified as patients at increased risk of cardiovascular and renal mortality and morbidity. [42]

BP pattern of hypertensive patients taking antihypertensive medication and presenting metabolic syndrome, were also studied by Tartan et al. using the MS-Score. A predictive role of high MS-Score for the non-dipping pattern of BP was proved. [43]

In addition, abdominal obesity associated with increased level of uric acid in patients with MS was closely related to non-dipper blood pressure profile as it was suggested by Tutal et colab. [44]

Metabolic syndrome pathophysiology that underlies the increase of arterial stiffness in non-dipper patients, determine aortic function damage, through exposer of the aortic wall to additional pressure load. Non-dipper obese hypertensive patients had an enlarged thoracic aortic diameter compared to dipper patients. [45]

Karaagac et al. demonstrated that for non-dipper hypertensive patients associating MS, specific 12-lead electrocardiogram parameters are valuable for assessing the increased cardiovascular morbidity and mortality risk. The interval between the peak (Tp) and the end (Te) of T wave, Tp-Te interval, is an index of total dispersion of myocardial repolarization. Combined with Tp-Te/QT ratio (QT- extension of the time between the beginning of the Q wave, and the intersecting descending part of T wave to isoelectric line) and Tp-Te/QTc ratio (QTc- corrected QT) these markers of potential ventricular arrhythmias were significantly higher and very strong associated to the non-dippers with metabolic syndrome. [46]

Obstructive sleep apnea

Obstructive sleep apnea (OSA) syndrome severity directly influence the non-dipping pattern. Despite mild severity of OSA, transient hypoxemia and hypercapnia caused by recurrent episodes of apnea, trigger changes in SNS activity and disrupt night sleep. [10] An increased OSA severity reduces the dipping pattern, augmenting the non-dipper number of OSA patients. [47]

Normotensive and hypertensive OSA patients are more affected by impaired nocturnal BP decrease than regular normotensive or hypertensive patients. [48] Apnea-hypopnea index has a significant association to non-dipper profile and no association to age or body mass index. [49]

Wolf et al. summarized the potential pathways of causative relation of OSA to non-dipper pattern: endothelium damage, oxidative stress, high levels of plasma asymmetric dimethyl arginine concentration and elevated L-selectin, ICAM-1 (intercellular adhesion molecule-1) and VCAM-1(vascular cell adhesion molecule-1). [50]

In obese patients with MS and OSA syndrome treatment with continuous positive airways pressure, upper airway surgery or stimulation, can improve BP circadian variations and reduces cardiovascular risk. [47,51]

Hormones

Routledge et al. analysed 47 menopausal women ABPM recordings. Non-dipper female patients (34%) were older and had lower stress scores compared to dipper participants. [52]

Research in perimenopausal hypertensive women reported 61.5% non-dipper profile in a sample of 130 participating women. Non-dipper profile in perimenopausal women more often associates obesity, postural hypotension, microalbuminuria, and elevated glycosylated haemoglobin, fibrinogen, C-reactive protein. [53]

Thyroid

Systolic and diastolic BP were investigated in relation to serum thyrotropin (TSH), in patients presenting elevated TSH levels with typical disease manifestation or subclinical form of hypothyroidism. Within normal TSH limits, a positive association was found between both, systolic (>160 mm Hg) and diastolic (>95 mm Hg) hypertension, and TSH serum. [54]

Nevertheless, a recent study presented results supporting the theory that hypertensive patients with subclinical hypothyroidism more frequently experience a diastolic non-dipper profile. [55]

Possible pathway of determining the non-dipper pattern in hypothyroidism is the result of increase vascular resistance under SNS influence. [10]

Concerning other thyroid hormones, an independent association of non-dipping pattern to low level of free triiodothyronine was reported in a study by Kanbay et al. investigating patients with no thyroid hormone disorder. [56]

Parathyroid hormone

Kanbay et colab. reported that non–dipper pattern in hypertensive patients is significantly associated to higher levels of phosphate, Calcium x Phosphorus product, and parathyroid hormone level. [57]

Chronic kidney disease

Prospective and cross-sectional studies in chronic kidney disease (CKD) patients have demonstrated changes in 24-h BP pattern that may support kidney function worsening. Mechanisms involved include RAAS provocation, endothelin activation, inflammation, altered baroreceptor sensitivity and SNS activity. [58]

CKD patients have a higher prevalence of non-dipping pattern and elevated mean systolic overnight BP, results shown by monitoring 1805 patients in Ancona Hypertension Centre, Italy. [59] Aggregate data confirm that personalised therapy (selection of drugs, dosage and chronotherapy) is needed in order to prevent long-term cardiovascular events and TOD in abnormal BP variations of CKD hypertensive patients. [58,59]

Non-dipper pattern is a negative determinant in the nephropathy evolution in type 1diabetes patients and a risk factor for microalbuminuria evolution. [60]

In renal transplantation patients, assessing the Doppler renal resistive index proved to be a strong predictor for non-dipper profile. [61] Presence of abnormal circadian BP variation at one year in kidney transplantation patients, increases the risk of transplanted kidney failure in the next 3-4 years. [62]

Non-dippers with reduced left ventricular ejection fraction (LVEF) have increased incidence of renal and cardiovascular worse outcomes, compared to dippers. In non-diabetic patients affected by decreased glomerular filtration

rate and progression of renal disease, 24-h ABPM and LVEF can be used as prognostic markers. [63]

Extended research is also conducted for identification of potential pathways of salt-induced non-dipper profile in hypertensive patients.

Data derived from a study conducted on 115 young never treated hypertensives patients, suggested that G972R insulin receptor substrate-1 gene (IRS-1) polymorphism, is associated with insulin resistance, salt sensitivity and non-dipper hypertension. [64] Heterozygous carriers exposed to high salt diet develop non-dipper hypertensive profile.

Decrease in night-time BP was identified in groups of salt sensitive hypertensive patients with sodium intake restriction. [65]

Elevated concentrations of plasma norepinephrine, CKD with attenuated inhibition of RAAS, impaired renal sodium excretion and genetic factors were recognized as responsible for salt-induced non-dipper hypertensive profiles. [66]

Therapeutic strategy

Current guidelines on treatment and management of hypertension do not recommend personalised treatment in dipper and non-dipper hypertensive patients. [6]

However, the 2016 European Guideline on cardiovascular disease prevention in clinical practice, indicates the assessment of dipping pattern or suspicion of non-dipping in CKD or diabetes mellitus patients or OSA patients. [67]

Table II. Current laboratory predictive biomarkers of non-dipper hypertension profile

Author, year	Participant number	Biomarker	Results in non-dipper subjects	Study conclusion
Tosu, 2014. [74]	120	Uric acid	Significantly higher levels compared to dippers/control group (p < 0.05)	In non-dippers, increased inflammatory markers can be the reason of advanced end-organ failure in conjunction with cardiovascular morbidity and mortality.
		C-reactive protein		
		Red blood cell distribution width (RDW)	Significantly higher than dippers/normotensives (p<0.05)	
Buyukkaya, 2016. [75]	170	RDW	Higher values compared to dippers 14.5 ± 0.87 vs.12.7 ± 0.66, p<0.001	High values of commonly hematological element (RDW) may be linked to inflammatory state, in normotensive and hypertensive non-dipper patients.
		hs-CRP	Higher hs-CRP levels 0.99 ± 0.52 vs.0.63 ± 0.43, p<0.001	
Kaya, 2010. [76]	126	Mean platelet volume (MPV)	Higher levels compared to normotensives and dippers 9.72+/-0.52fl vs 8.92+/-0.42fl and 9.38+/-0.33fl, p<0.05	Inflammatory activity and raised platelet activation pattern are present in non-dipper hypertensive patients, increasing the atherosclerotic risk.
		hs-CRP	Significantly raised compared to normotensives and dippers 4.9+/-1.7mg/l vs 2.7+/-0.8mg/l and 3.8+/-1.5mg/l, p<0.05	
Ortakoyluoglu, 2016. [77]	171	Gamma-glutamyl-transferase (GGT)	Significant increased versus dipper 36.2±13.8 vs 20.5±8.8 U/L, p=0.03	GGT positively associated to non-dipper profile; negatively related to night-to-day BP variation.
Tabara, 2016. [78]	1020	BNP plasma	Positively related to circadian BP variations (p<0.001)	Mild elevation of BNP plasma level identified as marker of atypical BP circadian variation along with abnormal pattern of nocturnal BP.
		Oxygen desaturation (SpO2)	Positive association OR:1.04, p=0.001	
Bakirci, 2015. [79]	80	YKL-40 levels	Significantly greater compared to dippers 183.1 ± 59.1 versus 125.9 ± 50.3 pg/mL, p<0.001	Higher level of YKL-40 serum, hs-CRP together with epicardial adipose tissue thickness were independently markers of non-dipper pattern; it can constitute an improved prediction tool for prevention and immediately treatment of high risk non-dipper induced cardiovascular outcome.
Ji, 2017. [80]	60	CD4+ effector T (Teff) cells: Th1, Th2, Th17	Th1 and Th17 notably higher compared to the dippers Th2 level significantly higher in dippers when compared to non-dippers	Th1 and Th17 subsets response were independently associated with the non-dipper pattern. TOD, hypertension prevention and treatment could benefit out of adjusting the CD4+ effector T cells.
Kadoya, 2014. [81]	250	Plasma BDNF	Intermediate value compared to highest value determined in reverse-dippers	Connection between autonomic nervous system activity, evaluated through BDNF plasma level, with abnormal BP circadian pattern.
Meric, 2014. [82]	90	Chemerin (tazarotene-induced gene 2 protein-TIG2)	Higher levels 219.7±16.3 vs. 182.4±21.4 ng/ml -dipper; p<0.001 219.7±16.3 vs. 85.4±38.1 ng/ml-normotensive; p<0.001	By determining the chemerin level in non-dipper hypertensives, clinical decision can be improved in high risk hypertensive patients. Results reinforced the inflammation role in pathophysiology of high BP.
Zheng, 2014. [83]	116	Sphingomyelin	Significance of systolic and diastolic BP night decline was negatively correlated to plasma SM level; r=-0.42, p<0.01 for systolic BP and r=-0.31, p<0.01 for diastolic BP	SM levels assessment may be applied in detection of high risk cardiovascular patients, linked to non-dipper pattern.
Cayli, 2013. [84]	317	Serum hs-cTnT	Absolute predictor for non-dipper pattern OR:1.409; 95% CI, 1.276–1.556; p<0.001	Serum hs-cTnT and NT-proBNP value demonstrate autonomous prediction for non-dipper profile in hypertensive patients; serum hs-cTnT values are related to night time systolic BP. Newly diagnosed hypertensive patients, can benefit from the potency of high sensitivity cardiac troponin T marker.
		NT-proBNP	Independent marker of non-dipper pattern; OR:1.012; 95% CI, 1.005–1.020; p=0.001	

Nevertheless, treatment strategies based on dipper/non-dipper status is an emerging novel concept for high-risk hypertensive patients. Recent studies contribute with close supporting evidence for personalized treatment approach in the non-dipper BP pattern. [68]

For the non-dippers, initiation or treatment adjustment along with choronotherapy, would determine highest benefit of antihypertensive therapy. Reducing the number of the non-dipping hypertensive patients will ensure long-term protection over cardiovascular events in patients presenting TOD, insulin resistance and increased proteinuria and fibrinogen levels. [69]

Bedtime administration of novel calcium channel blockers (barnidipine, cilnidipine) prove to restore normal dipping profile in most of OSA /non-OSA hypertensive patients. [24,25]

Kario et al. showed that administration of an alpha-adrenergic blocker (doxazosin) at bedtime reduced blood pressure overnight in non-dipper pattern. [70]

In a systematic review conducted by Wang on 3732 patients, chronotherapy could invert non-dipper profile in hypertensive CKD subjects with no significant disparities for cardiovascular and all-cause mortality. [71]

In CKD hypertensive patients, bedtime valsartan administration in non-dippers provided renal protection by slowly decreasing the glomerular filtration rate and low 24 hours proteinuria. A better protection of the target organs was computed. [72] Diuretic therapy or salt restriction in CKD patients, restore the dipper pattern by normalising sodium excretion. [73]

Research efforts are focused on the detection of dipper/non-dipper profile predictive markers. (Table II)

Conclusions

At present, available data support the evidence of certain clinical conditions related to non-dipper pattern.

Confirming the circadian BP variation with specific night-to-day BP ratio, is appropriate to investigate possible secondary causes of high BP, medication adjustment (dosage and chronotherapy) along with therapeutic drug and response monitoring. Antihypertensive therapeutic approach dependent on dipper/non-dipper profile represents an innovative, advanced concept aimed to maximize treatment response in high risk hypertensive patients.

Further studies are needed to reinforce the clear position of circadian blood pressure profile in practical aspects of hypertension.

Conflict of interest

None to declare.

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References

1. Mills KT, Bundy JD, Kelly TN, et al – Global disparities of hypertension prevalence and control a systematic analysis of population - based studies from 90 countries. Circulation. 2016;134(6):441-450.
2. Lloyd-Sherlock P, Beard J, Minicuci N, Ebrahim S, Chatterji S – Hypertension among older adults in low- and middle-income countries: prevalence, awareness and control. International Journal of Epidemiology, 2014;43(1):116-128.
3. Dorobantu M, Onciul S, Darabont R, et al – Arterial Hypertension Epidemiology: Romania among the Balkan Countries – Data from SEPHAR Surveys. Medicina Moderna. 2014;21(1):10-16.
4. Dorobantu M, Tautu OF, Dimulescu D, et al – Perspectives on hypertension's prevalence, treatment and control in a high cardiovascular risk East European country: data from the SEPHAR III survey. J Hypertens. 2017. doi:10.1097/HJH.0000000000001572. [Epub ahead of print]
5. Hermida RC, Smolensky MH, Ayala DE, Portaluppi F – Ambulatory blood pressure monitoring (ABPM) as the reference standard for diagnosis of hypertension and assessment of vascular risk in adults. Chronobiol Int. 2015;32(10):1329–1342.
6. Mancia G, Fagard R, Narkiewicz K, et al – 2013 ESH/ESC Guidelines for the management of arterial hypertension: the Task Force for the management of arterial hypertension of the European Society of Hypertension (ESH) and of the European Society of Cardiology (ESC). J. Hypertens. 2013;31:1281–1357.
7. Routledge FS, McFetridge-Durdle JA, Dean CR – Night-time blood pressure patterns and target organ damage: A review. Can J Cardiol. 2007;23(2):132-138.
8. Krzych LJ, Bochenek A – Blood pressure variability: epidemiological and clinical issues. Cardiol J. 2013;20(2):112–120.
9. Birkenhäger AM, van den Meiracker AH. – Causes and consequences of a non-dipping blood pressure profile. Neth J Med. 2007;65(4):127-131.
10. Dubielski Z, Zamojski M, Wiechecki B, et al. – The current state of knowledge about the dipping and non-dipping hypertension. Arterial Hypertension. 2016; 20(2):33–43.
11. Letizia C, Ferrari P, Cotesta D, et al. Ambulatory monitoring of blood pressure (AMBP) in patients with primary hyperparathyroidism. J Hum Hypertens 2005;19(11):901-916.
12. Krzyzanowska K, Schnack C, Mittermayer F, et al – High prevalence of abnormal circadian blood pressure regulation and impaired glucose tolerance in adults with hypopituitarism. Exp Clin Endocrinol Diabetes. 2005;113(8):430-434.
13. Pechanova O, Paulis L, Simko F – Peripheral and central effects of melatonin on blood pressure regulation. Int J Mol Sci 2014;15(10):17920–17937.
14. Pietrobelli DJ, Akopian M, Olivieri AO, et al – Altered circadian blood pressure profile in patients with active acromegaly. Relationship with left ventricular mass and hormonal values. J Hum Hypertens. 2001;15(9):601-605.
15. Covic A, Goldsmith DJ, Gusbeth-Tatomir P, et al – What added value does ambulatory blood pressure monitoring brings to the management of post renal transplantation hypertension? Rev Med Chir Soc Med Nat Iasi 2003;107(1):89-97.
16. Ohashi N, Isobe S, Ishigaki S, Suzuki T, Motoyama D, Sugiyama T, et al – The effects of unilateral nephrectomy on blood pressure and its circadian rhythm. Intern Med. 2016;55(23):3427–3433.
17. Kario K, Eguchi K, Nakagawa Y, Motai K, Shimada K – Relationship between extreme dippers and orthostatic hypertension in elderly hypertensive patients. Hypertension. 1998;31(1):77-82.
18. Briasoulis A, Silver A, Yano Y, Bakris GL – Orthostatic hypotension associated with baroreceptor dysfunction: treatment approaches. J Clin Hypertens (Greenwich). 2014;16:141–148.
19. Mellman TA, Brown DD, Jenifer ES, Hipolito MM, Randall OS – Posttraumatic stress disorder and nocturnal blood pressure dipping in young adult African Americans. Psychosom Med. 2009;71(6): 627–630.
20. Huang Y, Mai W, Hu Y, et al – Poor sleep quality, stress status, and sympathetic nervous system activation in nondipping hypertension. Blood Press Monit. 2011;16(3):117–123.
21. Xuan AP – Investigate damages in target organs in two groups of patients who have dipper and non-dipper hypertension. J Hypertens. 2011;29:e11.
22. Nesukay H, Polenova N, Titov E – Ambulatory blood pressure monitoring (ABPM) and myocardium deformation parameters in patients with hypertension. J Hypertens. 2016;34:e122-e123.
23. Sousa F, Neves J, Ferreira R, et al. – In hypertension the change from a non-dipper to a dipper pattern is associated with a better cardiovascular

prognosis than the persistence within the non-dipper pattern. J Hypertens. 2015;33(1):e6.
24. Crippa G, Zabzuni D, Cassi A, Bravi E – Effect of bedtime dosing of barnidipine hydrochloride in non-dipper hypertensive patients with obstructive sleep apnoea not treated with continuous positive airway pressure. Eur Rev Med Pharmacol Sci. 2016;20(2):339-344.
25. Kario K, Nariyama J, Kido H, et al – Effect of a novel calcium channel blocker on abnormal nocturnal blood pressure in hypertensive patients. J Clin Hypertens (Greenwich). 2013;15(7):465-472.
26. Esler M. The sympathetic system and hypertension. Am J Hypertens. 2000;13:99S–105S.
27. Dauphinot V, Gosse P, Kossovsky M, et al – Autonomic nervous system activity is independently associated with the risk of shift in the non-dipper blood pressure pattern. Hypertens Res. 2010;33:1032–1037.
28. Quijada SG, Sáez MC, Gutiérrez CD, et al – Factores cardiovasculares y hormonales asociados a la falta de descenso nocturno de la presión arterial en sujetos mayores de 55 años. Rev Clin Esp. 2002;202(5):264-268.
29. Grassi G, Seravalle G, Quarti-Trevano F, et al – Adrenergic, metabolic, and reflex abnormalities in reverse and extreme dipper hypertensives. Hypertension. 2008;52:925-931.
30. Voichanski S, Grossman C, Leibowitz A, et al – Orthostatic hypotension is associated with nocturnal change in systolic blood pressure. Am J Hypertens. 2012;25(2):159-164.
31. Alquadan KF, Singhania G, Koratala A, Eja AA – Office orthostatic blood pressure measurements and ambulatory blood pressure monitoring in the prediction of autonomic dysfunction. Clin Hypertens. 2017;23:3.
32. Hossain P, Kawar B, El Nahas M – Obesity and diabetes in the developing world – a growing challenge. N Engl J Med 2007;356(3):213–215.
33. Faulkner JL, Bruder-Nascimento T, Belin de Chantemèle EJ – The regulation of aldosterone secretion by leptin: implications in obesity-related cardiovascular disease. Curr Opin Nephrol Hypertens. 2017;doi:10.1097/MNH.0000000000000384. [Epub ahead of print]
34. Ruano M, Silvestre V, Castro R, et al – Morbid obesity, hypertensive disease and the renin-angiotensin-aldosterone axis. Obes Surg 2005;15:670–676.
35. Westcott KV, Huang BS, Leenen FH – Brain renin-angiotensin-aldosterone system and ventricular remodelling after myocardial infarct: a review. Can J Physiol Pharmacol. 2009;87:979–988.
36. Stepniakowski KT, Goodfriend TL, Egan BM – Fatty acids enhance vascular alpha-adrenergic sensitivity. Hypertension. 1995;25:774–778.
37. de Courten M, Zimmet P, Hodge A. et al – Hyperleptinaemia: the missing link in the metabolic syndrome? Diabet Med. 1997;14:200–208.
38. Kang YS – Obesity associated hypertension: new insights into mechanism. Electrolyte Blood Press. 2013;11:46–52.
39. Kanbay M, Turgut F, Uyar ME, et al – Causes and mechanisms of nondipping hypertension. Clin Exp Hypertens. 2008;30:585–597.
40. Fabbian F, Smolensky MH, Tiseo R, Pala M, Manfredini R, Portaluppi F – Dipper and non-dipper blood pressure 24-hour patterns: circadian rhythm-dependent physiologic and pathophysiologic mechanisms. Chronobiol Int. 2013;30(1-2):17–30
41. Ayukusuma AR, Rohman MS, Lukitasari M et al – No difference of serum IL-6 level among dipper and non dipper hypertensive patients. J Hypertens. 2015; doi: 10.1097/01.hjh.0000469858.46811.f4
42. Duggal A, Bal BS, Singh N – Study of dipping and non-dipping patterns in patients of type 2 diabetes mellitus with hypertension and its association with microalbuminuria. Ann Int Med Den Res. 2017;3(2):20-24.
43. Tartan Z, Uyarel H, Kasikcioglu H, et al – Metabolic syndrome as a predictor of non-dipping hypertension. Tohoku J Exp Med. 2006;210:57–66.
44. Tutal E, Sayın B, Ertugrul DT, et al – Is there a link between hyperuricemia, morning blood pressure surge, and non-dipping blood pressure pattern in metabolic syndrome patients? Int J Nephrol Renovasc Dis. 2013;6:71–77.
45. Karaagac κ, Tenekecioglu E, Yontar OC, et al – Effect of non-dipper and dipper blood pressure patterns on Tp-Te interval and Tp-Te/QT ratio in patients with metabolic syndrome. Int J Clin Exp Med. 2014;7(5):1397-1403.
46. Karaagac K, Vatansever F, Tenekecioglu E, et al – The relationship between non-dipper blood pressure and thoracic aortic diameter in metabolic syndrome. Eurasian J Med. 2014;46:120-125.
47. Ma Y, Sun S, Peng CK, Fang Y, Thomas RJ – Ambulatory blood pressure monitoring in chinese patients with obstructive sleep apnea. J Clin Sleep Med. 2017;13(3):433–439.
48. Suzuki M, Guilleminault C, Otsuka K, Shiomi T – Blood pressure "dipping" and "non-dipping" in obstructive sleep apnea syndrome patients. Sleep. 1996;19(5):382–387
49. Nabe B, Lies A, Pankow W, Kohl FV, Lohmann FW – Determinants of circadian blood pressure rhythm and blood pressure variability in obstructive sleep apnoea. J Sleep Res. 1995;4(S1):97–101
50. Wolf J, Hering D, Narkiewicz K – Non-dipping pattern of hypertension and obstructive sleep apnea syndrome. Hypertens Res. 2010;33(9):867–871.
51. de Paula Soares CF, Cavichio L, Cahali MB – Lateral pharyngoplasty reduces nocturnal blood pressure in patients with obstructive sleep apnea. Laryngoscope. 2014;124:311–316.
52. Routledge FS, McFetridge-Durdle JA, Dean CR – Stress, menopausal status and nocturnal blood pressure dipping patterns among hypertensive women. Can J Cardiol. 2009;25(6):e157-e163.
53. Refaie WR, Refaie E, Abd El Hady N, et al – Cardiovascular changes in dipper and nondipper hypertension in perimenopausal women (time-dependent effects of antihypertensives). Egypt J Int Med. 2012;24(2):37-42.
54. Iqbal A, Figenschau Y, Jorde R – Blood pressure in relation to serum thyrotropin: The Tromsø study. J Hum Hypertens. 2006;20:932–936
55. Canbolat IP, Belen E, Bayyigit A, et al – Evaluation of Daily Blood Pressure Alteration in Subclinical Hypothyroidism. Acta Cardiol Sin. 2017;33(5): 489–494.
56. Kanbay M, Turgut F, Karakurt F, et al – Relation between serum thyroid hormone and 'nondipper' circadian blood pressure variability. Kidney Blood Press Res. 2007; 30(6): 416–420.
57. Kanbay M, Isik B, Akcay A et al – Relation between serum calcium, phosphate, parathyroid hormone and 'nondipper' circadian blood pressure variability profile in patients with normal renal function. Am J Nephrol. 2007;27(5):516–521.
58. Velasquez MT, Beddhu S, Nobakht E, Rahman M, Raj DS – Ambulatory blood pressure in chronic kidney disease: ready for prime time? Kidney Int Rep. 2016;1(2):94–104.
59. Fedecostante M, Spannella F, Cola G, et al – Chronic kidney disease is characterized by "double trouble" higher pulse pressure plus night-time systolic blood pressure and more severe cardiac damage. PLoS One 2014;9(1):e86155.
60. Lurbe E, Redon J, Kesani A, et al – Increase in nocturnal blood pressure and progression to microalbuminuria in type 1 diabetes. N Engl J Med. 2002;347(11):797–805.
61. Sezer S, Karakan S, Çolak T, Haberal M – Nocturnal nondipping hypertension is related to dyslipidemia and increased renal resistivity index in renal transplant patients. Transplant Proc. 2011;43(2):530–532.
62. Wadei HM, Amer H, Griffin MD, Taler SJ, Stegall MD, Textor SC. Abnormal circadian blood pressure pattern 1-year after kidney transplantation is associated with subsequent lower glomerular filtration rate in recipients without rejection. J Am Soc Hypertens. 2011;5(1):39-47.
63. Choudhary L, Gupta P, Gupta A, et al – 24 hour ambulatory blood pressure monitoring and left ventricular ejection fraction- prognostic markers in chronic kidney disease. Int J Adv Med. 2016;3(2):402-408.
64. Dziwura J, Bińczak-Kuleta A, Miazgowski T, et al – The associations between G972R polymorphism of the IRS-1 gene, insulin resistance, salt sensitivity and non-dipper hypertension. Hypertens Res. 2011;34:1082–1086.
65. Uzu T, Ishikawa K, Fujii T, et al. – Sodium restriction shifts circadian rhythm of blood pressure from nondipper to dipper in essential hypertension. Circulation, 1997;96:1859–1862.
66. Campese VM, Romoff MS, Levitan D, et al. – Abnormal relationship between sodium intake and sympathetic nervous system activity in salt-sensitive patients with essential hypertension. Kidney Int. 1982;21:371–378.
67. Piepoli MF, Hoes AW, Agewall S, et al – 2016 European Guidelines on cardiovascular disease prevention in clinical practice. Eur Heart J. 2016;37:2315-2381.
68. Chen Y, Liu JH, Zhen Z, et al. – Assessment of left ventricular function and peripheral vascular arterial stiffness in patients with dipper and non-dipper hypertension. J Investig Med. 2017; pii: jim-2017-000513
69. Mahabala C, Kamath P, Bhaskaran U, et al – Antihypertensive therapy: nocturnal dippers and nondippers. Do we treat them differently? Vasc Health Risk Manag. 2013;9:125–133.
70. Kario K, Schwartz JE, Pickering TG - Changes of nocturnal blood pressure dipping status in hypertensives by nighttime dosing of alpha-adrenergic blocker, doxazosin: results from the HALT study. Hypertension. 2000;35(3):787-794.
71. Wang C, Ye Y, Liu C, et al – Evening versus morning dosing regimen drug therapy for chronic kidney disease patients with hypertension in blood pressure patterns: a systematic review and meta-analysis. Intern Med J. 2017;47(8):900-906.

72. Wang C, Zhang J, Liu X, et al – Effect of Valsartan with bedtime dosing on chronic kidney disease patients with nondipping blood pressure pattern. J Clin Hypertens (Greenwich). 2013;15(1):48-54.
73. Fukuda M, Kimura G. Salt sensitivity and nondippers in chronic kidney disease. Curr Hypertens Rep. 2012;14(5):382-387.
74. Tosu AR, Demir S, Selcuk M, et al – Comparison of inflammatory markers in non-dipper hypertension vs. dipper hypertension and in normotensive individuals: uric acid, C-reactive protein and red blood cell distribution width readings. Postepy Kardiologii Interwencyjnej. 2014;10(2):98–103.
75. Buyukkaya E, Erayman A, Karakas E, et al – Relation of red cell distribution width with dipper and non-dipper hypertension. Medicinski Glasnik. 2016;13(2):75-81.
76. Kaya MG, Yarlioglues M, Gunebakmaz O, et al. – Platelet activation and inflammatory response in patients with non-dipper hypertension. Atherosclerosis. 2010;209(1):278-282.
77. Ortakoyluoglu A, Boz B, Sitki DO, et al – The association of serum gamma-glutamyl transpeptidase level and other laboratory parameters with blood pressure in hypertensive patients under ambulatory blood pressure monitoring. Ther Clin Risk Manag. 2016;12:1395-1401.
78. Tabara Y, Igaseb M, Mikia T, et al – B-type natriuretic peptide is a determinant of the nocturnal increase in blood pressure independently of arterial hypertrophy and hypoxia. J Hypertens. 2016;34:2393-2401.
79. Bakirci EM, Degirmenci H, Hamur H, et al. – New inflammatory markers for prediction of non-dipper blood pressure pattern in patients with essential hypertension: Serum YKL-40/Chitinase 3-like protein 1 levels and echocardiographic epicardial adipose tissue thickness. Clin Exp Hypertens. 2015;37:1–6.
80. Ji Q, Cheng G, Ma N, et al – Circulating Th1, Th2, and Th17 levels in hypertensive patients. Dis Markers. 2017; 7146290
81. Kadoya M, Koyama H, Kanzaki A, et al – Plasma brain-derived neurotrophic factor and reverse dipping pattern of nocturnal blood pressure in patients with cardiovascular risk factors. PLoS One. 2014;9(8): e105977.
82. Meric M, Soylu K, Avci B, et al – Evaluation of plasma chemerin levels in patients with non-dipper blood pressure patterns. Med Sci Monit. 2014;20:698–705.
83. Zheng H, Xie X, Xie N, Xu H, et al – Sphingomyelin levels in nondipper and dipper hypertensive patients. Exp Ther Med. 2014;7:599-603.
84. Cayli M, Gur M, Elbasan Z, et al – High-Sensitivity Cardiac Troponin T Predicts Nondipper Hypertension in Newly Diagnosed Hypertensive Patients. J Clin Hypertens (Greenwich). 2013;15(10):731-736.

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RESEARCH ARTICLE

Clinical and Therapeutic Trial for the Efficacy of Narrow Band - UVB Phototherapy versus Systemic Therapy in Moderate and Severe Atopic Dermatitis of the Adult

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Objectives: The aim of this clinical and therapy study was to evaluate the efficacy of NB-UVB phototherapy versus systemic therapy in moderate-to-severe atopic dermatitis of the adult. **Material and methods**: The subjects of the study were divided into two groups of 25 adult patients with moderate and severe atopic dermatitis according to the inclusion criteria. The first group of 25 patients were treated with systemic corticosteroids while the second group of 25 patients were treated with NB-UVB phototherapy. At the end of the study, after all the data were centralized, we performed a statistical analysis of the results, comparing the two groups as well as the efficacy of the different therapies. **Results**: In group I the clinical efficacy of the systemic corticosteroid treatment was achieved, on average, at 4 weeks in patients with moderate atopic dermatitis and at 6 weeks in patients with severe atopic dermatitis. In group II the clinical effecacy of NB-UVB phototherapy was achieved, on average, at 6 weeks for patients with moderate atopic dermatitis and at 8 weeks for those with the severe form. In both groups, the total IgE serum levels were elevated at the beginning, and they became normal throughout the clinical improvement. Remarkable therapy-related side effects were found in the first study group. **Conclusion:** We conclude that NB-UVB phototherapy had similar efficacy in treating moderate-to-severe atopic dermatitis with minimal side effects compared to systemic corticosteroid therapy.

Keywords: atopic dermatitis, narrow band UVB, systemic treatment

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Introduction

In 1801, Ritter discovered ultraviolet rays (UV); in 1820, Home describes how solar radiation causes erythema, regardless the power of the emitted heat, and points out the fact that the pigments of dark skinned persons protect against inflammation caused by sunlight [1]. In 1858, the inflammatory effect of UV radiation is proven as a result of a mere laboratory accident. Finsen in 1900 recognized the therapeutic effect of UV radiation, therefore he was awarded the third Nobel Prize in 1903 [2]. UV radiation has both positive and negative biological effects on the human skin. The beneficial effects include: germicidal effect, pigmentogen effect, stimulation of the vitamin D synthesis, and the therapeutic effects on certain skin diseases. The immunomodulatory-suppresive effect of the UV radiation can be both advantageous and harmful [3]. In the 90's Parrish demonstrated that the erythema-causing effect and the therapeutic effects of the UV-B radiation are caused by different wavelengths [4]. In 1984, Fischer demonstrated that the wavelength of 313 nm is the most effective in the treatment of psoriasis. Consequently, UV-B emission lamps appeared with a wavelength of 311nm +/- 2nm in narrowband, named narow-band UVB, NB-UVB.

Atopic dermatitis is an immuno-allergic, inflamatory, non-infectious, itchy skin condition, with chronic evolution, that appears mainly at genetically predisposed individuals. Atopic dermatitis is a widespread skin disease, and its prevalence increases steadily from year to year. Men and women are equally affected, moreover, it accounts for approximately 10% -20% of the dermatological consultations. This skin condition typically affects children in their early childhood but can occur at any age. At the adults, the onset of the disease is between the ages of 20-40 years and it persists with exacerbations and remissions throughout the patient's lifetime. Moderate and severe forms of atopic dermatitis at adults are estimated to vary between 5-10% of the cases. The treatments used in these clinical forms includes emollients and antipruritic agents, systemic corticosteroids, cyclosporine, biological treatment and either UVB or NB-UVB phototherapy. Phototherapy is rather inaccessible for the patients due to the fact that hospitals and private clinics are not equipped with the ultimate NB-UVB phototherapeutic devices. [5].

Objectives

The aim of this clinical and therapy study was to evaluate the efficacy of NB-UVB phototherapy versus systemic therapy in moderate and severe atopic dermatitis of the adult. We also wanted to monitor the total IgE serum levels, as a paraclinic evaluation for the efficacy of the therapy, the side effects that appeared during the study and the long-term adverse effects reported at the end of the study, for all the patients treated with phototherapy.

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Material and methods

The subjects of the study were selected from the patients who presented themselves at the Dermatology Clinic and Outpatient Mures County Clinic Hospital of Tirgu Mures between November 2015 and August 2016. We obtained the study agreement of the Ethics Commission of the Mures County Clinic Hospital and of the Ethics Committee for Research of the University of Medicine and Pharmacy Tirgu Mures, with the decision no.1537 / 2016 respectively, 24/2016.

Patients were selected according to the followng inclusion/exclusion criteria:

Inclusion criteria:

– Clinically confirmed onset or acute exacerbation of moderate or severe atopic dermatitis
– Age over 20 years

Exclusion criteria:

– Acute morbid conditions
– Decompensated chronic diseases
– Age under 20 years
– Patients who did not sign the informed consent.

A study datasheet was performed every patient's case containing: personal information, medical history, both clinical and dermatological examination, treatments and investigations that are going to be performed, as well as the evolution of the affected skin area during the study. The weekly clinical evaluation, the adverse effects, and the possible new flares were monitored. All patients obtained topical treatment with emollients. In both groups, we monitorized the total serum IgE levels at the beginning and at the end of the treatment, as a paraclinic evaluation of treatment efficacy, as it is a well-known the fact that IgE serum levels are maximum in acute exacerbations, decreasing under therapy. We used the immunoturbidimetric method to determine IgE serum levels, and the device used was an Abbot-type Immunoassay, model c 2000 Architect. The subjects were divided into two groups of minimum 25 adult patients with moderate or severe atopic dermatitis according to the above mentioned criteria. The 25 patients from *group I* were treated with *systemic therapy,* using systemic corticosteroids (0.5 to 1 mg/kgprednisone) until clinical response was achieved: 0.5 mg/kg in medium forms and 1 mg/kg for those with severe forms. The systemic treatment was stopped at clinical recovery and the topical treatment was continued as a maintenanc therapy. The 25 patients from *group II* were treated with *NB-UVB phototherapy*. A minimal erythema dose (MED) was carried out for the optimal dosing, knowing that different skin phototypes require different UV doses. The phototherapeutic treatment dosing protocol was as follows:

– The starting dose is 70% of the MED at 1000W power of the device
– Increasing the dose with 10-30%
– Weekly exposure: 3 times per week
– Total exposure: 15-30 (5-10 weeks) of exposures depending on clinical response. For performing the phototherapeutic treatment and to obtain minimal erythema dose, it was used Dr Honle 1000 W NB-UVB phototherapeutic device, which could irradiate an entire hemibody, the patients being irradiated in the front and in the back, with a 1000 W transmit power. At the end of the study, after all the data were centralized, we performed an analysis of the results, comparing the two groups as well as the efficiency of the different therapies.

Results

Group I

Out of the 25 patients included in our study, with relapses, 18 were women (72%) and 7 were men (28%), including 16 suffering from moderate (64%), and 9 from severe form (46%). Of whom, 19 patients (3 men and 16 women) had phototype I (76%), 6 patients (4 men and 2 women) had phototype II (46%). In 5 cases (20%) the treatment was stopped due to gastrointestinal symptoms such as vomiting, nausea, gastric hyperacidity, and in 3 cases a symptomatic treatment was prescribed, while continuing the corticosteriod therapy. The average age of the study population was 29.4 years, all of them were suffering from atopy from childhood, the average of the onset being 16.5 years. The clinical efficacy of the systemic corticosteroid treatment was obtained on average at 4 weeks in patients with moderate atopic dermatitis and at 6 weeks for the patients with severe atopic dermatitis. (Figure 1) The IgE serum levels were elevated at the beginning, and they became normal with clinical improvement. For those with severe forms the average value of total IgE was of 486 IU/ml while for those with moderate form the average value was 280 IU/ml. (Figure 2)

Group II

During the study period 25 patients with relapses were included, 15 women (60%) and 10 men (40%), of whom 15 suffered from moderate forms (60%), and 10 suffered of severe forms, (40%). Out of them, 17 patients (9 male, 8 female) had phototype I (68%), and 8 had phototype II (32%) (6 male and 2 female). The average age of the subjects was 27.2 years, all suffered from atopy from childhood, the average of the onset of the disease being 15.7 years. The clinical efficacy of NB-UVB phototherapy was achieved, on average, at 6 weeks for those with moderate atopic dermatitis and at 8 weeks in those with the severe forms. (Figure 3) Aside from one case (female, phototype I, moderate form), the total serum IgE levels were elevated at the beginning, and they became normal through the clinical improvement. For those with severe froms, the average value of the total IgE was of 526 IU/ ml, while for those with moderate forms the average was 320 IU/ml. (Figure 4) Immediate side effects were observed at two women, with phototype I and severe clinical forms; the phototherapy was interrupted, for two sessions, because of grade I burn, after which we continued according to the proto-

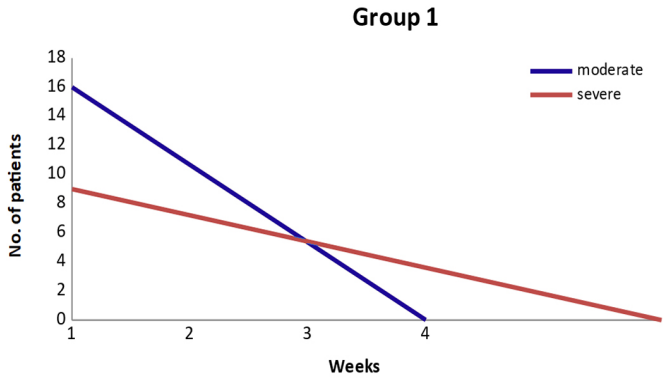

Fig. 1. Clinical efficacy of the treatment at group 1

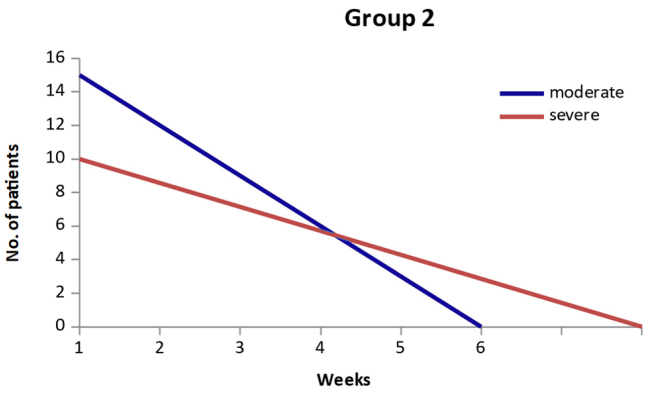

Fig.3. Clinical efficacy of the treatment at group 2

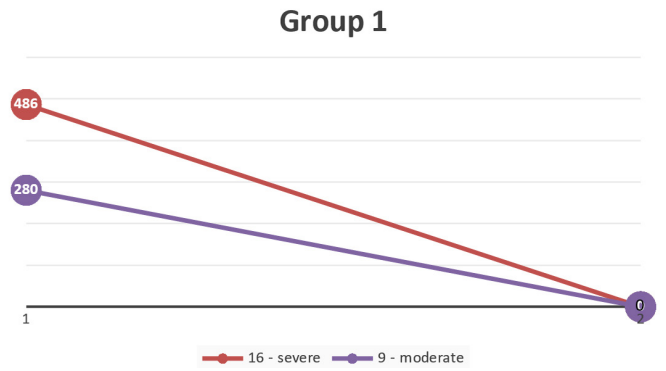

Fig.2. The dynamics of IgE levels at the clinical endpoint for group 1 (IU/ml)

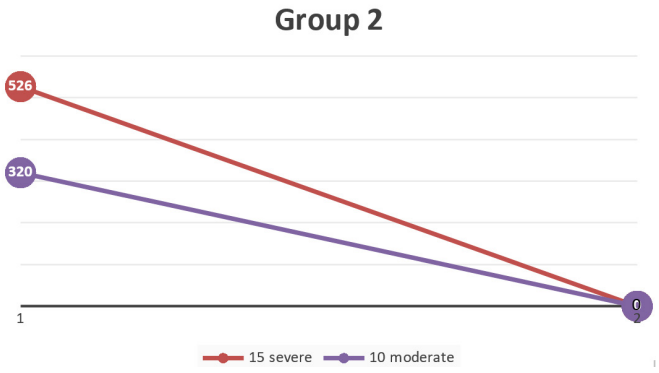

Fig. 4. The dynamics of IgE levels at the clinical endpoint for group 2 (IU/ml)

col. No adverse effects were detected in 6 months after the treatment.

Discussions

UV radiation are divided in function of the radiation wavelength into the UV A, B and C. Generally, the UV-C effect appears only as an inflammation. The UV-B radiation is responsible for the therapeutic effect. The effects of the UV-A radiation are mainly pigmentogene effects, erythematous manifestations occuring in approximatively 1000 x higher dose than the UV-B radiation. UV-B radiation therapies are used as monotherapy or in multiple combinations with local agents used for more than a half century with documented results, particularly in psoriasis, vitiligo and some chronic dermatoses [6]. The adverse effects can be divided into short-term effects such as sunburn, induced hyperkeratosis and pigmentogenesis, and long-term effects like the induction and development of skin cancers and photodermatoses [7]. The beneficial effects of UVB consist in blocking DNA synthesis in hyperproliferative conditions, like in psoriasis, induction of apoptosis in keratinocytes in eczema, and immunomodulatory effect by induction of IL-10, and by decreasing the NK-cell activity in atopic dermatitis [8]. The clinical effects of these new therapies are documented by several studies, and they have proved to be superior to broadband UV treatment, due to the shorter exposure, with longer remissions and with more moderate side effects [9]. In the phototherapeutic treatment of the atopic dermatitis a well-defined protocol for psoriasis is used, with the indication of moderate and severe atopic dermatitis of the adult. Phototherapy is not indicated under the age of 12 years [10]. NB-UVB therapy can also be used with protocols adjusted to several chronic skin conditions, such as: chronic urticaria, nodular prurigo, different etiologies of pruritus, alopecia areata, lichen sclero-atrophic, seborrheic dermatitis, mycosis fungoides, etc. [11]. In addition to the treatments used for these clinical forms, systemic treatment with corticosteroids, cyclosporine, and biological therapies are also used. [12-15]. In the systemic treatment of the study population, in group I we used systemic corticosteroids, since they are the most accessible systemic therapies. Atopic dermatitis is considered to be a multifactorial disorder, whose clinical expression depends on complex interactions like: hereditary predisposition, functionally altered skin barrier, immunological and neuro-endocrine abnormalities and a series of trigger or aggravating factors of skin lesions. Both sexes are equally affected. The study subjects were chosen to include men and women, approximately equal. The clinical and laboratory characteristics of these patients are: intense cutaneous pruritus, marked xerosis, the association of other allergic diseases, increased IgE serum levels, as well as the appearance of typical skin lesions of a chronic eczema covering large areas of the skin [16]. Concerning the effi-

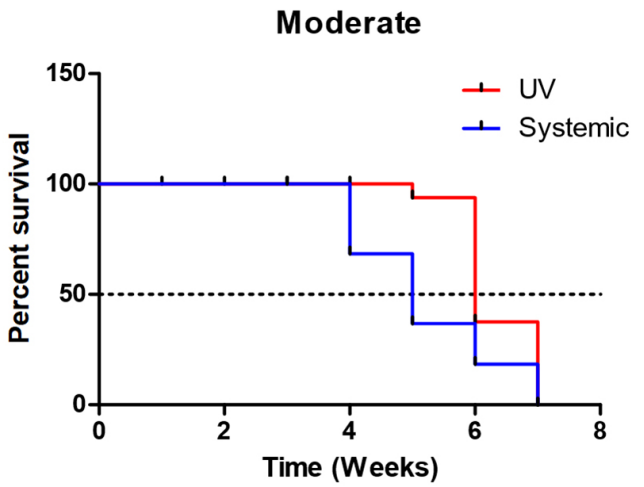

Fig. 5. Survival analysis test for patients with moderate clinical form

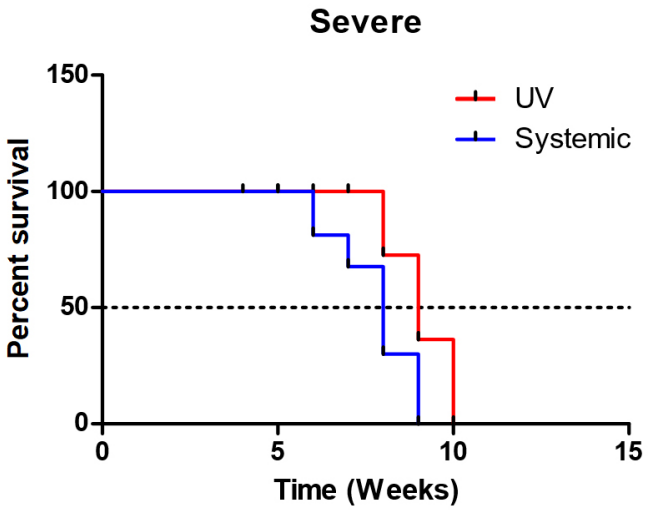

Fig. 6. Survival analysis test for patients with severe clinical form

cacy of the treatments both of them were efficient, and the difference of the time until recovery between the two groups has been clinically significant. We done the survival analysis Log-rank test to compare the efficacy of the treatment at patients with moderate and severe clinical forms from both groups. At patients with moderate clinical forms the survival curves were significant different with p value of 0.0036. (Figure 5)

At patients with severe clinical forms the survival curves were significant different with p value of 0.0013. (Figure 6) The systemic treatment is more practical, the phototherapeutic therapy requiring a more difficult management of the patients. During the study, the dynamics of IgE serum levels were directly correlated with the clinical evolution and efficacy of the treatment, it was elevated at the beginning, and it became normal throughout the clinical improvement. Similarly, IgE serum levels were also correlated with the clinical form of the disease, having been more elevated in the severe forms of the disease that corresponds with the literature datas [17]. Regarding adverse effects, in group I with patients on systemic corticosteroids, we have had a drop to 20% of the patients due to the gastrointestinal side effects. In group II there was no evidence of short-term side effects, while the long-term adverse effects will be followed and documented.

Conclusions

As a conclusion we can say that phototherapy had similar efficacy with minimal side effects compared to systemic drug therapy. The management of patients treated with phototherapy is much more difficult. The dynamics of serum IgE levels during the study are directly correlated with the clinical evolution and efficacy of the treatment. Side effects are more severe in patients receiving systemic treatment than in those who are treated with phototherapy, this type of treatment being an effective alternative. At the moment, phototherapy is rather inaccessible for patients, due to the fact that hospitals and private clinics are not equipped with the ultimate NB-UVB photohterapeutic devices.

Conflict of interest

None to declare.

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References

1. Ritter J W. - Kurze Mitteilung uber die Endeckung der Uv-Strahlen. Ann. Phys 1801,7:527.
2. Hölze E. - Photodermatosen und Lichtreaktionen der Haut. Wissenschaftliche V mbH, Stuttgart, 2003,31-33.
3. Horkay Irén - Klinikai photodermatologia, Ed. Medicina, Budapest, 2008, 29-39.
4. Parrish JA, Jaenicke KF. - Action spectrum for phototherapy of psoriasis. J Invest. Dermatol.,1981: 76:359.
5. Brown SJ. - Atopic eczema. Clin Med (Lond), 2016,16(1):66-69.
6. Vangipuram R, Feldman SR. - Ultraviolet phototherapy for cutaneous diseases: a concise review. Oral Dis., 2016, 22(4):253-259.
7. Rodenbeck DL, Silverberg JI, Silverberg NB.- Phototherapy for atopic dermatitis. Clin Dermatol. 2016, 34(5):607-613.
8. Fernández-Guarino M, Aboin-Gonzalez S, Barchino L, et al. - Treatment of moderate and severe adult chronic atopic dermatitis with narrow-band UVB and the combination of narrow-band UVB/UVA phototherapy. Dermatol Ther. 2016, 29(1):19-23.
9. Horkaylrén - Ultraibolya fény okozta kórélettani változások a bőrben. Bőrgyógy. Vener. Szl., 2005, 81: 95-97.
10. Sokolova A, Lee A, D Smith S. - The Safety and Efficacy of Narrow Band Ultraviolet B Treatment in Dermatology: A Review. Am J Clin Dermatol, 2015, 16(6):501-531.
11. Patrizi A, Raone B, Ravaioli GM. - Management of atopic dermatitis: safety and efficacy of phototherapy. Clin Cosmet Investig Dermatol, 2015, 5;8:511-520.
12. Notaro ER, Sidbury R. - Systemic Agents for Severe Atopic Dermatitis in Children. Paediatr Drugs, 2015, 17(6):449-457.
13. Totri CR, Eichenfield LF, Logan K, et al. - Prescribing practices for systemic agents in the treatment of severe pediatric atopic dermatitis in the US and Canada: The PeDRA TREAT survey. J Am Acad Dermatol, 2017, 76(2):281-285.
14. Simon D, Bieber T. - Systemic therapy for atopic dermatitis. Allergy, 2014, 69:46-55.

15. Cornish KS, Gregory ME, Ramaesh K. - Systemic cyclosporin A in severe atopic kerato-conjunctivitis. Eur J Ophthalmol, 2010, 20(5):844-851.
16. Weinhold A, Obeid R, Vogt T, Reichrath J. - Prospective Investigation of 25(OH)D3 Serum Concentration Following UVB Narrow Band Phototherapy in Patients with Psoriasis and Atopic Dermatitis. Anticancer Res, 2016, 36(3):1439-1444.
17. Sidbury R, Davis DM, Cohen DE, et al. - American Academy of Dermatology. Guidelines of care for the management of atopic dermatitis: section 3. Management and treatment with phototherapy and systemic agents. J Am Acad Dermatol, 2014, 71(2):327-349.

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RESEARCH ARTICLE

Sciatic Nerve Regeneration in Wistar Albino Rats Evaluated by *in vivo* Conductivity and *in vitro* 1H NMR Relaxometry

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Objective: The aim of this study was to evaluate and quantify functional and structural nerve regeneration after reconstruction using either direct suture or silicon graft. **Methods**: Thirty-two adult Wistar Albino rats were divided in two equal groups. The left sciatic nerve was cross-sectioned and reconstructed using either direct suture (DS group) or a silicone graft (SG group). At 4, 6, 8 and 10 weeks two rats were randomly chosen from each group for in vivo measurement of nerve electric conductivity and subsequently sacrificed together with other two rats from the same group for in vitro 1H NMR relaxometry measurements. The T2 distributions were assigned to 1H located in different pools corresponding to the nerve structure. **Results**: In the injured nerve we observed a significant increase in the stimulation threshold and a decrease in conduction velocity when compared with the healthy nerve in both groups. Whereas the conduction velocity increased progressively from 4 to 10 weeks in the DS group, the opposite evolution was observed in the SG group. In both groups, the first two peaks corresponding to water bound to collagen and epineurium had smaller transverse relaxation times in the injured nerves, while there was no change in the peaks corresponding to perineurium and free water between healthy and injured nerves. **Conclusions**: Significant differences were observed between direct suture and nerve graft reconstructions from both a functional and structural point of view. In the case of direct suture reconstruction, the nerve was functionally healed at 10 weeks after injury.

Keywords: rat's sciatic nerve regeneration, electric conductivity, 1H NMR relaxometry

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Introduction

In the last twenty years, the treatment methods regarding patients with a peripheral nerve injury has significantly evolved. Due to better understanding of the structure and function of the peripheral nerves, new intraoperative diagnosis, reconstructive surgery is being optimized permanently. Peripheral nerves are designed to receive and relay input from the environment to the central nervous system. They are associated with three separate tissue sheaths, the endoneurium, perineurium and epineurium. These three elements have been investigated over time and it has been demonstrated that they are providing multiple mechanical and physiologic functions [1].

The retrograde cell death of motor and sensory neurons is one of the main problems associated with peripheral nerve injury. Compared to the central nervous system, injured peripheral axons are able to self-regenerate. Sometimes misdirected growth of nerve fibres can occur, even if the axonal regeneration across the injury site was successful. In most cases of peripheral nerve injuries, the nerve ends can be sutured, and the neurological recovery can be reasonable. However, if we have a nerve defect, the primary suture is not possible and nerve grafts are required to bridge the defect [2].

In vivo specific methods of examination of peripheral nervous system are based on electrodiagnosis and nuclear magnetic resonance imaging or spectroscopy. Electrodiagnosis is the observation and interpretation of the electrical signals derived from the depolarization and repolarization of the peripheral nerves. Voluntary muscle contractions can produce these action potentials, or they can be evoked by electrical stimulation [1]. In the early 90s, Howe et al [3] and Filler [4] proposed an MRI (magnetic resonance imaging) examination, called neurography, focused on the visualization of the peripheral nervous system with dedicated sequences optimizing the contrast between nerves and their environment. The neuronal ultrastructure is examined by a phased-array coils into a high field imager significantly improved the spatial resolution and the signal to noise ratio into an MR image. Such MR picture is spatially encoded by a specific NMR (nuclear magnetic resonance) parameter called, transverse relaxation time, T_2 which is specific to each tissue. The T_2- weighted spin-echo sequences, have good contrast resolution, but they have been replaced by the neurography sequences which are highly weighted T_2 sequences using long echo times combined with fat signal suppression. MRI findings are consistent with the peripheral nerve`s anatomy [5]. Only few studies were reported on the degeneration/injury of the peripheral nerve on animals. Thus, Does and Snyder use the multi-

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exponential T_2 relaxation distribution on the amphibian Xenopus laevis of which sciatic nerve was crushed [6]. Recently the ^{1}H NMR relaxometry method combined with inverse Laplace analysis [7, 8] was successfully used to assess the ovariectomy induced osteoporosis on Wistar Albino rats [9-12]. The measured T_2-distribution on different section of rats femur allowed the calculation of the pores sizes distributions as well as the effects of treatment with simvastatin or fenofibrate.

The purpose of this study was to evaluate the Wistar Albino rats' sciatic nerve regeneration after injury through *in vivo* electrodiagnosis and *in vitro* ^{1}H NMR relaxometry.

Methods

The Ethics Committee of The University of Medicine and Pharmacy of Targu Mures approved the experiment and study protocols, according to document 45/2017. For this study we have used thirty-two Wistar Albino rats, with an average weight of 500 grams. The animals were divided into two groups, each having sixteen rats. For all animals the left sciatic nerve was interrupted using a No. 11 blade (Fig. 1a). In the first group the nerve was directly sutured under magnification loupes (2.5X Keeler) with 8.0 sutures (Fig. 1b). The left sciatic nerve of rats belonging to the second group, by interruption, a 7 mm defect was created and the reconstruction was performed using a silicone graft. The proximal and distal nerve stumps were inserted 1 mm into the tube to leave a 5 mm gap and they were fixed to the graft with two epineural sutures (8.0 Prolene) (see Fig. 1c). The right sciatic nerve for both groups was used as the control group.

In vivo electric conductivity measurements (Fig. 2) were performed for two randomly selected rats at 4, 6, 8 and 10 weeks after surgery. Electrical nerve stimulation was carried on using an external programmable pulse generator based on a PIC16F877 microcontroller produced by Microchip. Inc. , Arizona USA. The rectangular pulses were delivered with a period of 1 second, a 1 ms the pulse-width, having an amplitude of 2× threshold for each nerve. The stimulation electrode was bipolar type, using two parallel pure silver wires at 2mm distance, placed approximately 1cm proximal to the lesion.

The nervous influx was recorded using bipolar silver electrodes placed distal to the lesion, amplified using an AD621 instrumentation amplifier produced by Analog Devices and fed into a National Instruments acquisition board. LabView software was used to view, store and analyze the recorded signals. Using a digital vernier calliper, we measured the distance between the stimulation and the receiving electrode. The nerve conduction time was measured in LabView and the conduction speed was calculated. Ten measurements of distance, conduction time and stimulus amplitude were performed for each nerve, and the average values were reported.

At 4, 6, 8 and 10 weeks after the injury and reconstruction, 4 animals from each group were randomly selected and euthanized with an overdose of ketamine and xiline (8-10 mg/kg). Both sciatic nerves were removed and preserved into a solution of formaldehyde until the NMR

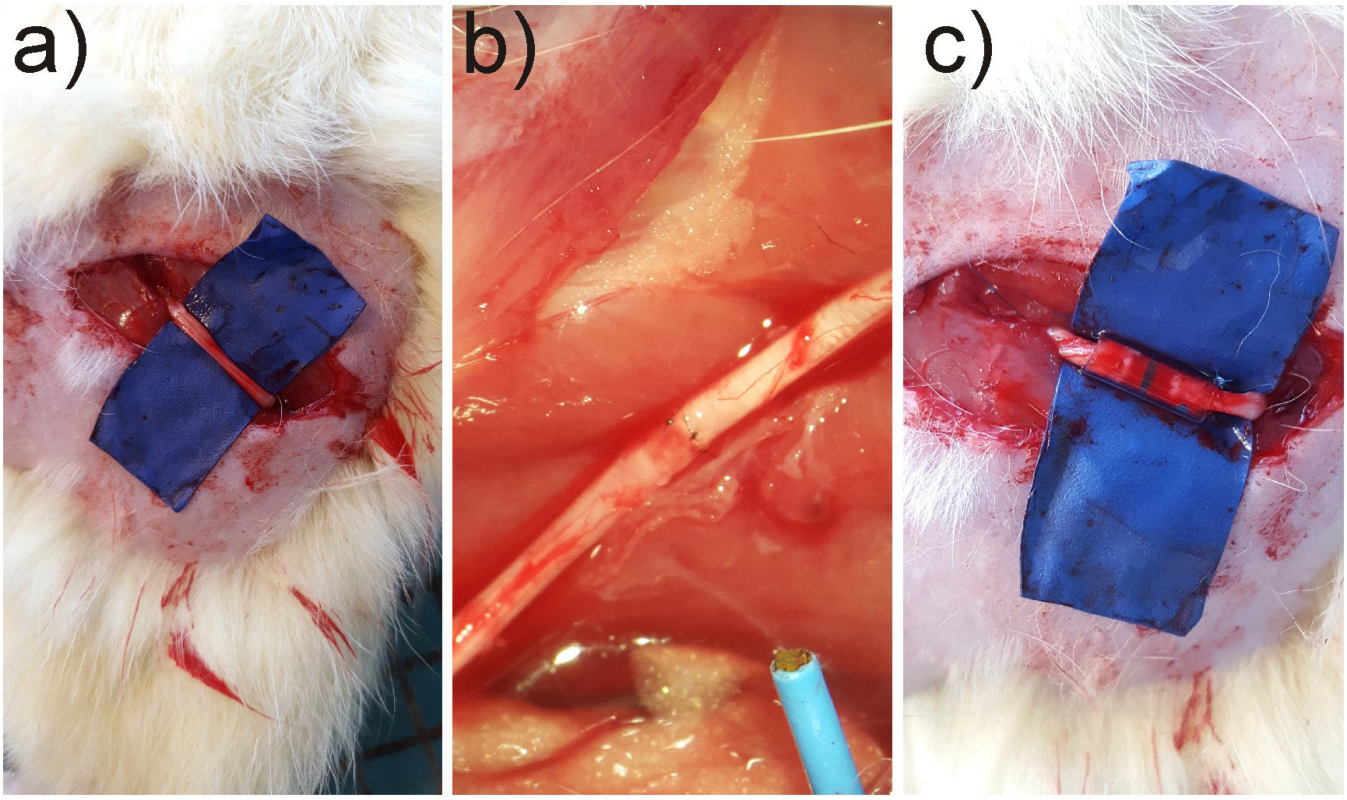

Fig. 1. Picture of a) healthy nerve; b) direct suture method and c) silicone nerve graft method for Wistar Albino rats.

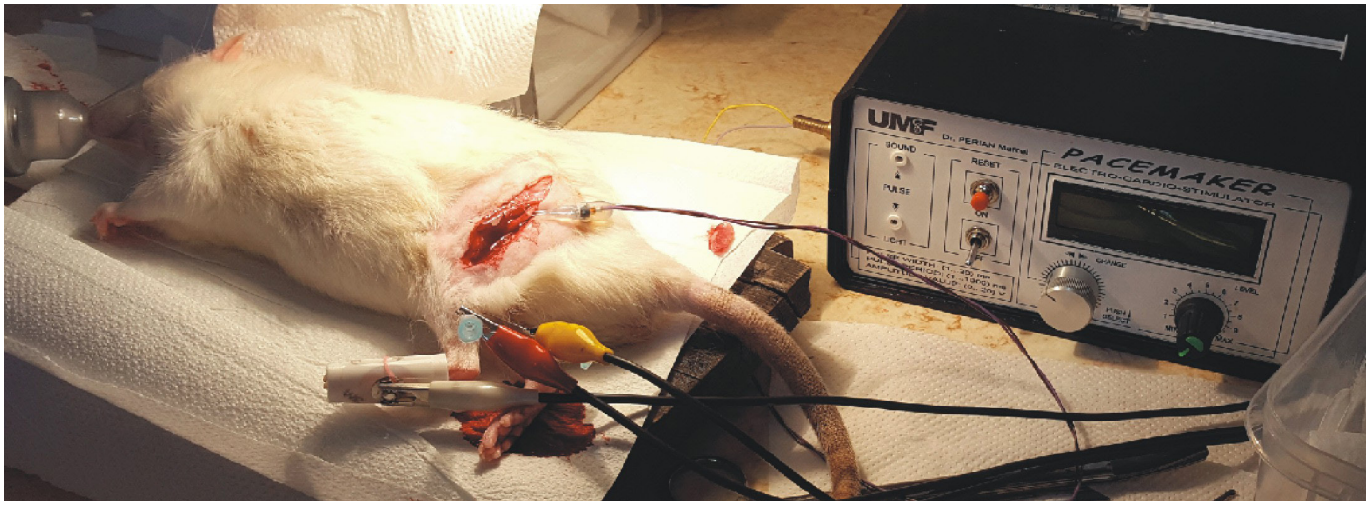

Fig. 2. Picture of the experimental set-up for the in vivo electric conductivity measurement of rat's sciatic nerve.

measurements were performed. The low field NMR measurements were performed using the BRUKER MINISPEC mq20 spectrometer working at 19.6712 MHz. The inter-echo time in CPMG pulse sequence was set at 0.25 ms. The tipping pulse length was 9 μs and refocusing pulses with the same duration was set double in amplitude. A total number of 3000 echoes were recorded (see Fig. 3a). The decay of transverse magnetization during the CPMG echoes train was assumed to be multi-exponential and hence can be described by equation depicted in Fig. 3. Here T_2 is the transverse relaxation time of each particular statistical sub-ensemble of protons and $f(T_2)$ is the distribution function. The interpretation of 1D experimental data request to find the transverse relaxation time distribution $f(T_2)$. To this purpose the experimental data was analyzed using a Laplace inversion algorithm. The mathematically details were detailed elsewhere [7, 8]. An example of such T_2-distribution is presented in Fig 3b for the injured sciatic nerve of the rat No. 8 sacrificed at 6 weeks after direct suture (group DS). Usually, the association of each distinct peak with specific hydrogen reservoirs (mostly water) is based on the water molecule mobility, then the most mobile ^{1}H are characterized by a large transverse relaxation time, T_2 while the most rigid ^{1}H are characterized by the smallest T_2.

Results

The average values of response voltage, response time and response velocity are presented in Fig. 4 for both groups (direct suture and silicone graft reconstruction methods) function of time elapsed from reconstruction. In each case parameters measured for the healthy nerve (red bars) are compared with those measured for injured sciatic nerve (blue bars).

A clear differentiation can be observed between the healthy nerve's average voltage response (2.8 – 4.2 V) and under-recovery sciatic nerve with the corresponding values between 4 – 6.5 V (see Fig. 4a). The net differentiation is

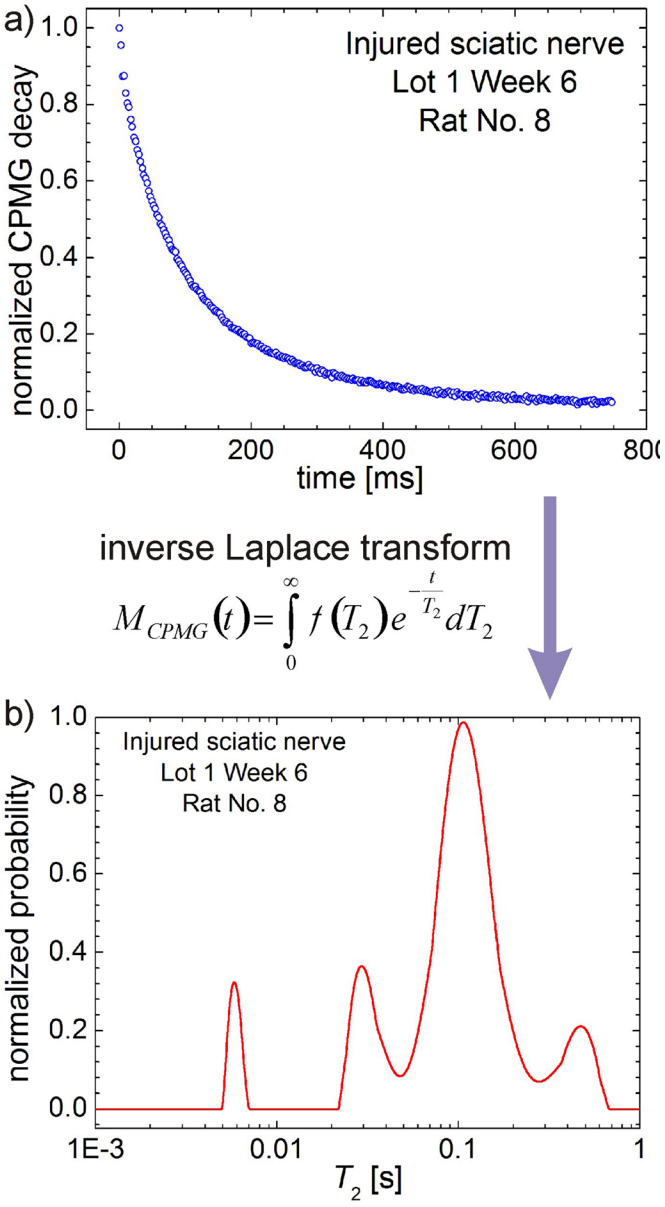

Fig. 3. a) The normalized CPMG decay measured after 6 weeks from injury and direct suture reconstruction of Rat's No. 8 sciatic nerve and b) the corresponding T2-distribution obtained by inverse Laplace transform.

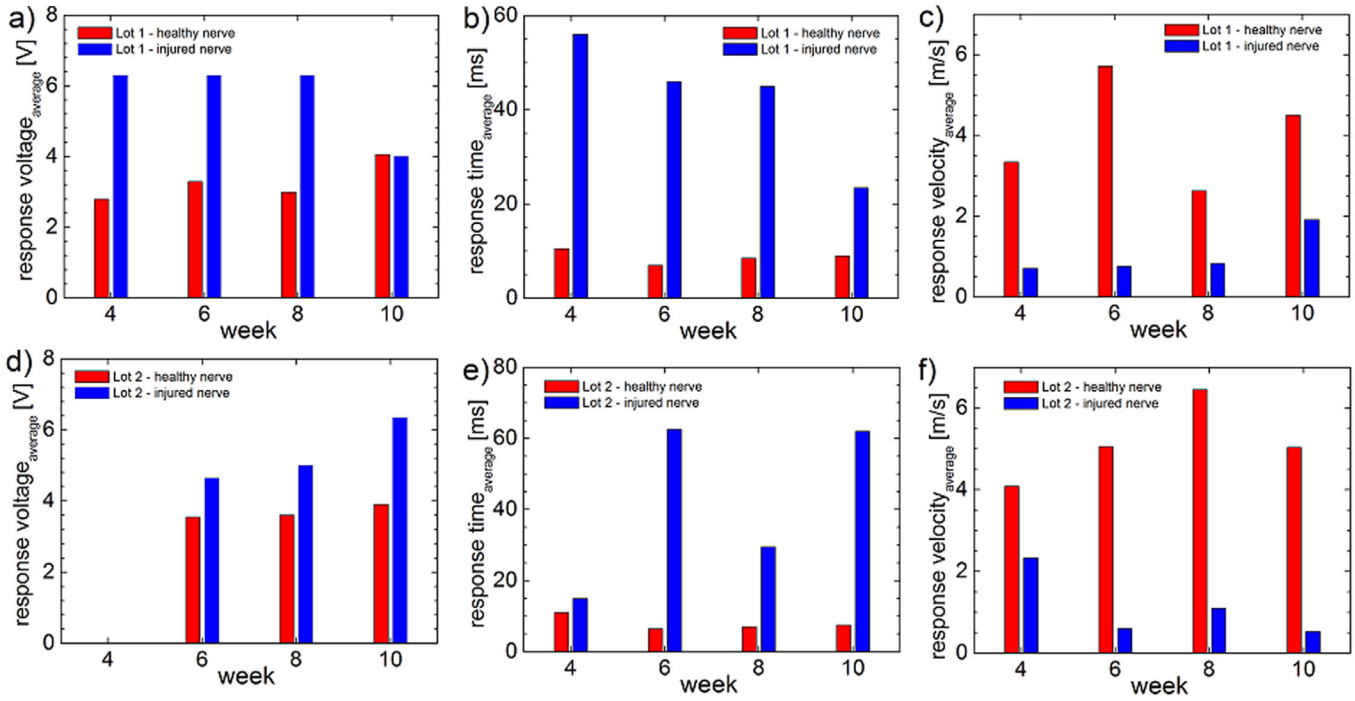

Fig. 4. In vivo electric conductivity comparison between the direct sutured group – a, b, c; and the silicon grafted group – d, e, f. Measured parameters were average voltage response –a, d; time response – b, e; and velocity response – c, f.

observed for the sciatic nerve measured at an early and medium recovery time (4 – 8 weeks after injury and suture). At 10 weeks after injury the average voltage response values are the same for injured and healthy nerve. The average time response for the healthy nerve is much shorter (< 11 ms) compared with the same average time measured for the injured and directly sutured sciatic nerve (>23 ms)(Fig. 4b). A fast response time is associated with a large average conduction velocity (see Fig. 4c). The large difference between the measured values characterizing the *in vivo* electric conduction of the healthy and injured sciatic nerve of the group DS, overcome the small fluctuations of the corresponding values measured in time for the healthy nerve (see the left column of Fig. 4).

The healing behavior, as resulted from *in vivo* electric conductivity measurement on Wistar Albino rats from group SG was unexpected. Large differences were observed for all three parameters, the average voltage, time and velocity, measured at 10 weeks after surgery (see the right column in Fig. 4). In fact it appears that, for this group, the sciatic nerve functionality is decaying while time passes, since smaller differences between measured parameters are observed at an early healing process. Due to the fluctuation of values along the recovery time, the average response time and velocity appears not to be able to describe satisfactorily the healing process (see the blue bars in Fig. 4e and 4f).

The electric conductivity measurement is an expression of the nerve function while the ^{1}H NMR relaxometry measurement can describe the regeneration process from a structural point of view. The average normalized T_2-distributions measured for the healthy and recovering after injury and direct suture Wistar Albino rats' sciatic nerves (group DS) are presented in Fig. 5 for rats sacrificed at 4, 6, 8 and 10 weeks. All T_2-distributions presents four peeks associated with characteristic protons' mobility to i) ^{1}H bounded to collagen fibrils characterized by the smallest T_2 values between 1.35 ms – 11.2 ms for the healthy nerve (Fig. 5a) and between 1.19 ms – 7.09 ms for the injured nerve (Fig. 5b); ii) ^{1}H from water located in epineurium characterized by T_2 values between 7.64 ms – 48.89 ms for the healthy nerve and between 7.00 ms – 45.37 ms for the injured nerve; iii) ^{1}H from water located in perineurium characterized by T_2 values between 25.7 ms – 266.2 ms for the healthy nerve and between 23.3 ms – 410.0 ms for the injured nerve; iv) ^{1}H from water from blood vessels characterized by T_2 values between 139.0 ms – 926.6 ms for the healthy nerve and between 284 ms – 1160 ms for the injured nerve. At 6 weeks the peaks appear to be better resolved while at 8 weeks after surgery the peaks characterized by the larger T_2 value merges with the main peak.

The average normalized T_2-distributions measured for the healthy and recovering after injury and reconstruction of sciatic nerves with silicone graft (group SG) are presented in Fig. 6. As in the previous case all T_2-distributions present the same four peeks with the same association. Then, the ^{1}H bounded to collagen fibrils presents T_2 values between 1.91 ms – 8.5 ms for the healthy nerve (Fig. 6a) and between 0.1 ms – 4.19 ms for the injured nerve (Fig. 6b). The ^{1}H from water located in epineurium are characterized by T_2 values between 6.72 ms – 46.37 ms for the healthy nerve and between 5.08 ms – 41.2 ms for the injured nerve. The ^{1}H from water located in perineurium have T_2 values between 27.41 ms – 261.33 ms for the healthy nerve and between 20.91 ms – 466.0 ms for the injured nerve; Finally, the ^{1}H from water from blood vessels is characterized by T_2 values between 143 ms – 816

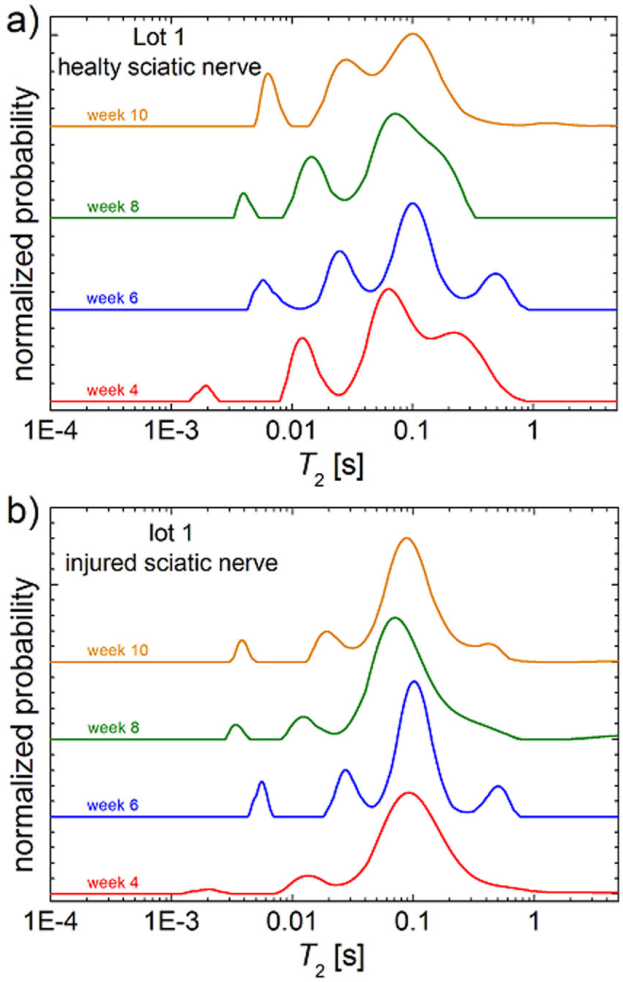

Fig. 5. Compared normalized T2 – distributions measured for the rat's sciatic nerve a) healthy and b) at 4, 6, 8 and 10 weeks after direct suture.

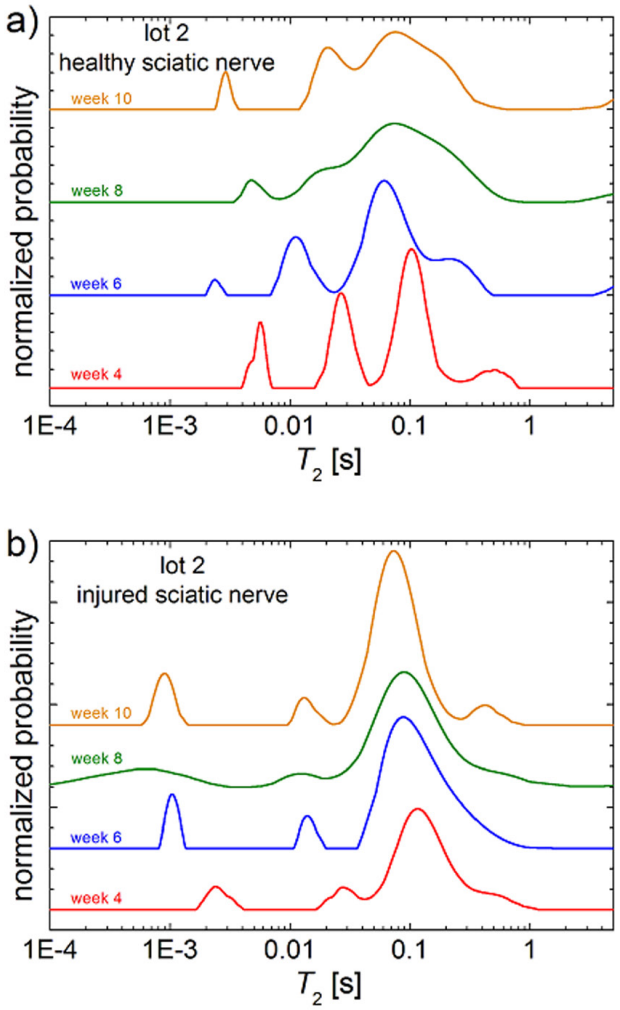

Fig. 6. Compared normalized T2 – distributions measured for the rat's sciatic nerve a) healthy and b) at 4, 6, 8 and 10 weeks after silicone grafting.

ms for the healthy nerve and between 276 ms – 1185 ms for the injured nerve. As in the case of the group DS, in group SG the T_2-distributions presents broaden peaks at 8 weeks after surgery.

Discussion

The response voltage is an important parameter which can be associated with the healthy state of the sciatic nerve. Thus, a healthy nerve will respond (carrying the electric signal) to a lower stimulating voltage.

The *in vivo* electric conductivity measurement for group DS presents a clear recovery after injury and direct sciatic nerve reconstruction. Therefore, form the average voltage response point of view, the rat's sciatic nerve is healed at 10 weeks after injury. Contrary, from the average time response and consequently from average velocity response, the rats' sciatic nerve is not healed therefore the nerve function is not completely restored. Nevertheless, the overall behavior of the electric conduction parameters show that the nerve is in the healing process since these values become closer to the corresponding values measured for the healthy nerve. Moreover, the recovering nerve (left one) presents a more predictable behavior than the more fluctuating behavior observed for the healthy nerve (the right one of the same rat). The sudden change in the values of all three measured electric conduction parameters between the week 8 and week 10 can be explained by the healing process which takes place from proximal to distal part of the nerve [1]. One can consider, that until the 8th week, the healing process did not reach the injury site while sometime after 8 weeks the process passes over the lesion and the functionality of the sciatic nerve is faster restored.

As a general observation the T_2-distributions measured for the injured sciatic nerve for both groups, presents a main peak (the majority of ^{1}H characterizing a certain pool) associated with water located in the perineurium. The evolution in time of this peak can lead to the interpretation of healing process in terms of structural changes at the nerve level. In all cases an interesting process was observed at 8 weeks which leads to a broadening of the T_2-distributions. It is possible to associate this phenomenon with the front of the healing process which is closer to the injured site.

Conclusion

In vivo electric conductibility and ^{1}H NMR relaxometry techniques are sensitive and can be used to evaluate the

nerve healing process from direct point of view of functionality and structural changes. Significant differences were observed between the two types of reconstruction, direct suture or nerve graft. In this sense, the electric conductivity showed that the functionality of the sciatic nerve is restored faster, in the case of direct suture compared to the silicone graft. The ^{1}H NMR relaxometry and electric conductivity highlights the 8^{th} week, when we assumed that the frond of healing process is closer to the former injury site.

Conflict of inerest

None to declare.

References

1. Tenzis K. Julia, Smith L. Kevin - Repair and Grafting of the Peripheral Nerve. McCarthy G. Joseph, Plastic Surgery Volume 1 General Principles, W. B. Saunders Company, p;630-670.
2. Pettersson J., Kalbermatten D., McGrath A., Novikova L. - Biodegradable fibrin conduit promotes long-term regeneration after peripheral nerve injury in adult rats. Journal of Plastic, Reconstructive & Aesthetic Surgery. 2010;63:1893-1899.
3. Howe FA, Filler AG, Bell BA, Griffiths JR. - Magnetic resonance neurography. Magn Reson Med. 1992;28(2):328-38.
4. Filler AG, Kliot M, Winn HR, Tsuruda JS, Hayes CE, Howe FA, et al. - Magnetic resonance neurography. Lancet 1993;341(8846):659-61.
5. Ohanaa, M., Moserb T., Moussaouïc A., et al. - Current and future imaging of the peripheral nervous system. Diagnostic and Interventional Imaging. 2014;95: 17-26.
6. Does M., Snyder R. - Multiexponential T2 Relaxation in Degenerating Peripheral Nerve. MRM. 1996;35:207-213.
7. Venkataramanan L., Song Y., Hurlimann M. - Solving Fredholm integrals of the first kind with tensor product structure in 2 and 2.5 dimensions. IEEE Trans Sign Proc. 2002;50:1017-1026.
8. Song Y., Venkataramanan L., Hürlimann M., et al. - J. Magn. Reson. 2002;154:261-268.
9. Sipos R., Fechete R., Moldovan D., Sus I., Szasz S, Pávai Z. - Assessment of femoral bone osteoporosis in rats treated with simvastatin or fenofibrate. Open Life Sci. 2015;10:379-387.
10. Sipos R, Fechete R, Chelcea R, et al. - Ovariectomized rats' femur treated with fibrates and statins. Assessment of pore-size distribution by 1H-NMR relaxometry. Rom J Morphol Embryol. 2015;56 (2 Suppl):743-752.
11. Chelcea R., Sipos R., Fechete R., et al. - One-Dimensional Laplace Spectroscopy Used For The Assessment Of Pore-Size Distribution On The Ovariectomized Rats Femur. STUDIA UBB CHEMIA. 2015; LX 1:57-70.
12. Sipos R., Fechete R., Moldovan D., et al. - Ovariectomy-Induced Osteoporosis Evaluated by 1H One-and Two-Dimensional NMR Transverse Relaxometry. Applied Magnetic Resonance. 2016;47 (12):1419-1437.

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RESEARCH ARTICLE

Comparative Enantioseparation of Amlodipine by HPLC and Capillary Electrophoresis

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University of Medicine and Pharmacy from Tirgu Mures, Romania

Objective: The purpose of this study was to separate the enantiomers of amlodipine by High Performance Liquid Chromatography (HPLC) using ovomucoid (OVM) as chiral selector, respectively by Capillary Electrophoresis (CE) using cyclodextrines and to evaluate the analytical performance of the both proposed methods. **Material and methods**: HPLC enantioseparation of amlodipine was performed on an HPLC Agilent Technologies 1100 series using as chiral stationary phase an Ultron ES OVM, 150x4.6 mm column with ovomucoid as chiral selector. The stereoselective CE analysis of amlodipine was achieved on Agilent Technologies 7100 CE using uncoated fused-silica capillaries 48 cm x 50 mm and different type of cyclodextrins as chiral selectors. **Results**: A mobile phase consisting of 80% Na2HPO4 10 mM at a pH level of 5.0 and 20% ACN, isocratic elution at a flow of 1 ml/min turned to be the optimal experimental conditions for HPLC analysis (R=5.51; α=1.71) with retention times shorter than 10 minutes for the two isomers, tR (S-AML) = 4.63 (min); tR (R-AML) = 5.54 (min). The migration times for amlodipine enantiomers were tm (S-AML) = 8.15 (min) and tm (R-AML)= 8.45 (min) and the optimum CE conditions have proven to be a buffer solution containing 25 mM H3PO4 at pH 3.0 and 20 mM α-CD as chiral selector and a capillary temperature set at 15°C (R=1.51; α=1.03). **Conclusion**: The analytical performances of the chromatographic method using OVM as chiral selector are superior to the electrophoretic analysis method but the CE method is more economical and may represent an alternative to the HPLC chromatographic separation.

Keywords: amlodipine, enantioseparation, ovomucoid, cyclodextrins, HPLC, CE

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Introduction

During the past years, one of the main research direction in the pharmaceutical industry is the implications of chirality in the pharmacological effect and therapeutic use, respectively the development of modern enantioseparation methods for chiral drugs. Although high performance liquid chromatography (HPLC) remains a powerful technique in analysis of chiral drugs, capillary electrophoresis (CE) offers advantages in terms of low consumption of organic solvents and the wide variety of chiral selectors that can be easily added into background electrolyte solutions [1].

In the past years, chromatographic methods have been the first choice for the enantioseparation of chiral molecules. Nowadays, CE using cyclodextrins (CD) as chiral selectors presents many advantages compared to HPLC. Worth to mention that in CE, the consumption of organic solvents is relatively low; the type, ionic strength, concentration of electrolytes and the pH of the background electrolyte (BGE), can be easily changed; the buffers that can be used may be polar or non-polar. More importantly is that, in CE, the chiral selector can be easily changed [2,3]. HPLC is a very well known technique offering high robustness and reproducibility. Nevertheless, in LC related chiral analysis methods, the chiral selector usually can be found at the level of the stationary phase. Thus beeing said, the versatility in changing the chiral selector resides in changing the chromatographic column, which involves high costs due to the high purchasing prices of chiral columns [4].

An interesting drug molecule with optical activity is amlodipine. Amlodipine (AML), (3-ethyl 5-methyl-2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-6-methyl-1,4-dihydropyridine-3,5 dicarboxylate), a leader among dihydropyridine class, is used alone or in combination with other active pharmaceutical ingredients in various cardiovascular pathologies [5]. Amlodipine has one chiral center, due to the presence of an asymmetric carbon atom in position 4, which automatically generates two optical isomers (Figure 1). Amlodipine is available in pharmaceutical dosage forms as racemic mixture, although, the eutomer, levamlodipine (S-AML) was reported to be 1000 times more active than R-AML [6]. In few countries amlodipine is available as S enantiomer tablets.

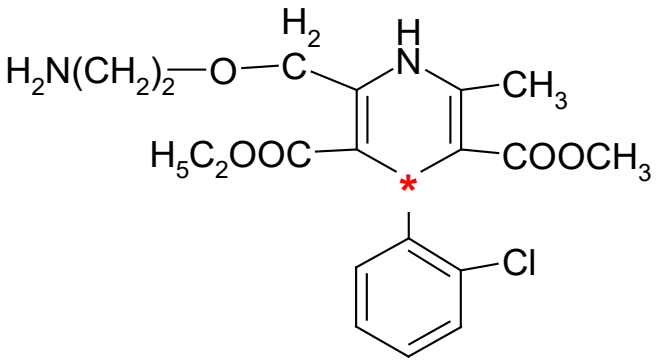

Fig. 1. Amlodipine chemical structure (* denotes the asymmetric carbon atoms)

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In recent years, only few HPLC chiral methods have been reported for the chiral discrimination of AML enantiomers, such as an enantioseparation with the aid of a Lux Cellulose-4 chiral column by using a mobile phase composed of acetonitrile (ACN), ethanol and diethylamine [7]. Another HPLC method for amlodipine separation was developed by Luksa et al. [8]. They used α1- acid glucoprotein immobilized on 5 μm silica gel column and a mobile phase consisting of acetate buffer and propranol. The ovomucoid has been also used in the past by Ansari et al. [9] for its ability to distinguish between the enantiomers of many drugs. They developed an isocratic HPLC method for the enantiopurity quantitation of S-AML by using an OVM chiral column and a mobile phase consisted of phosphate buffer and acetonitrile.

In terms of chiral electrophoretic analytical methods developed for the enantioseparation of amlodipine, there are many methods reported in the literature where cyclodextrins were used as chiral selectors. Zandkarimi et al. [10] have succeeded the enantioseparation of amlodipine using highly sulfated cyclodextrins (HS-β-CD) as chiral selector. Another derivatized anionic cyclodextrin used by Owens et al. [11] aimed to solve the chiral separation of amlodipine was sulfobutylether-β-cyclodextrin (SBE-β-CD). Neutral hydroxypropyl-β-CD (HP-β-CD) were used by Wang et al. [12] for the enantioseparation of amlodipine. The use of randomly methylated β-CD (RAMEB-β-CD) as chiral selector by Hancu et al. [13] allowed the chiral separation of amlodipine with a resolution greater than 2. Native CD, α-CD, has been used for AML enantiomers separation using CE by Small et al. [14] with the migration times longer than 25 minutes for both enantiomers.

Our study was focused on investigating the chiral enantioseparation behaviour of AML, with the help of two different newly developed methods. The chosen methods were based on HPLC and CE techniques, aiming in the separation of amlodipine enantiomers in less than 10 minutes analysis time.

Materials and methods

Chemicals and reagents

Racemic amlodipine besylate and its pharmacologically active enantiomer, S-amlodipine used as reference substances were purchased from Sigma Aldrich (Germany). Acetonitrile (ACN), methanol (MeOH) and ethanol (EtOH) (LC Grade, Merck, Germany) were used as solvents in the composition of mobile phases or as solvents for standard solutions preparation. Sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium hydroxide and orto-phosphoric acid (Merck, Germany) were used for the preparation of the HPLC mobile phases and for the ECZ buffers solutions. A Millipore Direct Q water purification system was used to obtain ultra-pure water. As chiral selectors for ECZ analysis various types of cyclodextrins were used: α-cyclodextrin (α-CD), 2-hydroxypropyl-β-cyclodextrin (2 HP-β-CD), randomly methylated-β-cyclodextrin (RAMEB-β-CD), γ-cyclodextrin (γ-CD) (Cyclolab, Hungary).

Instruments

Chromatographic conditions

The HPLC stereoselective method of amlodipine was developed on an Agilent 1100 Series System (Agilent Technologies, USA) equipped with an UV-VIS detector. As chiral stationary phase, an Ultron ES OVM was used, packed in a 150x4.6 mm column (5 μm particles) (Shinwa Chemical Industries LTD, Agilent Technologies). The chiral selector consisted of ovomucoid glycoprotein immobilized on an aminopropylsilane-derivatized silica column. Different mobile phases were tested, containing different proportions of aqueous component as phosphate buffers (Na2HPO4; NaH2PO4) and various proportions of organic modifiers (ethanol, methanol and acetonitrile). The mobile phases were degassed by ultrasonication (Elma Transsonic bath) for 15 minutes prior to use.

Electrophoretic conditions

The CE enantioseparation of amlodipine besylate was performed on an Agilent Technologies 7100 CE equipped with a diode array UV detector. The equipment was assisted by ChemStation 7.01 software. The AML solutions were hydrodynamically injected at the anodic end of the capillary. Uncoated fused silica capillaries (50 cm x 50 mm - Agilent, Germany) were used, while different types of cyclodextrins have been screened for stereospecific interactions with amlodipine: α-CD, 2-HP-β-CD, RAMEB-β-CD and γ-CD. BGEs were prepared by dissolving the appropriate amount of buffer (H3PO4; Na2HPO4; NaH-2PO4) in ultrapure water and adjusting the pH, when it was necessary, with NaOH 1 M or H3PO4 10%. The capillary was conditioned with NaOH 0.1 M for 30 minutes, followed by purified water for 15 minutes and finally with the BGE for 15 minutes. Between runs, the capillary was preconditioned with purified water for one minute and with the BGE, for two minutes.

The detection undertook for both methods at the wavelength value of 238 nm, where amlodipine recorded maximum absorbance. The Terminal 740 (Inolab) pH meter, previously calibrated, was used for pH buffer adjustments of mobile phases (HPLC) and for BGE solutions (CE). An ultrasonic bath T700H (Elma Transsonic) was used for the preparation and degassing of the mobile phases.

Samples, mobile phases and BGE preparations

AML stock solutions were prepared by weighing a suitable amount of powder and disolving it in methanol in order to give a concentration level of 1000 μg/mL in a volumetric flask. Stock solutions were later diluted to the appropriate concentration with the mobile phase mixture. S-AML stock solution was prepared also in methanol, at a concentration level of 10 μg/mL. CDs solutions were prepared at a concentration level of 50 mM by dissolving the CD in

BGE. All samples, CDs and buffer solutions, were filtered through a 0.45 µm syringe filter and ultrasonicated for five minutes prior to use. The detection took place at the cathode end of the capillary.

Results and discussions

HPLC method development and optimization

The proposed HPLC method is based on the separation due to the interaction with chiral stationary phase CSP. The chiral selector belongs to the protein class, chemically bonded to spherical silica gel particles. The ovomucoid, a chiral polymer is able to present stereoselective interactions with a large number of pharmacologically active compounds, exhibiting a very complex recognition mechanism. The chiral selector contains chiral recognition sites for a pair of enantiomers, and the chiral separation mechanisms are based on unique combinations of hydrophobic and polar interactions between the chiral analyte and ovomucoid molecules [15].

Since the overall charges both for the ionizable enantiomers and the proteic stationary phases are influenced by the pH, one of the most important parameters for enantiomeric separation on an ovomucoid is the pH of the mobile phase by taking in consideration to the pKa of the analyte (pKaAML = 9.10).

OVM has an isoelectric point (pI) of 4.1, therefore, it is expected for the stationary phase to be negatively charged when the mobile phase pH is above the pI values. The decrease of the mobile phase pH towards the ovomucoid pI reduces the negative charges of the stationary phase, resulting in shorter retention for the basic compounds, whereas increasing in the mobile phase pH towards the pI reduces the positive charge of the stationary phase, resulting thus in shorter retention times for acidic compounds [15,16].

Taking into account these facts, the HPLC method was developed and improved by changing some of the critical chromatographic conditions such as the pH of the mobile phase's aqueous component, proportion of the mobile phase's organic modifier (ethanol, methanol or acetonitrile), the chromatographic column temperature, all in an univariate manner.

The influence of the mobile phase's aqueous component pH was studied at different pH values between 3 and 7. It is worth mentioning that the tested pH interval fell within the manufacturer recommended pH usage range of the column.

The optimal pH value with whom the chiral resolution and selectivity are associated with enantioseparation of amlodipine, is 5.0 (R=5.51; α=1.71). The obtained retention times were shorter than 10 minutes for the two isomers, tR (S-AML) = 4.90 (min); tR (R-AML)= 7.18 (min). (Figure 2)

Taking into account that the temperature has an important role on all chromatographic techniques and due to the fact that both the thermodynamics and kinetics of

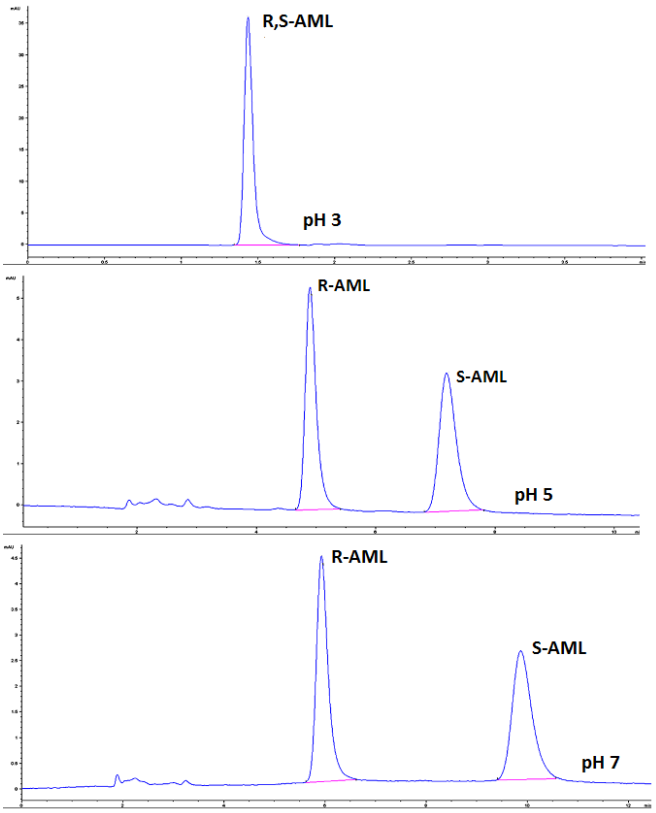

Fig. 2. Chromatograms of the enantioseparation for racemic AML at different pH values (50µg/ ml) (HPLC conditions: Ultron ES-OVM column; 25°C; mobile phase: A – Na_2HPO_4 10 mM 78%, B – ACN 22%, flow 1 ml/min, isocratic elution).

adsorption processes are temperature dependant, column temperature was varied between 20-35°C. This temperature range is supported by the stationary phase, according to the producer statement. In this study, lowering the elution temperature produces a decrease in retention time and an improving, consecutively, of the peaks shape. Elevated temperatures are able to decrease the viscosity of the mobile phase and to increase solubility and diffusivity, improving thus the separation resolution and the selectivity of the method. (Table I)

Optimal HPLC experimental conditions were established at a column temperature of 30°C, the percentage of the aqueous buffer component of the mobile phase was 80% (Na2HPO4 10mM) at a pH level of 5.0. The organic modifier, consisting of ACN, turned to be optimal when it is used under a proportion of 20%. The elution mode was isocratic at a flow of 1 ml/min. The established chromatographic conditions allowed the separation of the enantiomers of amlodipine, in less than 7 minutes.

Table I. Variation of separation parameters (t_R, N, R, α) of amlodipine depending on the elution temperature

Column temperature	Amlodipine					
	t_{R1}	t_{R2}	N_1	N_2	R_s	α
20 °C	5.02	7.66	3706	2740	5.76	1.79
25 °C	4.89	7.18	3801	3157	5.51	1.71
30 °C	4.63	5.54	3894	3263	5.04	1.65
35 °C	4.57	6.01	4248	3688	4.26	1.50

CE method development and optimization

The selectivity for capillary electrophoresis depends primarily on the electrical charge, which can be induced by changing the pH of the buffer solution and the ionic strength. The buffer composition and the ionic strength can influence the enantiorecognition, because the mobility of the analytes and cyclodextrins, respectively, are strongly affected. In the case of CE, cyclodextrins play the role of chiral selectors, the separation of chiral molecules can be achieved in CE, given the fact that it forms inclusion complexes with different stability and mobilities for the two isomers [2,3].

To improve the selectivity in CE, using cyclodextrins as chiral selectors, the optimum buffer concentration was determined by recording the electropherograms of racemic amlodipine, under identical conditions, at different concentrations of the phosphate buffer: 25 mM, 50 mM, 75 mM or 100 mM. High separation selectivity was observed when using the most diluted phosphate buffer solution, 25 mM.

The pH of the BGE also turned to be a critical parameter. This happened due to the fact that it influenced the electric charge of both the analyte and the cyclodextrin, modifying thus the electrostatic interactions between the chiral selector and the two isomers, in the end resulting in the different mobility of the complexes. Considering the pKa value of amlodipine (9.1) the electropherograms have been recorded in a pH range comprised between 2.0 to 7.0. The best stereoresolution of the amlodipine enantiomers was achieved at a pH value of 3.0.

To improve the selectivity CE, the optimum BGE concentration was determined by recording the electropherograms of racemic AML, using different concentrations of the phosphate buffer between 25-100 mM. High separation selectivity was observed when using 25 mM phosphate buffer solutions.

Once the optimum composition and concentration of the BGE had been established, the enantiomeric resolution of AML was further studied using several types of native CD: α-CD; β-CD and γ-CD and derivatized β-CD: 2-HP-β-CD, RAMEB-β-CD. The CDs were added in different concentrations relative to the used buffers in order to observe the enantiomeric separation mechanism, taking in consideration chiral resolutions. (Figure 3)

According to the Dalgliesh's three-point interaction rule, chiral recognition is possible in a context where at least three simultaneous interactions between the enantiomer and the used chiral selector must exist.

Although AML is a relatively large molecule presenting two aromatic rings in its chemical structure, the CD with the smallest inner cavity, α-CD was the only one able to support the complete separation under the electrophoretic conditions mentioned above. Regarding the AML molecule from 3D perspective with molecular energy optimization the 2 aromatic rings are almost orthogonal one with respect to the other and the benzene ring has no steric hindrance in the S-enantiomer, therefore a higher binding energy between S-AML and cyclodextrin is more probably. [3]

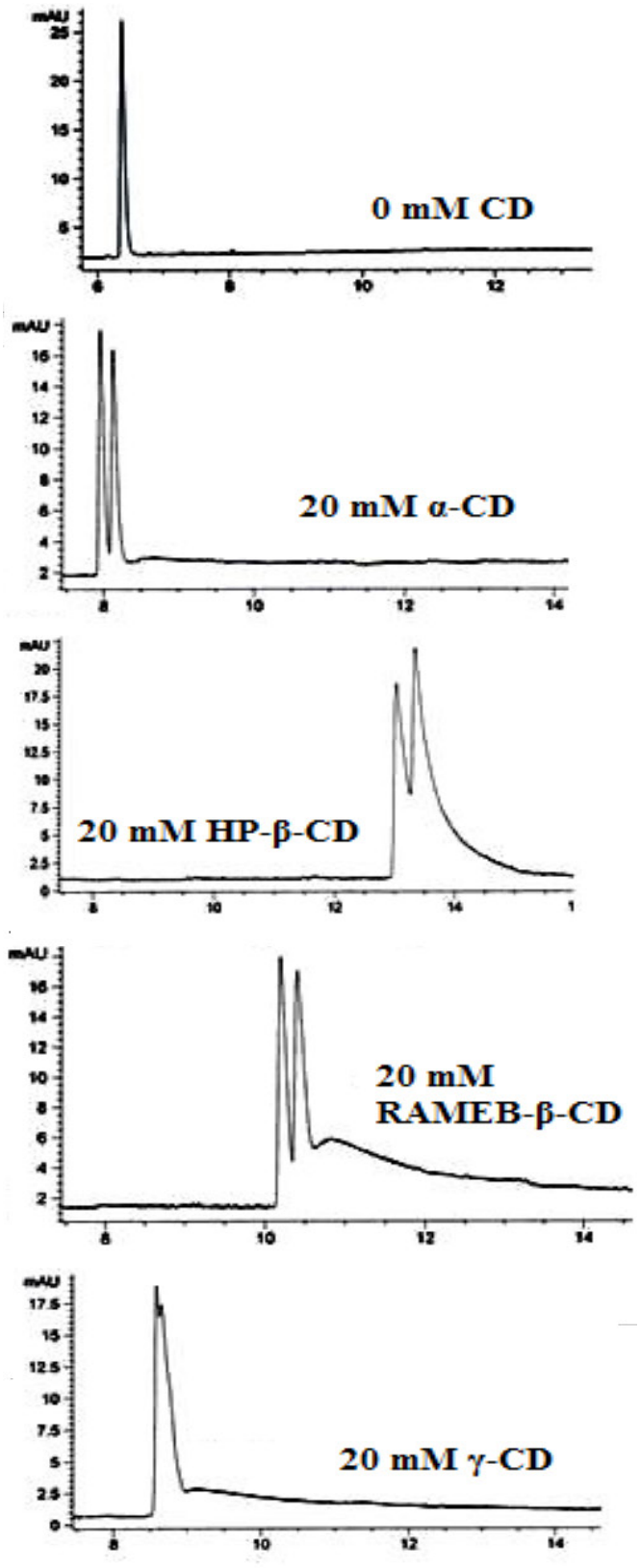

Fig. 3. Electropherograms of the chiral separation for racemic AML using different CD as chiral selectors (CE conditions: phosphate buffer 25 mM, pH 3, injection pressure/time: 30mbar/3sec, voltage +25kv, 25°C, CD concentration 20mM: a)α CD; b)2HP-β-CD; c) RAMEB-β-CD; d)γ-CD).

After the establishment of the optimum chiral selector, the capillary temperature was varied between 15°C and 30°C. This was done only to observe if the capillary temperature in the case of electrophoretic determination may represent a critical parameter as in the case of chromatographic determination.

The increase in capillary temperature leads to a decreased viscosity of the buffer solution, altering both the chiral AML and CD-analyte complex mobility. Whereas the chromatographic method was negatively influenced by the increase of the temperature as both the resolution and the selectivity were lowered, in the case of the electrophoretic method, the variation of capillary temperature affects only the resolution, without affecting the enatiomeric selectivity. (Table II)

The optimum CE conditions seemed to be based on using a buffer solution containing 25 mM H3PO4 at pH 3.0 and 20 mM α–CD as chiral selector. The optimum separation underwent under an applied voltage of + 25 kV, a capillary temperature set at 15°C and UV detection at 238 nm. Under these parameters, the separation for the two enantiomers of amlodipine succeeded in approximately 10 minutes, with a resolution of 1.51 and a stereoselectivity factor of 1.03. (Figure 4)

The elution order and the migration time of the enantiomers were established by injecting the solution of the pure enantiomer, S-AML, in the same optimiezed chromatographic/electrophoretic conditions, and was S-AML followed by R-AML.

Table II Electrophoretic parameters (R, α) recorded following of enantioseparation of amlodipine

Capillary temperature	α-CD		2 HP-β-CD		RAMEB-β-CD	
	R	α	R	α	R	α
15 °C	1.51	1.03	1.05	1.02	0.94	1.02
18 °C	1.46	1.03	1.04	1.02	0.91	1.02
21 °C	1.32	1.03	1.01	1.02	0.90	1.02
24 °C	1.25	1.03	0.96	1.02	0.89	1.02
27 °C	1.22	1.03	0.72	1.02	0.86	1.02
30 °C	1.09	1.03	0.69	1.02	0.84	1.02

Table IV Analytical performance for the proposed HPLC and CE methods

Enantiomer	HPLC			CE		
	Retention time (min)	R	α	Migration time (min)	R	α
R-AML	4.63	5.04	1.65	8.15	1.51	1.03
S-AML	5.54			8.45		

The evaluation of the analytical performances of the two proposed chiral methods

Calibrations of the two methods were achived by fitting a linear curve to five concentration points covering ranges between 20-500 μg/mL for AML enantiomers (HPLC method) and 50-500 μg/mL (CE method), respectively. Three independent injections were performed at each concentration level.

The limit of detection (LOD) for the two isomers was established in terms of peak height to background noise signal ratio, which must not be less than three times the noise signal intensity for both of the proposed methods. The limit of quantification (LOQ) was set at the lowest concentration at which the peak's height of the analytes were 10 times more intense than the noise signal intensity. As can be seen, the limits of detection and the limits of quantification for the two developed methods are substantially different. (Table III and IV)

The two proposed methods were statistically evaluated by comparing the variances of the retention times (tR-HPLC) and migration times (tm-CE) of the amlodipine enantiomers (F test, N=5, α=0.05). (Table V)

F test revealed that, there is no statistical difference between the variances of the retention times obtained with the help of HPLC method compared to the variances of the migration times obtained when using the CE method, for both enantiomers, R-AML and S-AML, respectively.

Conclusions

Both developed methods turned to comply to the desired separation performance parameters in terms of resolution, selectivity and migration times. Analysis time is was short-

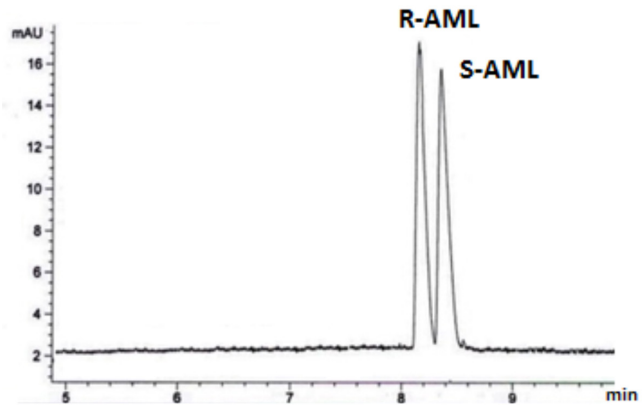

Fig. 4. Typical electropherogram for racemic amlodipine (100μg/ml) (analytical conditions: 25 mM phosphate buffer, pH 3.0, 20 mM α-CD chiral selector, voltage + 25 kV, temperature 15°C, injection pressure/time: 30mbar/3sec, UV detection 238 nm)

Table III The limit of detection (LOD) and limit of quantitation (LOQ) for the proposed HPLC and CE chiral method

Enantiomer	HPLC				CE			
	Regression equation	R^2	LOD (μg/ml)	LOQ (μg/ml)	Regression equation	R^2	LOD (μg/ml)	LOQ (μg/ml)
R-AML	y= 4.835x-31.34	0.999	2.02	6.31	y=9.016x-29.69	0.998	10.06	29.69
S-AML	y = 4.829x-31.83	0.999	2.12	6.43	y=9.013x-31.58	0.997	10.33	30.67

Table V Comparison between qualitative precision of the two methods, HPLC and CE

R-AML HPLC (t_R) vs CE (t_m)	Variable 1	Variable 2	S-AML HPLC (t_R) vs CE (t_m)	Variable 1	Variable 2
Mean	4.592	8.282	Mean	5.484	8.492
Variance	0.00082	0.00772	Variance	0.00328	0.00152
CV%	0.6236	1.0443	CV%	1.0609	0.4591
Observations	5	5	Observations	5	5
F	0.1062		F	2.1579	
P(F<=f) one-tail	0.0517		P(F<=f) one-tail	0.4745	
F Critical one-tail	0.1565		F Critical one-tail	6.3882	

er than 7 minutes for the HPLC method and shorter than 10 minutes in the case of CE method.

The analytical performances of the chromatographic method using OVM as chiral selector (R-5.04; α – 1.71; LOD –2μg/mL) are superior to the electrophoretic analysis method (R-1.51; α – 1.03; LOD –10μg/mL). Although analysis times from the literature published in previous studies are similar with those obtained in this study, the enantioresolution of our method (R=5.04) is clearly superior (R=1.43).

The proposed electrophoretic method, using α-CD as chiral selector to the BGE, has been successfully applied for the steric discrimination of AML enantiomers, with good repeatability of the migration time and peaks areas. More to be added, the method is more economical may represent an alternative to the HPLC chromatographic separation.

Although the evaluation of the analytical performances of the two methods indicates that the HPLC method is more efficient in enantioseparation of AML, due to low solvent, analyte and chiral selector consumption and lower operational cost, the CE proposed method represents a viable alternative for the quantitative determination of AML emantiomers in pharmaceutical forms.

Conflict of interest

None to declare.

Acknowledgement

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References

1. Zhang Y, Yao S, Zeng H, Song H - Chiral Separation of Pharmaceuticals by High Performance Liquid Chromatography. Curr Pharm Anal. 2010;6:114-130.
2. Saz JM, Marina ML - Recent advances on the use of cyclodextrins in the chiral analysis of drugs by capillary electrophoresis, J Chromatogr A. 2016;1467:79–94.
3. Al Azzam KM, Abdallah HH, Abdul Halim HN, Ahmad MA, Shaibah H – Host-Guest Inclusion Complexes between Amlodipine Enantiomers in the Biphasic Recognition Chiral Extraction System using Tartaric Acid and β-Cyclodextrin Derivatives as Positive Confirmation by using their Enantioselective Extraction. Sci Pharm 2015;83(4):683-698.
4. Bi C, Zheng X, Azaria S, Beeram S, Li Z, Hage DS - Chromatographic Studies of Protein-Based Chiral Separations. Separations 2016;3(3):27.
5. Arrowsmith JE, Campbell SF, Cross PE, Stubbs JK, Burges RA, Gardiner DG. et al. - Long-acting dihydropyridine calcium antagonists. 1. 2-Alkoxymethyl derivatives incorporating basic substituents. J Med Chem. 1986;29(9):1696–1702.
6. Hotha KK, Roychowdhury S, Mullangi R, Ravindranath LK - Rapid quantification of amlodipine enantiomers in human plasma by LC-MS/MS: Application to a clinical pharmacokinetic study. Biomed Chromatogr. 2013,27:1192–1199.
7. Kannappan V, Mannemala SS - Simultaneous enantioseparation and purity determination of chiral switches of amlodipine and atenolol by liquid chromatography. J Pharm Biomed Anal. 2016; 120:221–227.
8. Luksa J, Josíc D, Podobnik B, Furlan B, Kremser M - Semi-preparative chromatographic purification of the enantiomers S-(-)-amlodipine and R-(+)-amlodipine. J Chromatogr B Biomed Sci Appl. 1997;693(2):367-375.
9. Ansari KA, Pagar KP, Vavia PR - Development and validation of HPLC method for the determination of S (-) Amlodipine Besylate and its related substance in tablet formulation by using chiral separation. Asian J Biomed Pharm Sci. 2012;2(12):60-64.
10. Zandkarimi M, Shafaati A, Foroutan SM, Lucy CA - Rapid enantioseparation of amlodipine by highly sulfated cyclodextrins using short-end injection capillary electrophoresis. DARU. 2009;17(4):269-275.
11. Owens PK, Fell AF, Coleman MW, Berridge JC - Effect of charged and uncharged chiral additives on the resolution of amlodipine enantiomers in liquid chromatography and capillary electrophoresis. J Chromatogr A. 1998;797:187–195.
12. Wang R, Jia Z, Fan JJ, Chen LR, Xie H, Ma J, Ge X, Zhang Q, Ao Y, Wang J - CE, with hydroxypropyl-β-cyclodextrin as chiral selector, for separation and determination of the enantiomers of amlodipine in the serum of hypertension patients. Chromatographia. 2007;65:575-579.
13. Hancu G, Budău M, Kántor LK, Cârje A, Cyclodextrin screening for the chiral separation of amlodipine enantiomers by capillary electrophoresis. Adv Pharm Bull. 2015;5(1):35-40.
14. Small SS, Fell AF, Coleman MW, Berridge JC - Central composite of ruggedness and design for the rapid optimisation chiral separation of amlodipine in capillary electrophoresis. Chirality. 1995;7:226-234.
15. Hermansson J - Direct liquid chromatographic resolution of racemic drugs by means of α1-acid glycoprotein as the chiral complexing agent in the mobile phase. J Chromatogr A. 1984; 316:537-546.
16. Oda Y, Mano N, Asakawa N, Yoshid Y, Sato T, Nakagawa T - Comparison of Avidin and Ovomucoid as Chiral Selectors Resolution of Drug Enantiomers by High-Performance Liquid Chromatography. Anal Sci. 1993;9:221-228.

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RESEARCH ARTICLE

An Insight into Histopathologic Examination as a Gold Standard for the Diagnosis of Chronic Apical Periodontitis

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Objective: The purpose of our study was to determine the level of correlation between histopathologic results after surgery for chronic apical periodontitis and the radiographic and clinical diagnosis. The status of gold standard technique of histologic examination was evaluated in the diagnosis of apical radiolucency in necrotic teeth.

Methods: Out of 154 patients with incorrect root fillings and apical radiolucency included in an endodontic retreatment protocol, 87 patients (108 teeth) were scheduled for apical surgery at 3-6 months control recall. Clinical and radiographic exams were completed prior to surgery and compared to the histological results of apical biopsies. The collected data were statistically analyzed with the SPSS version 20.0 and the Chi-square test was used to determine the associations between clinical and histologic diagnosis. A value of $p < 0.05$ was considered statistically significant.

Results: There was a statistically significant difference between the number of cases diagnosed as granulomas or cysts during clinical and radiological evaluation compared to histologic evaluation of tissue samples, with 40.9% to 75.9% and 54.2% to 16.8% respectively ($p<0.05$).

Conclusions: The final diagnosis was obtained only after histologic examination of apical tissue samples, which means that the observations made based on radiologic investigations must be confirmed by biopsy.

Keywords: apical periodontitis, granuloma, apical cyst, radiography, apical surgery

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Introduction

Chronic apical periodontitis is defined as an inflammatory disease usually caused by microorganisms located inside the root canals of necrotic teeth. Therefore, the main objectives of endodontic treatment are the thorough cleaning of the infected endodontic system and the adequate aseptic control, aiming to create conditions for the final filling of the entire radicular space with a hermetic tridimensional seal. The success rate of the primary endodontic treatment is considered to be between 86%-98% and its failures are generally caused by one of the following biological factors: persistent infection in the apical third of the root canal, development of an extra-radicular infection, over-filling with endodontic materials responsible for foreign body reactions, cystic lesions and healing by scar tissue [1, 2]. Unsuccessful cases are always scheduled for conservative retreatment but the success rate in these cases is lower, around 80%-85% [3, 4]. If non-surgical retreatment is not feasible, the best choice remains the apical surgery that assures optimum conditions for healing. Diagnosis of chronic apical periodontitis is based on clinical and radiological examination, which represents important steps in determining the best treatment approach and in cases submitted to apical surgery it should always be confirmed by the histopathologic examination of the periapical tissues. Since many years, histology has been considered as the gold standard by which the true diagnosis of different forms of apical pathology can be confirmed and differentiate, contributing to the development of clinical treatment options in endodontics [5-7]. The purpose of our study was to evaluate the importance of histopathologic examination in the diagnosis of chronic apical periodontitis, by comparison with clinical and radiological findings. As imaging techniques digital radiographs will be used in all cases and cone beam computed tomography only for well selected cases, in order to protect the patients from exposure to higher doses of radiation. The null hypothesis to be tested is that there is a good correlation between clinical, radiographic and histologic examinations and an accurate apical diagnosis can be obtained without histologic evaluation of biopsy specimens.

Methods

A total of 154 patients with chronic apical periodontitis were examined at the Center for Integrated Dental Medicine of the Faculty of Dental Medicine, University of Medicine and Pharmacy Tîrgu Mureş, from October 2015 to

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June 2017 and consecutively enrolled in this study. Prior to any medical procedure, a written consent was obtained and the investigation was conducted based on principles of the Declaration of Helsinki. Based on clinical and radiological examination using digitally captured radiographs, an indication of endodontic retreatment was made in 138 cases that were considered to have an inadequate primary root canal treatment (incorrect length of the endodontic filling, presence of broken instruments in the apical third of the root canal, poor condensation of endodontic sealer, persistence or enlargement of the apical lesions). At 3-months follow-up, we noted an improvement in 46 cases which therefore had been excluded from the study. The cases without apical radiolucency but persistent clinical symptoms or cases with no signs of improvement were scheduled for CBCT examination. The remaining 108 teeth (87 patients) were scheduled for surgical treatment and used for the final examination protocol that included the recording of age, sex, tooth type, lesion size and the result of histologic examination carried out after apical surgery. The indication for apical surgery was given for teeth with persistent apical radiolucency at 3-6 months follow-up examinations based on the following aspects: persistence of clinical symptoms after endodontic retreatment (pain during mastication, swelling of vestibular gingiva, presence of broken instruments in the apical third that impeded a correct treatment, overfilling with sealer or gutta-percha, presence of posts or bridge abutments that showed lack of healing after endodontic retreatment. Each patient was examined by two authors previously calibrated (MM and AS) that used clinical tests and digital dental radiographs in order to determine the diagnosis; these observations were compared with the histological results of apical tissue biopsies. In 83 cases we obtained enough apical tissue in order to conduct a histologic evaluation, according to a protocol that included: fixation in 4% buffered formalin solution, embedment in paraffin, cutting into 5 micrometers thick sections that were colored with hematoxylin and eosin stain. The specimens were evaluated by two experienced pathologists (SM and CM) and classified into apical granulomas, cysts or abscesses. The collected data were statistically analyzed with the SPSS version 20.0 and the Chi-square test was used to determine the associations between clinical and histologic diagnosis. A value of $p < 0.05$ was considered to be statistically significant.

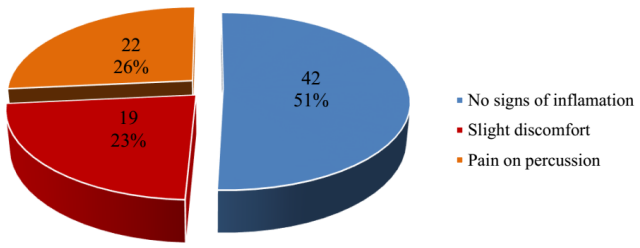

Fig 1. Distribution of symptoms observed during clinical examination prior to apical surgery

Results

The mean age of the patients included in this study was 31.4+/-10.5 years, ranging between 17 -58 years. The distribution of tooth type included 29 (34.9%) upper anterior teeth, 12 (14.6%) upper premolars, 6 (7.3%) upper first molars, 19 (22.8%) mandibular anterior teeth, 14 (16.8%)

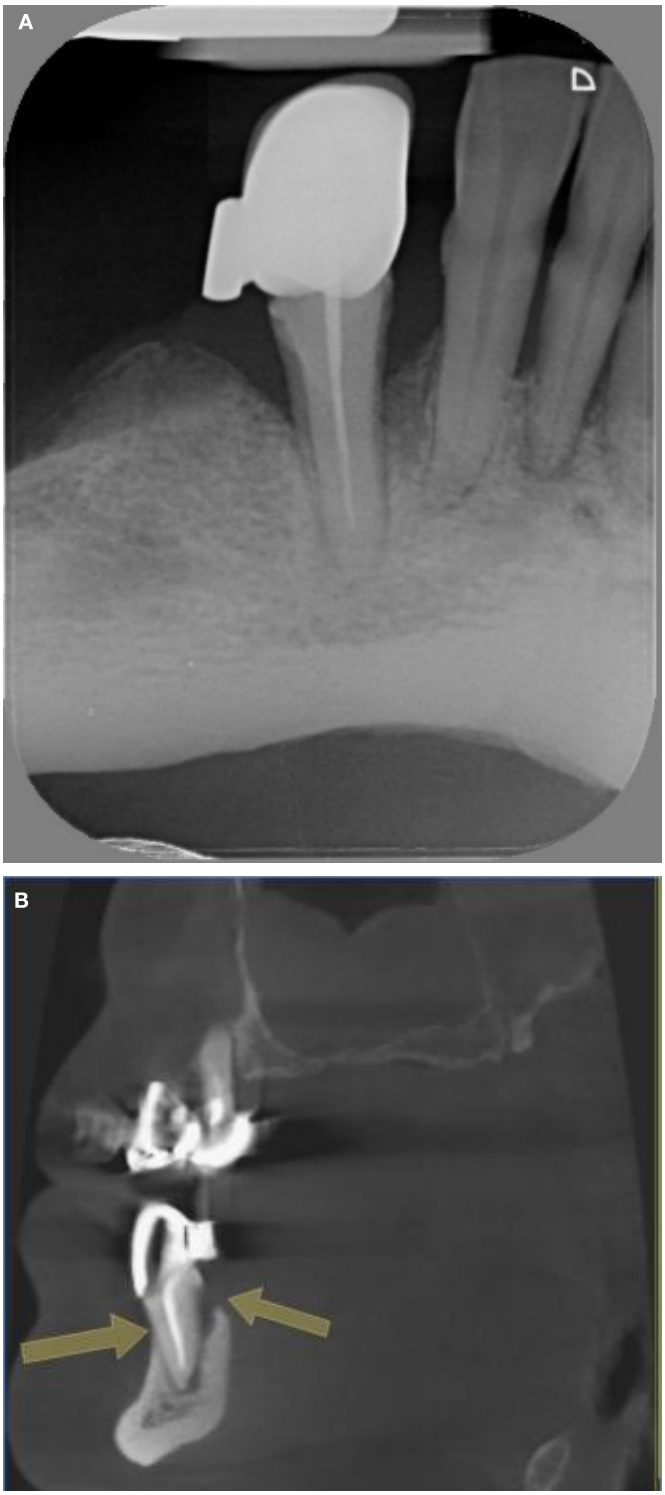

Fig. 2. The radiographic examination after the endodontic retreatment of a lower canine showed an incomplete root filling without apical radiolucency, but the persistent clinical symptoms as slight discomfort during mastication determined us to refer the patient for further investigation. On CBCT the apical and lateral bone defects were evident and the patient was submitted for apical surgery.

mandibular premolars and 3 (3.6%) lower first molars (Table I). The majority of affected teeth belonged to the anterior group with a total of 48 cases (57.7%) followed by the upper first molars. The clinical examination identified no signs of inflammation in 42 cases (50.6%), slight discomfort during mastication and sensation of tooth egression in 19 cases (22.8%) and in 22 cases (26.6%) pain on axial percussion was recorded. The results of clinical, radiologic and histopathologic examinations are presented in Table II and Figures 1-4. There was a statistically significant difference between the number of cases diagnosed as granulomas or cysts during clinical and radiological evaluation compared to histologic evaluation of tissue samples, with 40.9% to 75.9% and 54.2% to 16.8% respectively ($p<0.05$). The majority of the cases were diagnosed as cysts (54.2%) based on clinical and radiographic methods but after histopathologic examination the majority (75.9%) proved to be granulomas (Table II).

Discussion

The use of histological samples obtained after apical surgery has a long history in endodontic practice and proved to be

Table I. The distribution of tooth type submitted to apical surgery

Upper/lower jaw	Anterior teeth	Premolars	Molars
Maxillary teeth	29 cases (36%0	12 cases (15%)	7 cases (9%)
Mandibular teeth	19 cases (21%)	12 cases (14%)	3 cases (6%)

Table II. Comparative results after digitally captured radiographs and histopathological examination

Diagnosis	Radiographic	Histopathologic	p-value
Granuloma	34 (40.9%)	63 (75.9%)	0.026*
Cyst	45 (54.2%)	14 (16.8%)	0.001*
Abscess	4 (4.81%)	6 (7.2%)	0.398

*statistically significant difference

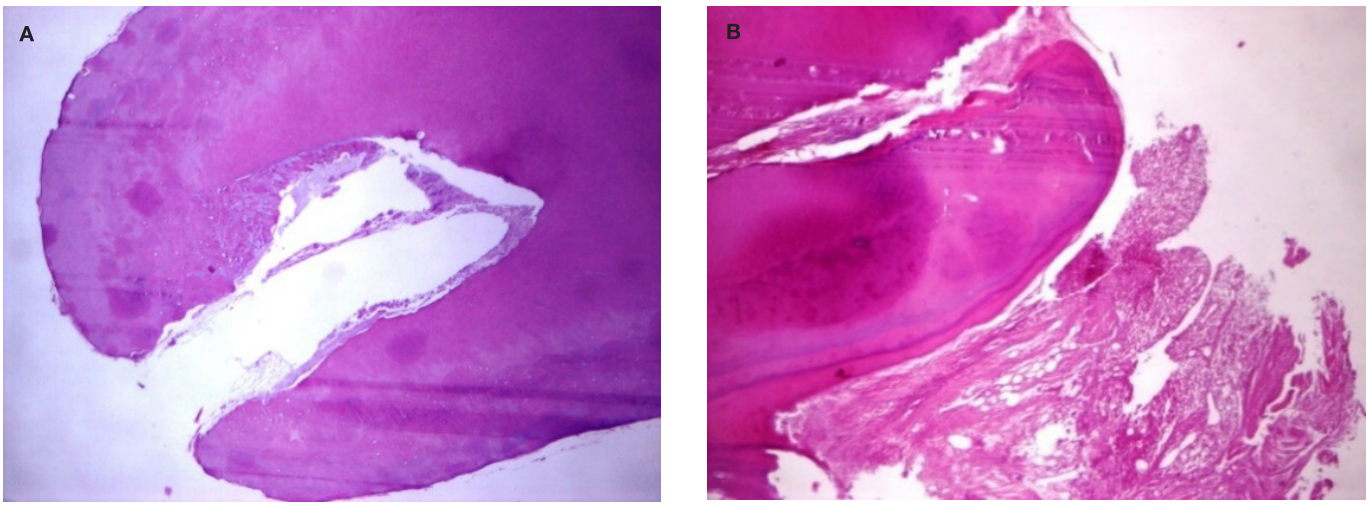

Fig. 3. Histologic specimens obtained from the apical third of the root. A. Apical fragment with radicular dentin, necrotic radicular pulp tissue and inflammatory cells located inside the root canal. B. Granulation tissue attached to the outer surface of the apex and inside the root canal.

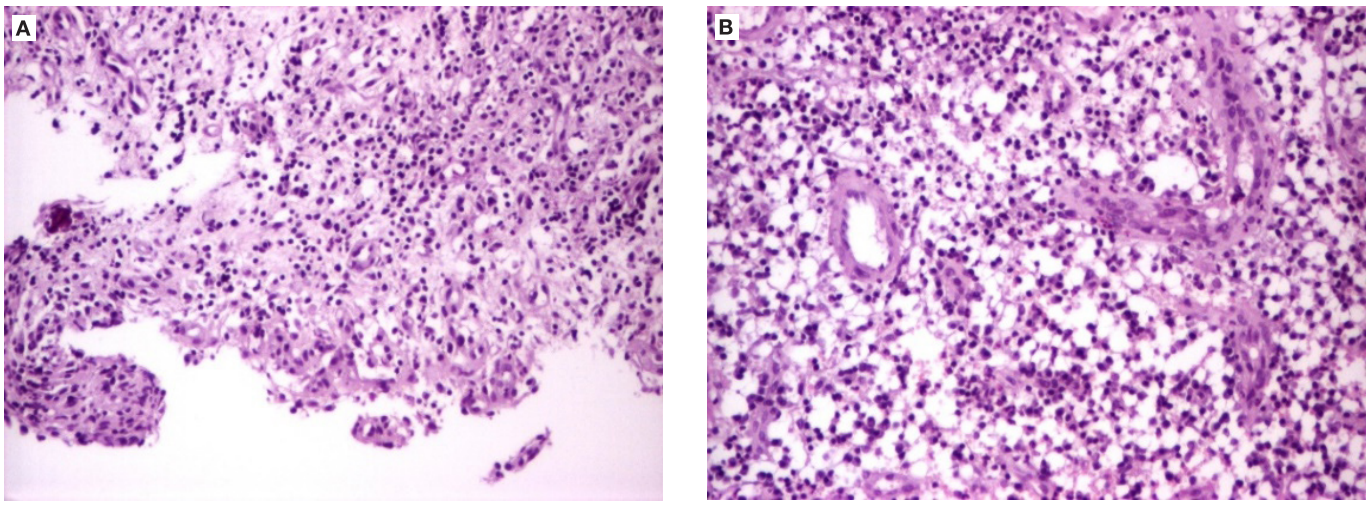

Fig.4. Conjunctive apical granuloma. A. Numerous fibroblasts, lymphocytes and macrophages (HE stain X 10) B. Granulation tissue with rich inflammatory infiltrate and numerous blood vessels, with epithelial islands inside the connecting tissue, representing the indication of a potential apical cyst development. (HE stain, X 10).

of outmost importance for the development of treatment protocols in cases with chronic apical periodontitis. In our study, we recorded histological results in only 7.3% and 6.3% upper and lower molars respectively, which could be attributed to a low submission rate for this type of examination after apical surgery or a preference for extraction in multi-rooted teeth with apical pathology. Diagnosis can be defined as the art of science aiming to distinguish deviations from the state of health; it involves the use of subjective information, clinical tests and imaging techniques. This complex process is carried out also in the case of apical pathology and represents the cornerstone upon which the treatment decisions are based on. Radiographic examination is an essential part in the diagnosis of chronic apical periodontitis; furthermore, it has an important role in the management of endodontic pathology, from treatment plan to the evaluation of therapy outcome. The drawbacks of conventional and digitally captured radiographs may be overcome by using cone beam computed tomography (CBCT) which offers a three-dimensional image of the tooth and its surrounding structures [8-11]. Data from literature confirms that after histologic examination, the majority of cysts diagnosed using conventional radiography proved to be granulomas. In a study conducted by Bornstein et al (2015), the radiographic evaluation of periapical lesions overestimated cystic lesions by 28.4% compared to CBCT [7]. Patel et al (2012) found that in teeth with primary endodontic disease, the prevalence of apical lesions detected by conventional radiographs and CBCT was 20% and 48% respectively [8]. In teeth with irreversible pulpitis, the radiography identified the presence of apical pathology in 3% and the CBCT in 14% of the cases [9]. Nevertheless, according to the American Association of Endodontists the CBCT must be considered an adjunct to 2-dimensional imaging techniques reserved only for special cases [12]. Campello et al (2017) found that the accuracy of CBCT was much higher than the digital radiography in detecting artificially created periapical lesions. The correct diagnosis recorded using CBCT was 73.6% and by radiograph 56.9% of the cases, the greatest differences being observed in maxillary (71.4% and 28.6%) and multi-rooted teeth (83.3% and 33.3%) respectively [12].

In clinical practice, the digital retroalveolar radiographs are the most common type of investigation used in the case of apical lesions [9, 13]. It is generally accepted that radiography is not a perfect diagnostic method, as it gives a bi-dimensional image of a three-dimensional structure and furthermore, specific clinical and biological aspects my not be visible on radiographs. Thus, the presence of an apical lesion sometimes is not directly evident and the extension and spatial relationship with anatomical landmarks are not always visualized. With all the limitations of conventional radiographs in showing small bone lesions, a systematic review conducted by Petersson et al [14] concluded that this type of examination has a high capacity in detecting normal apical conditions. In our study the radiographic examination was based on specific criteria in differentiating the cysts from granulomas, as the size of the lesion, degree of radiolucency, aspect of the borders. Accordingly, a radiolucent lesion with irregular periphery and less than 10 mm diameter was considered granuloma, whereas intense radiolucent defects with well-defined borders and more than 10 mm diameter were considered cysts.

There were numerous attempts in the scientific literature to distinguish between granulomas and apical cysts based entirely on their radiographic aspect: the granuloma has an indistinct border while cysts are characterized by a visible limit. The presence of a proliferating epithelial tissue without a cystic cavity was considered to have the potential of cystic transformation [15-17]. According to the scientific literature, the incidence of granuloma and cyst is between 9.3%-87.1% and 6%-55% respectively, due to different classification criteria used in histologic studies [17-19]. In the study conducted by Kondori et al (2011) the endodontists were not able to reach the correct diagnosis of apical granulomas in 42.2% of the cases with apical pathology, compared to 45.9% error diagnostics made by general dentists. This had probably led to unnecessary surgery, as apical granulomas can be successfully treated by conservative endodontic therapy. Many studies have emphasized the need to subject all periapical lesions removed during surgery to histopathological examination [5, 20]. The results of our study confirmed as well that histopathology is superior to radiography in the accurate diagnosis of apical granulomas and cysts. Histopathologic examinations that identify other pathologic conditions than granulomas, cysts, abscesses or scar tissue are estimated to represent 0.7%-5.0% of all periapical biopsies [21]. These can be benign aggressive lesions as central giant-cell granuloma, fibro-osseous lesions as periapical cemental dysplasia or many primary and metastatic malignant lesions.

Our histopathologic results showed the high incidence of apical granulomas which is in accordance to other studies [22, 23]. We found that 75.9% of specimens were granulomas and 16.8% were cysts. In 7.2% of the cases the results showed the presence of apical abscesses, compared to 4.8% after clinical and radiographic examination, results that were not statistically significant. A better correlation of diagnosis in these cases was determined probably due to specific clinical aspects of the apical abscess: deformation of alveolar bone, intense pain, slightly extruded tooth or mobility. Von Arx et al [24] discussed the prognostic factors of apical surgery, suggesting that the healing rates increase if there is no history of preoperative pain, the size of the lesion is less than 5 mm wide and the root filling is well condensed. Simon et al [6] compared CBCT images and histological results of periapical biopsies regarding the ability to differentiate apical cysts from granulomas, concluding that CBCT proved to be clinically more precise and more useful. Furthermore, when used for diagnostic purposes, it was noted that CBCT detects radiolucent changes of the apical bone more often and in initial stages than conven-

tional radiographs, which allows an earlier endodontic or surgical treatment. CBCT is a powerful tool for endodontic diagnosis, treatment and follow-up but its advantages must be correlated to the higher radiation dose compared to digital radiographs [25, 26]. Dental specialists need an evidence-based methodology based on further clinical studies, regarding the use of CBCT during endodontic treatment in order to make a correct medical decision on both treatment outcome or need for surgical interventions.

Conclusions

Periapical lesions represent the most frequent apical pathology diagnosed by conventional radiographic examinations. However, due to the limits of these methods, some cases remain misdiagnosed. The radiographic diagnosis of these osteolytic lesions may be hindered by their structural and evolution variations; the apical root area is difficult to be truly assessed on a two dimensional image and the third dimension information is essential in surgery planning.

Based on the results of our study, the retroalveolar digital radiography was characterized by a limited capacity in detecting periapical lesions when compared to histopathological examinations. Therefore, the null hypothesis was not confirmed as we could not reach the correct diagnosis of the apical lesions based only on clinical and radiographic examination. The poor correlation observed between the histopathologic diagnosis compared to clinical and radiological data led us to the conclusion that the former can be considered the gold standard diagnostic method in chronic apical periodontitis. It is recommended that all chronic apical lesions that did not respond to conservative therapy to be submitted to histopathologic examination, as there is a slight possibility to be the sign of other severe diseases.

In order to reduce unnecessary surgical interventions in teeth with adequate endodontic treatments but persistence of apical radiolucency, the use of advanced imaging techniques as CBCT might be a good option in well selected cases.

Conflict of interest

None to declare.

Acknowledgement

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References

1. Nair PN. On the causes of persistent apical periodontitis: a review. Int Endod J 2006; 39: 249-281.
2. Tanalp J, Gungor T. Apical extrusion of debris: a literature review of an inherent occurrence during root canal treatment. Int Endod J 2014; 47: 211-221.
3. De Chevigny C, Dao TT, Basrani BR, Marquis V, Farzaneh M, Abitbol S, Friedman S. Treatment outcome in endodontics: the Toronto study - Phase–4: initial treatment. J Endod 2008; 34: 258-263.
4. Torabinejad M, Corr R, Handysides R, Shabahang S. Outcomes of nonsurgical retreatment and endodontic surgery: a systematic review. J Endod 2009; 35: 930-937.
5. Ramanpreet B, Simarpreet SV, Rajat B, Amandeep B, Shruti G. Histopathological insight into periapical lesion: An institutional study from Pujab. Int Oral Maxillofac Pathol 2012; 3(3): 2-7.
6. Simon JHS, Enciso R, Malfaz JM, Roges R, Bailey-Perry M, Patel A. Differential diagnosis of large periapical lesions using cone beam computed tomography measurements and biopsy. J of Endodontics. 2006; 32: 833-837.
7. Bornstein MM, Bingisser AC, Reichart PA, Sendi P, Bosshardt DD, Von Arx T. Comparison between radiographic (2-dimensional and 3-dimensional) and histologic findings of periapical lesions treated with apical surgery. J Endod 2015; 41(6): 804-811.
8. Patel S, Wilson R, Dawood A, Mannocci F. The detection of periapical pathosis using periapical radiolucency and cone beam computed tomography – part 1: Pre-operative status. Int Endod J 2012; 45(8): 702-710.
9. Patel S, Durack C, Abella F, Shemesh H, Roig M, Lemberg K. Cone beam computed tomography in Endodontics – a review. Int Endod J 2015; 48: 3-15.
10. Fernandy R, Cadavid D, Zapata SM, Alvarez LG, Restrep FA. Impact of three radiographic methods in the outcome of nonsurgical endodontic treatment: a five-year follow-up. J Endod 2013; 39: 1097-1103.
11. Paula-Silva FWG, Wu M, Leonardo MR, Bezerra da Silva L, Wesselink PR. Accuracy of Periapical Radiography and Cone-Beam Computed Tomography scans in diagnosing apical periodontitis using histopathological findings as a gold standard. J Endod 2009; 35 (7):1009-1012.
12. Campello AF, Goncalves LS, Guedes FR, Marques FV. Cone-beam computed tomography versus digital periapical radiography in the detection of artificially created periapical lesions: A pilot study of the diagnosis accuracy of endodontists using both techniques. Imaging Sci Dent 2017; 47(1): 25-31.
13. Tewary S, Luzzo J, Hartwell G. Endodontic radiography: who is reading the digital radiographs? J Endod 2011; 37(7): 919-921.
14. Petersson A, Axelsson S, Davidson T, Frisk F, Hakeberg M, Kvist T. Radiological diagnosis of periapical bone tissue lesions in endodontics: A systematic review. Int Endod J 2012; 45(9): 783-801.
15. Saraf AP, Kamat S, Puranik RS, Puranik S, Saraf S, Singh BP. Comparative evaluation of immunohistochemistry, histopathology and conventional radiography in differentiating periapical lesions. J Conserv Dent 2014; 17(2): 164-168.
16. Shen J, Zhang H, Gao J, Du X, Chen Y, Han L. Short-term observation of clinical and radiographic results of periapical surgery: a prospective study. Biomedical Research 2016: 27(3): 923-928.
17. Matsuda V, Kadowaki CAN, Kataoka SHN, Caldeira CL. A comparison of clinical, histological and radiographic findings in periapical radiolucid lesions. Dental Press Endod 2011; 1(3): 17-21.
18. Kanagasingam S, Lim CX, Yong CP, Mannocci F, Patel S. Diagnostic accuracy of periapical radiography and cone beam computed tomography in detecting apical periodontitis using histopathological findings as a reference standard. Int Endod J 2017; 50(5): 417-426.
19. Kondori J, Mottin RW, Laskin DM. Accuracy of dentists in the clinical diagnosis of oral lesions. Quintessence Int 2011; 42: 575-577.
20. Goel S, Nagendrarely SG, Raju MS, Krishmojirao DJ, Rostogi R, Mohau RS, Gupta S. Ultrasonography with color Doppler and power Doppler in the diagnosis of periapical lesions. Indian J Radiol Imaging. 2011; 21:279-283.
21. Peters E, Lau M. Histopathologic examination to confirm diagnosis of periapical lesions: A review. J Can Dent Assoc. 2003; 69(9): 568-600.
22. Song M, Jung IY, Lee SJ, Lee CY, Kim E. Prognostic factors for clinical outcomes in Endodontic Microsurgery: A retrospective study. J Endod. 2011; 37: 927-933.
23. Song M, Chung W, Lee SJ, Kim E. Long-term outcome of the cases classified as successes based on short-term follow-up in Endodontic microsurgery. J Endod 2012; 38:1192-1196.
24. Von Arx T, Jensen SS, Hanni S, Friedman S. Five-year longitudinal assessment of the prognosis of apical microsurgery. J Endod 2012; 38:570-579.
25. Leonardi Dutra K, Haas L, Porporatti AL, Flores-Mir C, Nascimento-Santos J, Mezzomo LA. Diagnostic accuracy of cone-beam computed tomography and conventional radiography on apical periodontitis: a systematic review and meta-analysis. J Endod 2016; 42: 356-364.
26. Venskutonis T, Plotino G, Tocci L, Gambarini G, Maminskas J, Juodsbaly SG. Periapical and endodontic status scale based on periapical bone lesions and endodontic treatment quality evaluation using cone beam computed tomography. J Endod 2015; 41(2): 190-196.

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RESEARCH ARTICLE

Mediators of Inflammation as a Link Between Diabetes Mellitus and Periodontal Breakdown

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Our **objective** was to investigate immunological changes that occur in saliva of subjects with type 2 diabetes mellitus (T2DM) without signs of periodontal disease and to establish if salivary inflammatory cytokines are a possible link between diabetes mellitus and periodontal breakdown. **Material and methods**. Twenty T2DM subjects with no periodontal disease and twenty healthy controls were registered for the present study. TNF-α and IL-6 level from saliva and serum were measured. Periodontal tissue samples were histologically examined. **Results**: TNF-α and IL-6 levels were higher in T2DM subjects compared to controls, with an extremely significant difference in saliva ($p<0.001$). Significant inflammation, affecting both epithelial and connective tissues was present in periodontal biopsies. **Conclusions**: The subjects showed an increased TNF-α and IL-6 levels, both in serum and -mostly in -saliva of diabetics without signs of periodontal disease, confirming the hypothesis of immunological implication, as a correlation between periodontal disease incidence and diabetes mellitus. Histologic alterations, suggesting a local inflammatory state, were present in periodontal tissue of diabetics, confirming the above hypothesis. The study reveals that saliva analysis is a quite efficient method in testing the periodontal breakdown progression in the subjects with T2DM.

Keywords: diabetes mellitus, periodontal disease, TNF-α, Il-6, histopathology

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Introduction

Diabetes mellitus is a heterogeneous group of metabolic disorders that have in common hyperglycemia, beeing a worldwide major health concern, as the number of cases of both types of diabetes are constantly increasing. Chronic hyperglycemia is associated with irreversible complications such as nephropathy, retinopathy, neuropathy,cardiovascular diseases, peripheral vascular diseases,delayed healing and periodontal diseases [1]. It is still not completely understood the exact mechanism by which diabetes affects periodontal tissues [2, 3].

The aim of this study was to investigate immunological changes that occur in the saliva of subjects with type 2 diabetes mellitus without clinical signs of periodontal disease and the influence of these changes on periodontal tissues. We evaluated as well if salivary inflammatory cytokines are a possible link between diabetes mellitus and periodontal tissue alteration, and if salivary cytokines can play the role of an indicator in the progression of periodontal disease.

Material and methods

The study group included 20 type 2 diabetic subjects without any clinical signs of established periodontal disease. Twenty systemically healthy subjects served as controls. Diabetics were recruited from the Puls Health Center Tg. Mures, without the knowledge of the patients` glycaemic control level and they were chosen to participate in the study after a periodontal examination. The systematically healthy control group was recruited after a general dental examination, followed by a periodontal examination; they were chosen from the patients of the Odontology and Oral Pathology Department of UMF Tg Mures.

Inclusion criteria for the control group were as follow: ≥ 20 years of age; at least 20 periodontally healthy teeth; no attacment level > 2 and no probing pocket depth > 3mm. The subjects with Type 2 daiabetes were at least 20 years old, had a minimum of 20 teeth; no major diabetic complications; no periodontal changes; during the last six months without antibiotic, corticoid or immunosuppressive treatment in the medical history. Smokers, subjects who had medical conditions that would require antibiotic prophylaxis for routine dental procedures or influence the course of diabetes treatment were excluded from the study.

Clinical examination had the following procedure: information regarding the personal data of the subjects, contouring the medical history, extra- and intraoral examination. A trained clinician measured the following periodontal parameters at 6 sites per tooth (mesiolingual, lingual and distolingual, mesiobuccal, buccal, distobuccal), for all present teeth, third molars being excluded. Clinical parameters included: visible plaque accumulation(0 or 1), gingival redness (0 or 1), bleeding on probing (0 or 1), recession (mm) and poket depth (mm). The values were rounded to the nearest upper mm.

Ethical Comity of UMF Tg. Mures approved the procedure of the study as well. An informed consent was signed by the participants after a previous information about the purpose of the study.

For the detection of TNF-α and IL-6 in both saliva and serum, we used the same method as described in one of our previous studies [4]. Two ml of unstimulated saliva and

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venous blood were collected from each enrolled subject, than centrifugated, the supernatant filtered and refrigerated until examination. ELISA-sandwich method was used to determine the TNF-α levels both in saliva and serum. For this purpose it was chosen an easily accessible immunoassay kit (OptEIA human TNF-α, Pharmingen, USA). Manufacturer`s guidelines were strictly respected. TNF-α levels from both the saliva and serum were compared trough extrapolation with TNF-α standard, given in pg/ml. Detection of salivary and serum IL-6 levels were performed using an easily accessible enzyme-linked immunoabsorbent, sandwich ELISA test (DuoSet ELISA Development System, R&D Systems, USA), again manufacturer`s guidelines were severely respected. Results were given as total amount of IL-6 in pg/ml.

In case of each diabetic subject blood glucose and glycated haemoglobin (HbA1c) levels were determined with the purpose to detect their glycaemic control level.

For the statistical analysis of the data it was used the Mann-Whitney nonparametric test in order to determine if there are any statistically significant differences between salivary and serum levels of TNF-α and Il-6 in diabetics versus controls. Only p values over 0.05 were considered statistically significant.

From each diabetic subject, biopsy specimens were obtained from a dental-periodontal unit in the posterior region of dental arches and sent for histopathology study. Histopathological examination was performed using formalin fixed, paraffin embedded tissue fragments following standard protocols. The specimens were sliced in 4-5 micron thick tissue sections, stained with hematoxylin-eosin and digitally examined using Zeiss MiraxScan system.

Results

The diabetic subjects enrolled in the study had a mean age of 46.73 ± 7.38, records of at least 5 years of diagnosed Type 2 diabetes, and each of them showed oral antidiabetic drug treatment in the medical history.. Detection of HbA1c and blood glucose levels showed medium to poor glycaemic control in all enrolled diabetics, and and first degree obesity was evident in some of the patients, with a mean value of BMI around 27.95 (table 1). Biologic and clinical parameters of all subjects are presented in table 1.

Serum IL-6 levels in type 2 diabetics were significantly increased ($p<0.001$) compared to healthy controls (figure 1). Serum TNF-α levels in diabetics were higher than in healthy controls, without statistical relevance ($p=0.465$) (figure 2).

Both TNF-α and IL-6 levels showed a significant increase in the saliva of diabetic patients compared with the control group ($p<0.0001$)(figure 3 and 4).

Data concerning the correlation between serum and salivary levels of both cytokines and other clinical parameters in diabetic subjects are presented in table 2. No significant correlation was established, as p-value was higher than 0.05.

Table 1. Demographic, biologic and clinical parameters of subjects in the study groups

	T2DM (n=20) Mean±Std.dev.	Controls (n=20) Mean±Std.dev.
Age (years)	46.73±7.38	43.09±14.10
% males	40	38
BMI	27.95±2.38	25.06±4.74
Glycaemia (mg/dL)	168.46±21.63	82.61±15.65
HbA1C (%)	9.02±0.84	-
Pocket depth (mm)	2.74 ± 0.12	1.82 ± 0.24
Recession (mm)	0.14 ± 0.15	0.11 ± 0.11
% sites with:		
plaque	20 ± 3	22 ± 18
bleeding on probing	13 ± 6	10 ± 8
gingival redness	5 ± 2	3 ± 5

Table 2. Correlations between serum and salivary cytokines levels and biologic parameters in diabetic patients.

Correlation between	Cases	Correlation coefficient	p-value
Serum IL-6 level and age	20	0.491	0.063
Serum IL-6 level and BMI	20	0.084	0.766
Serum IL-6 level and glycaemia	20	0.098	0.727
Serum IL-6 level and HbA1C	20	0.219	0.433
Serum IL-6 and serum TNF level	20	0.345	0.208
Serum and salivary IL-6 level	20	0.500	0.057
Salivary IL-6 level and age	20	-0.296	0.284
Salivary IL-6 level and BMI	20	0.456	0.087
Salivary IL-6 level and glycaemia	20	0.465	0.081
Salivary IL-6 level and HbA1c	20	0.504	0.056
Serum TNF-α level and age	20	0.363	0.183
Serum TNF-α level and BMI	20	-0.167	0.553
Serum TNF-α level and glycaemia	20	-0.095	0.737
Serum TNF-α level and HbA1C	20	0.215	0.443
Serum and salivary TNF-α levels	20	0.674	0.006
Serum TNF-α and salivary IL-6 levels	20	0.345	0.208
Salivary TNF-α level and age	20	0.409	0.130
Salivary TNF-α level and BMI	20	-0.376	0.168
Salivary TNF-α level and glycaemia	20	0.070	0.805
Salivary TNF-α level and HbA1C	20	0.106	0.708
Salivary TNF-α and IL-6 levels	20	-0.100	0.723

Histological alterations in tissue sections obtained from diabetic patients were present in both the epithelium and the lamina propria of gingival tissue. Acanthosis and parakeratosis were noticed at epithelial level, with reduced quantities of acute inflammatory infiltrate composed mostly of polymorphonuclear leucocytes (segmented granulocytes) throughout its thickness and in superficially located microabscesses. A diffuse polymorphous inflammatory infiltrate consisting of lymphocytes, plasma cells and, to a lesser extent, granulocytes was present in the mildly fibrotic lamina propria, displacing collagen fibers and surrounding ectatic blood vessels and exteriorized erythrocytes (figure 5 and 6). Biopsies showed mitotic activity in the basal layer of

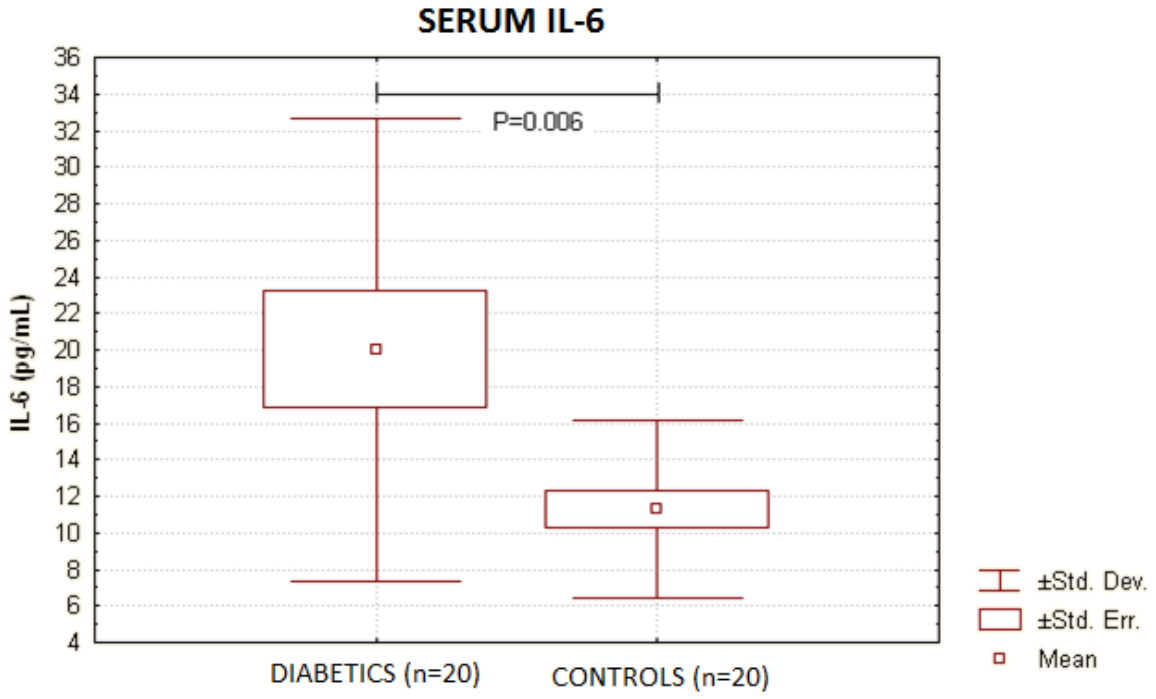

Fig. 1. Serum IL-6 in study groups

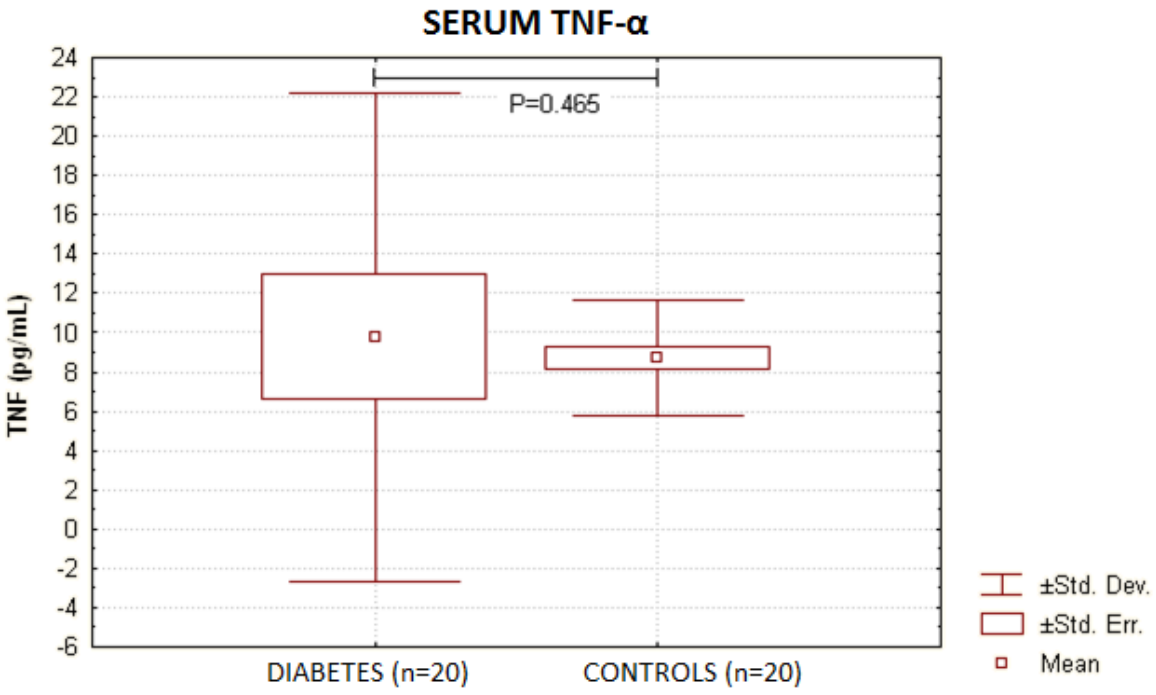

Fig. 2. Serum TNF-α in study groups

the epithelium that accompanies the inflammatory process. Stratum spinosum presented several cellular intra-epithelial vacuolization and acantholysis. In lamina propria a perivascular lymphoplasmocitic infiltrate surrounded dilated capillaries and venules. The connective tissue was degraded due to a rich inflammatory infiltrate and fibrosis.

Discussions

The hypothesis of the study was that diabetes mellitus can increase salivary TNF-α and IL- levels, contributing to periodontal breakdown. The serum of diabetic subjects showed elevated TNF-α and IL-6 levels versus the healthy control group, but very high differences of these cytokines were noticed in the saliva of diabetics. Sevaral studies on salivary TNF-α levels did not provide any evidence of association with periodontal disease, because salivary TNF-α levels are very low or nonexistent [5-11]; only few studies reported significantly elevated salivary TNF-α levels in subjects with periodontitis as compared to healthy controls, but the levels of TNF-α were very low (<4.3 pg/mL) [12].

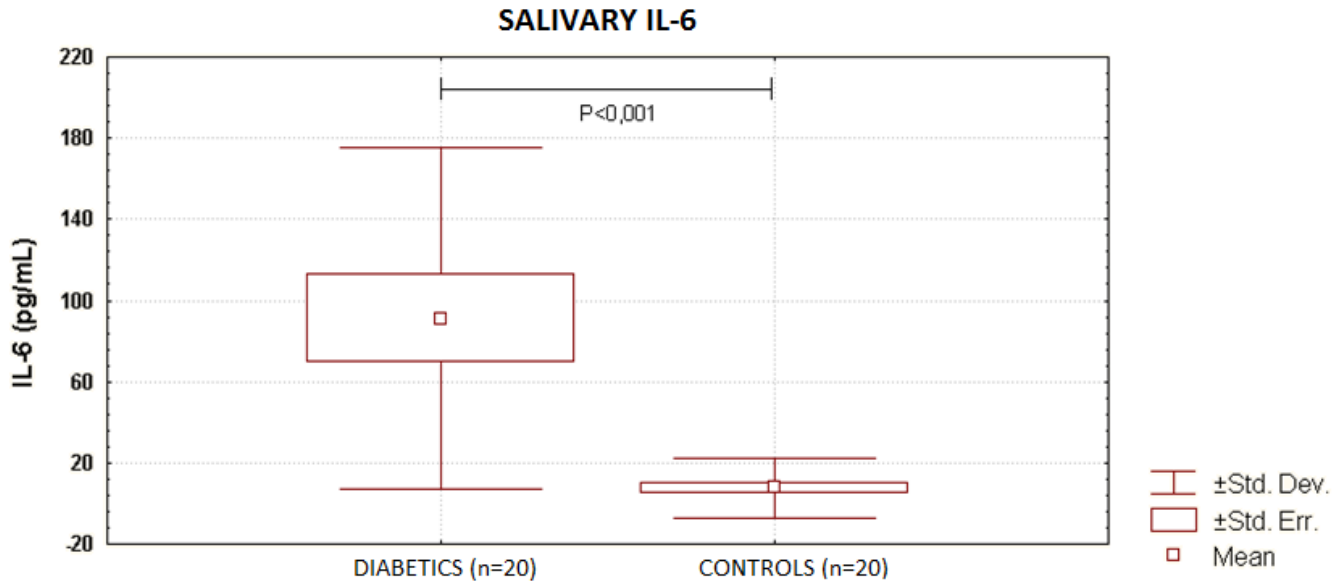

Fig. 3. Salivary IL-6 in study groups

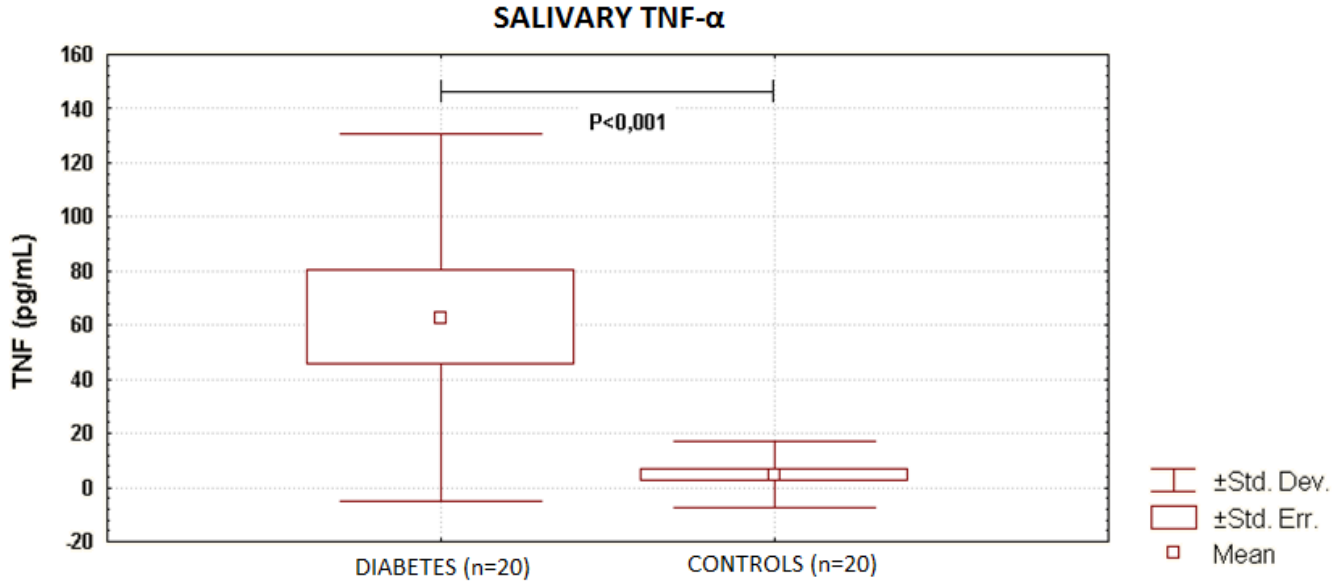

Fig. 4. Salivary TNF-α in study groups

Most of the studies on salivary IL-6 showed no association with periodontal disease [5, 7, 9, 10]. Opposite, in 3 reports significantly elevated salivary IL-6 levels were found in subjects with periodontitis [8, 13, 14]. The detected salivary TNF-α and IL-6 concentrations were significantly increased compared with both the control group of our own study and the mean TNF-α and IL-6 values described by others in systematically healthy subjects and almost equal to mean levels in systematically healthy parodontopatic patients [15-17]. This confirms the hypothesis of the present study, that diabetes mellitus might co-induce the onset, progression and prognosis of periodontal disease.

In our study salivary IL-6 showed a tendecy to be similar with HbA1c and blood glucose levels, but results are not powerful because of the small amount of study group. Thus, we may postulate that detection of IL-6 level in total saliva might be enough to evaluate the progression of periodontal disease in case of the patients who present type 2 diabetes mellitus.

Saliva, or more appropriate „oral liquid", is consisted of exocrine secretions of the oral salivary glands, gingival crevicular fluid, as well as elements from dental plaque and diet [18, 19]. It is already known that saliva might be a source for identifying useful biomarkers, as well as for developing convenient technologies to measure these in the clinic and (potentially) at home [20, 21]. The abundant amount of this liquid, its cheep collection method and its accessibility through noninvasive and painless procedures make clinical trial involvement more attractive for the patient. Also, it does not require specially trained medical or dental staff for collection. Different markers at low levels can be detected in saliva, although some salivary proteins that are in high quantities (e.g. albumin, immunoglobulins) may interfere with the detection of less abundant proteins [22]

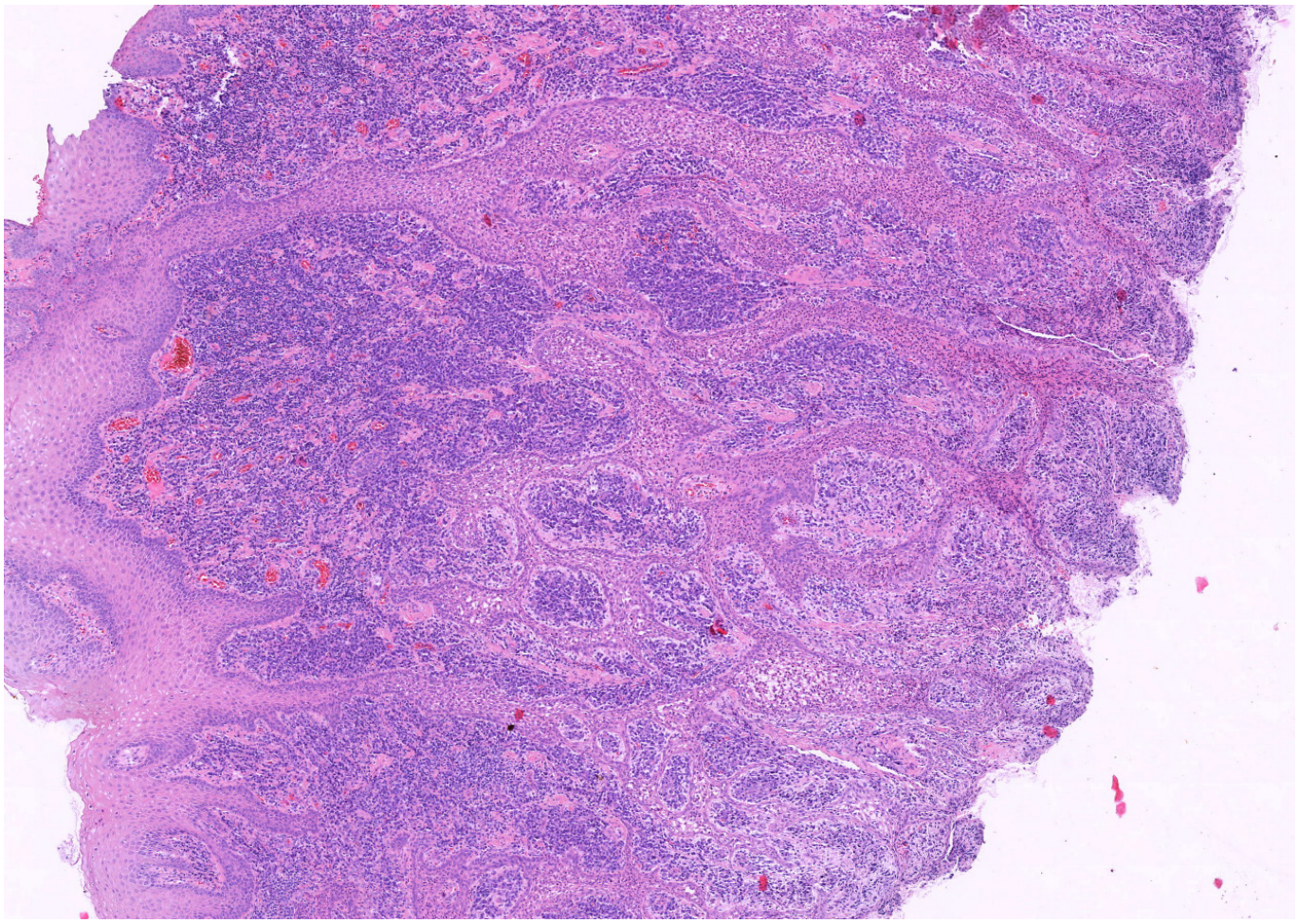

Fig. 5. Histological alterations present in both the epithelium and the lamina propria (gingival biopsy from diabetic, HE stain, 10x)

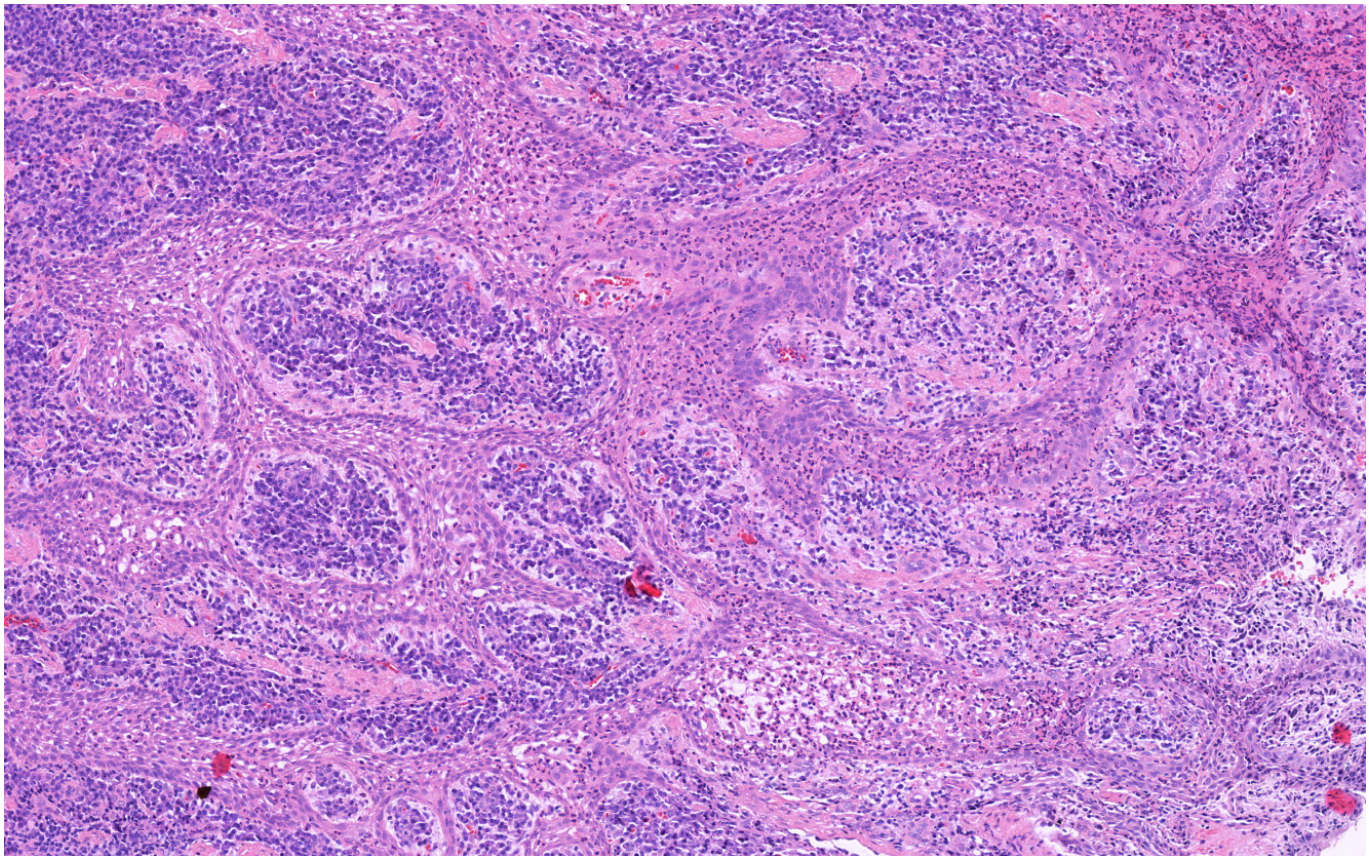

Fig. 6. Diffuse polymorphous inflammatory infiltrate (gingival biopsy from diabetic HE stain, 20x).

Our data suggest an important role of increased TNF-α and IL-6 concentrations regarding the progression of pathologic phenomena that characterise periodontal disease in diabetic subjects. The results sustain the utility of salivary immunologic examinations in case of periodontal disease, associated or not with different systemic conditions.

In our study, although no clinical signs of periodontal disease could be detected in diabetics, the microscopic changes of periodontal alterations were present. Our histological findings are in concordance with previously published data [23], and with those of other authors [24, 25], confirming the fact that hyperglycemia can induce alterations in periodontal structures, increasing the chance of periodontal disease occurrence [26-30]. Chronic hyperglycemia leads to irreversible complications such as neuropathy, retinopathy, nephropathy, cardiovascular and peripheral vascular diseases, delayed healing and periodontal diseases [1].

Periodontal diseases, including gingivitis (te reversible form), are highly prevalent in diabetic subjects. The changes that involve periodontal tissue alterations are mediated by the interaction between pathogens and the host immune-inflammatory response [31]. Periodontal pathogens are considered to be the initiative factor of the disease [32], but periodontal tissue destruction is the consequence of the host response to those pathogens [33]. It is still not fully elucidated by which exact mechanism diabetes affects periodontal tissue [34]. An altered immune-inflammatory response to bacterial pathogens has been suggested [33]. One means of investigating the local inflammatory status of the oral cavity is by analysis of saliva, a non-invasive approach for assessing the presence or absence of various inflammatory molecules.

In present study, increased salivary TNF-α and Il-6 levels were present in the same time with histological alterations in periodontal tissues, both in epithelium as well as connective tissue. Thus, we may confirm the hypothesis that immunological changes are involved in pathomechanism of periodontal disease in diabetic subjects.

Diabetic subjects included in our study had a history of at least 5 years of type 2 diabetes, with a poorely control glycaemic status, immunological and histological changes but no clinical signs of periodontal diesase. This might due to the fact that the main ethiological factor of periodontal disease (dental plaque) was absent, most of subjects mentaining a wery good oral hygiene. In this case, we conclude that type 2 diabetes can only co-induce periodontal breakdown through alteration of immune-inflammatory response to bacterial pathogens.

The clinical relevance of our study is that salivary detection of inflammatory cytokines can be a predictive tool for the development of periodontitis in diabetic subjects.

Limitations of the study are the small sample of subjects and the limited number of cytokines. Further research should include pro-inflammatory as well as anti-inflammatory cytokines, in order to get a more appropriate image about the complex immunological mechanism involved in periodontal breakdown in diabetics.

Further prospective studies are warranted to produce sufficient evidence to support the application of specific salivary biomarkers for prediction and prognosis of periodontal disease among patientswith diabetes.

Conclusions

The serum and mainly the saliva of patients with diabetes mellitus -without signs of periodontal idease- contained increased levels of TNF-α and IL-6, confirming the hypothesis of immunological implication, as a correlation between diabetes mellitus and periodontal disease. Histological alterations, suggesting a local inflammatory state, were present in periodontal tissue od diabetics, confirming the above hypothesis. The study reveals that saliva analysis is a quite efficient method in testing the periodontal breakdown progression in the subjects with T2DM.

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Conflict of interest

None to declare.

References

1. Sima C, Glogauer M. Diabetes mellitus and periodontal diseases. Curr Diab Rep. 2013; 13(3): 445–52.
2. Hanes PJ, Krishna R. Characteristics of inflammation common to both diabetes and periodontitis: are predictive diagnosis and targeted preventive measures possible? The EPMA journal. 2010; 1(1): 101–16.
3. 12. Taylor JJ, Preshaw PM, Lalla E. A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes. J Periodontol. 2013; 84(4 Suppl): S113–134.
4. Monea A, Gruber R, Nagy E, Beresescu G, Moldovn C, Monea M. Saliva and serum levels of TNF-alpha and IL-6 in a sample of Romanian adult subjects with Type 2 diabetes mellitus and periodontal disease. European Scientific Journal.2014; 10: 350–359.
5. Gursoy UK, Kononen E, V.-J. Uitto et al., "Salivary interleukin-1β concentration and the presence ofmultiple pathogens in periodontitis," Journal of Clinical Periodontology, 2009; 36(11): 922–927.
6. Mirrielees J, Crofford LJ, Lin Y et al., "Rheumatoid arthritis and salivary biomarkers of periodontal disease," Journal of Clinical Periodontology, 2010; 37(12): 1068–1074.
7. Rathnayake N, Akerman S, Klinge B et al., "Salivary biomarkers of oral health: a cross-sectional study," Journal of Clinical Periodontology, 2013; 40(2): 140–147.
8. Ebersole JL, Schuster JL, Stevens J et al., "Patterns of salivary analytes provide diagnostic capacity for distinguishing chronic adult periodontitis from health," Journal of Clinical Immunology,2013; 33(1): 271–279.
9. Teles RP, Likhari V, Socransky SS, and Haffajee AD. "Salivary cytokine levels in subjects with chronic periodontitis and in periodontally healthy individuals: a cross-sectional study,"Journal of Periodontal Research, 2009; 44(3): 411–417.
10. Ramseier CA, Kinney JS, Herr AE et al. "Identification of pathogen and host-response markers correlated with periodontal disease," Journal of Periodontology, 2009; 80(3): 436–446.

11. Aurer A, Jorgi´c-Srdjak K, Plan˘cak D, Stavljeni´c-Rukavina A, and Aurer-Ko˘zelj J. “Proinflammatory factors in saliva as possiblemarkers for periodontal disease,” Collegium Antropologicum, 2005: 29(2): 435–439.
12. Frodge BD, Ebersole JL, Kryscio RJ, Thomas MV, and Miller CS. “Bone remodeling biomarkers of periodontal diseasein saliva,” Journal of Periodontology, 2008; 79(10): 1913–1919.
13. Taba Jr. M, Costa PP, Trevisan GL; et al. “Salivary interleukin-6, matrix metalloproteinase-8, and osteoprotegerin in patients with periodontitis and diabetes,” Journal of Periodontology, 2010; 81(3): 384–391.
14. Prakasam S and Srinivasan M. “Evaluation of salivary biomarker profiles following non-surgical management of chronic periodontitis,” Oral Diseases, 2014; 20(2) :171-7..
15. Ng, P.Y., et al., Candidate salivary biomarkers associated with alveolar bone loss: cross-sectional and in vitro studies. FEMS Immunol Med Microbiol, 2007; 49(2): 252-60.
16. Frodge, B.D., et al., Bone remodeling biomarkers of periodontal disease in saliva. J Periodontol, 2008; 79(10): 1913-1919.
17. Coman M,L., Immunological research in periodontal disease, PhD Thesis, Department of Odontology and Periodontology. 2009, University of Medicine and Pharmacy: Tg.Mures, Romania.
18. Baum BJ, Yates JR 3rd., Srivastava S, Wong DT, and Melvin JE. “Scientific frontiers: emerging technologies for salivary diagnostics,” Advances in dental research, 2011; 23(4): 360–368.
19. Goodson JM. “Gingival crevice fluid flow,” Periodontology 2000; 31:43–54.
20. Wong DT. “Salivary diagnostics powered by nanotechnologies, proteomics and genomics,” Journal of the American Dental Association, 2006; 137(3): 313–321.
21. Mandel ID. “Salivary diagnosis: more than a lick and a promise,” The Journal of the American Dental Association, 1993; 124(1): 85–87.
22. Hu S, Li Y, Wang J et al. “Human saliva proteome and transcriptome,” Journal of Dental Research, 2006; 85(12): 1129–1133.
23. Monea A, Mezei T, and Monea M. “The influence of diabetes mellitus on periodontal tissues: a histological study.” Rom J Morphol Embryol 2012; 5(3): 491-495.
24. Dağ, A., et al. “Morphological Changes Caused by Streptozotocin-Induced Diabetes in the Healthy Gingiva of Rats.” Experimental and clinical endocrinology & diabetes: official journal, German Society of Endocrinology [and] German Diabetes Association, 2016; 124(03): 167-172.
25. Popescu, M. R., et al. “Histological Analysis of Collagen Fibers in Patients with Diabetes Mellitus and Periodontal Disease.” Journal of Cytology & Histology 2014; S4:2.
26. Silva JA, Lorencini M, Reis JR, Carvalho HF, Cagnon VH, Stach-Machado DR. „The influence of type I diabetes mellitus in periodontal disease induced changes of the gingival epithelium and connective tissue” Tissue Cell. 2008; 40: 283–292.
27. Hillman G, Dogan S, Guertsen W. “Histopathological investigation of gingival tissue from patients with rapidly progressive periodontitis”. J Periodontol, 1998; 69(2): 195-208.
28. Brownlee M, Cerami A, Vlassara H. „Advanced glycosilation end-products in tissue and biochemical basis of diabetic complications”. N Engl J Med 1998; 318: 1315-20.
29. Bucala R, Makita Z, Koschinsky T, Cerami A, Vlassara H. ‚Lipid advanced glycosylation: pathway for lipid oxidation in vivo”. Proc Natl Acad Sci U S A 1993; 90: 6434–8.
30. Schmidt A, Weidman E, Lall E, et al. „Advanced glycation end-products (AGEs) induce oxidant stress in the gingiva: a potential mechanism underlying accelerated periodontal disease associated with diabetes”. J Periodontal Res 1996; 31: 508–15.
31. Preshaw PM, Taylor JJ. ‚How has research into cytokine interactions and their role in driving immune responses impacted our understanding of periodontitis?’ J Clin Periodontol. 2011; 38(11): 60–84.
32. Kornman KS. „Mapping the pathogenesis of periodontitis: a new look”. J Periodontol. 2008; 79(8 Suppl): 1560–8.
33. Hanes PJ, Krishna R. „Characteristics of inflammation common to both diabetes and periodontitis: are predictive diagnosis and targeted preventive measures possible?”The EPMA journal. 2010; 1(1): 101–16.
34. Taylor JJ, Preshaw PM, Lalla E. ‚A review of the evidence for pathogenic mechanisms that may link periodontitis and diabetes.” J Periodontol. 2013; 84(4 Suppl): S113–134.

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Erratum

Due to an editing error of the authors, the article „Laparoscopic Greater Curvature Plication for Morbid Obesity: Indications, Results, Perspectives" published in Acta Medica Marisiensis, 2015;61(2):142-144 (DOI: 10.1515/amma-2015-0041) was published without the financial support statement. By the request of corresponding author Borz C, we publish the following eratum:

Disclosure. None of the authors reported conflict of interest regarding this study. The study was financially supported by the University of Medicine and Pharmacy Tirgu Mureș, grant no. 8918/2015.

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