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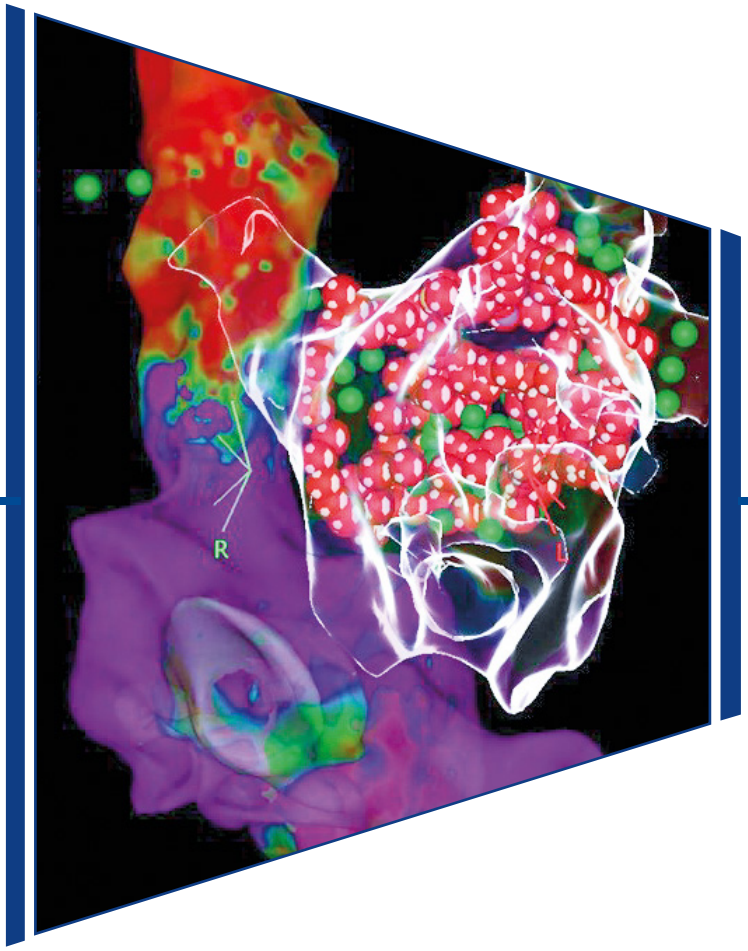

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Photo on the cover of the issue by Dariusz Rodkiewicz

Editorial Office

Medical University of Gdańsk
European Journal of Translational Medicine
Dębinki 7 Street, Building 1
80-211 Gdańsk, Poland

Phone: +48 58 349 15 37

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INVITED EDITORIAL

The King is Naked – a very subjective look at child and adolescent psychiatry

Agnieszka Wlazło

[1] Dr. Emil Cyran Memorial Regional Neuropsychiatric Hospital, Lubliniec, Poland

Abstract

For quite some time now an increasing number of voices can be heard in the public space regarding the decline of child and adolescent psychiatry: shortage of specialists, shortage of hospital wards and permanently insufficient financial support of this branch of medicine. However, in all the media hum about this problem and in the debates about how to solve it. are we not losing sight of its essence?

Keywords: adolescent · psychiatry · child · shortage

Citation

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For quite some time now an increasing number of voices can be heard in the public discourse regarding the decline of child and adolescent psychiatry: shortage of specialists, shortage of hospital wards and permanently insufficient financial support of this branch of medicine [1-2]. Certainly such news are highly frustrating, particularly in the context of psychiatric hospital bedspace shortages or long wait times for consultations with child and adolescent psychiatrist. However, despite all the media hum surrounding the claim „there are not enough child and adolescent psychiatrists" and the debates about how to improve this situation, are we not losing sight of the essence of this problem?

My professional experience includes both adult psychiatry (that was my first specialty) and child/adolescent psychiatry. In addition I am a trained psychologist and psychotherapist. I worked in various clinical environments ranging from outpatient psychiatry clinics, to open wards and acute psychiatric wards. I cooperated with psychology/pedagogy clinics, schools and courts. Over a year ago I returned to work at an acute psychiatric ward. Children with psychotic disorders or depressive episodes were always a small subset of my patient group. Indeed an increasing number of children and adolescents sought psychiatric help with diagnosing pervasive developmental disorders. However, the majority of the patients who sought help at my workplaces were children and teenagers with disharmonically developing personality or behavioral problems secondary to difficulties with regulating their emotions [3].

Corresponding author:
Agnieszka Wlazło, Dr. Emil Cyran Memorial Regional Neuropsychiatric Hospital, Lubliniec, Poland
e-mail: pogon4life@gmail.com
Available online: www.ejtcm.gumed.edu.pl

In the context of my clinical experience, I have several doubts. Are we certain that the main problem is the shortage of psychiatric health centers for children and adolescents? Is it necessary to build more child and adolescent acute psychiatric wards and outpatient clinics? Before we start trying to solve the problem, perhaps it is worth assessing what is the cause of it? Perhaps it is worth taking a look at the abnormal functioning of children and adolescents through the lens of causes, not effects?

Contrary to the mass-media headlines, majority of the teenagers who are admitted to the ward I work at are not patients with depression or psychosis. Instead, these patients are presenting deficits particularly in tolerating frustration and postponing satisfaction, which arose in the course of brain development, parenting and socialization. They don't need pharmacotherapy or even individual psychotherapy, but instead they need consistent correction of parenting and socio-therapeutic interventions, the fundamentals of pedagogical and psychological counseling [4].

The inability to cope with situations perceived as difficult and problems related to the teenager's attempts to define his or her own identity seem to more frequently result not in the weltschmerz of youth or in „feeling down" that is discussed with a fellow teenager and/or „treated" via listening to loud music, but instead in a suicide attempt. In addition, an increasing number of suicide attempts by teenagers seem to be instrumental or manipulative, e.g. „you took my cell phone (or other privilege), so I will kill myself." Also I observe an increasing ineffectiveness and powerlessness among the parents, teachers and even the staff of therapeutic centers, decreasing resilience of adults against manipulative statements and increasing shifting of responsibility for the child among parents, teachers, psychologists and psychiatrists. The final link of this chain of responsibility is always the acute psychiatric ward.

Someone might accuse me of trivializing a complex problem. No. I am not trivializing it. In my professional opinion the problems of children and adolescents should be treated seriously. However the focus, the center of gravity should not be on psychiatry because that leads us to medicalize problems for which help should be provided by supporting the family system, via interventions that support parents' competence. Such interventions are within the scope of practice of pedagogical and psychological counseling centers. In case of deeper problems in the family there should be easy access not to individual psychotherapy with the child, but to family therapy [5-6]. Instead, parents succumb to the illusion, amplified by the mass-media, that in case of problems with their child they should visit a psychiatrist who will solve these problems (or would solve them, only if there were more doctors with that specialty). It seems that children are no longer taught to solve problems, no longer taught to handle the consequences of their own behavior. Instead, teenagers exchange on social media and online forums tips about which medication is best for depression and arrange to see each other at „the psych ward." Isn't this sad? Doesn't this expose the inadequacy of our interventions?

We should not forget that once a child consults with a psychiatrist, the design of the healthcare system managed by the National Health Fund (NFZ) to some extent forces that psychiatrist to make a particular diagnosis. We doctors are well-aware of this situation and take it into consideration. However, once a child has medical documentation with a diagnosis whose ICD code begins with the letter „F," then its parents, teachers, etc. approach him or her as someone who should be treated, instead of raised or taught. Mass-media support the society's expectations by broadcasting images not of children whom nobody taught how to delay satisfaction, how to tolerate frustration or how to bear the consequences of own behavior, not showing images of teenagers with interpersonal problems or difficulties with building their own identities in an increasingly changing world. Instead, we are bombarded with media images of young patients who need psychiatric care.

Majority of children and adolescents with whom I had and continue to have professional contact with should have never entered the psychiatric healthcare system. On the contrary, we should make more effort so that they do not enter it at all. Psychiatry is a branch of medicine, therefore without any doubt its role is to help with differential diagnosis of mental problems. However, it is not the role of psychiatry to teach delayed gratification or coping with frustration, help develop a sense of causation and responsibility, etc. If we do not address the causes of children's and adolescents' problems with functioning, then in several years we will have to confront an increasingly powerless members of our society who will easily name their diagnosis (or diagnoses) and all the medications they were taking so far, but who will not be able to handle the typical difficulties of daily life.

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EUROPEAN JOURNAL OF TRANSLATIONAL
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RESEARCH ARTICLE

High-power and short-duration ablation with the Qdot+ algorithm for pulmonary vein isolation and the right superior ganglion plexus ablation without fluoroscopy

Edward Koźluk[1], Agnieszka Piątkowska[2,1], Dariusz Rodkiewicz[1], Grzegorz Opolski[1]

[1] I Chair and Department of Cardiology Medical University of Warsaw, Warsaw, Poland
[2] Department and Clinic of Emergency Medicine, Wrocław Medical University, Wrocław, Poland

Abstract

In this report we present pulmonary vein and posterior box isolation together with the right superior ganglion plexus ablation using the Qdot Micro catheter without fluoroscopy. We describe different possibilities of this new technology for catheter ablation. The main advantages of this catheter to potentially increase ablation safety and effectiveness are discussed. Specifically, the possibility to perform high-density mapping with the lowest available distance between points. Furthermore, the possibility to decrease the risk of collateral tissue damage and to improve atrial linear lesions contiguity, transmurality and durability due to the dominance of resistive heating supported by the feedback temperature control. Finally, the possibility to shorten the procedure and fluoroscopy duration due to the high shortening of application duration to 4 seconds only.

Keywords: atrial fibrillation · pulmonary vein isolation · high power short duration ablation · Qdot Micro catheter · ganglion plexi ablation · zero-fluoroscopy ablation

Citation

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Corresponding author:
Dariusz Rodkiewicz, I Chair and Department of Cardiology, Medical University of Warsaw, Poland
e-mail: elektrofizjologia@wum.edu.pl

Available online: www.ejtcm.gumed.edu.pl

Introduction

Isolation of the pulmonary veins is the most effective treatment for atrial fibrillation (AF)[1]. The reference method of this procedure is radiofrequency (RF) ablation with a single-point catheter using an electroanatomical system. Its main limitations are the long duration of the procedure and still unsatisfactory effectiveness [2]. Hence, methods are sought to increase its effectiveness and safety, combined with shortening of the procedure's duration. One of the ways is to use single-shot devices such as balloons or the pulmonary vein ablation catheter [2-3]. An alternative is to shorten the time of a single application. In part, it was possible to achieve this by controlling the pressure of the electrode against the tissue, especially in combination with the so-called "close" protocol [4]. The next big step seems to be increasing the power to 90W, which allows to shorten a single application to 3-4 seconds. The primary aim of this article is to present the new Qdot catheter, which is the first device to enable this procedure [5].

In recent years, there has been an increased interest in cardioneuroablation [6-10]. A beneficial effect has been demonstrated by this type of procedure for patients with reflex syncope [6-10]. Since in some patients with sinus node disease the syncope is functional (patients with rapid rhythm-induced pauses, drug-induced sinus node disease, athletes with bradycardia) [11], ablation of the parasympathetic ganglia is often effective for them and allows them to avoid pacemaker implantation [6, 12]. To the best to our knowledge, there have been no studies presenting cardioneuroablation using high-power short-duration ablation. Thus, our second aim is to present the first experience with Qdot+ protocol in pulmonary vein ablation together with cardioneuroablation.

Case presentation

To illustrate these aims, we present the case of a 77-year-old female after the right atrial inferior isthmus ablation because of typical atrial flutter 5 years ago. For about 2 years the patient experienced paroxysmal AF with EHRA score III [1]. The symptoms of arrhythmia consisted of palpitations, chest discomfort, shortness of breath, headache and dizziness. Concealed sinus node disease was also diagnosed (during treatment with a beta-adrenolitics an episode of syncope occurred and in 24-hour ECG holter monitoring there were pauses of automatism up to 4 seconds). These symptoms resolved after the discontinuation of antiarrhythmic drugs. The patient reported an episode of gastrointestinal bleeding. Since pharmacotherapy was not possible, the patient was qualified for pulmonary vein isolation together with parasympathetic ganglion plexi ablation. Before the procedure, thromboembolic material in the left atrium was excluded using transesophageal echo. Patent foramen ovale (PFO) was diagnosed with small interatrial leak. Because the transseptal puncture was not necessary, the patient was qualified for ablation without fluoroscopy using the technique we previously described [13].

At the beginning of the procedure the patient was on AF. The catheters were introduced into the coronary sinus and the right ventricle under the control of CARTO system. Using the Qdot catheter, long transseptal sheaths were introduced via the right femoral vein into the superior vena cava. The right atrial voltage map was made using the LassoNav catheter. On its basis, a Qdot catheter was introduced into the left atrium through the PFO. Over this catheter two transseptal sheaths were successively inserted into the left atrium. A map of the left atrium and proximal parts of the pulmonary veins was obtained using the CARTO system. The pulmonary vein potentials were diagnosed in all four pulmonary veins. With the use of the Qdot+ algorithm (90W for 4 s), all pulmonary vein isolation was performed according to the "close" protocol, verified with the LassoNav catheter (Fig. 1-3).

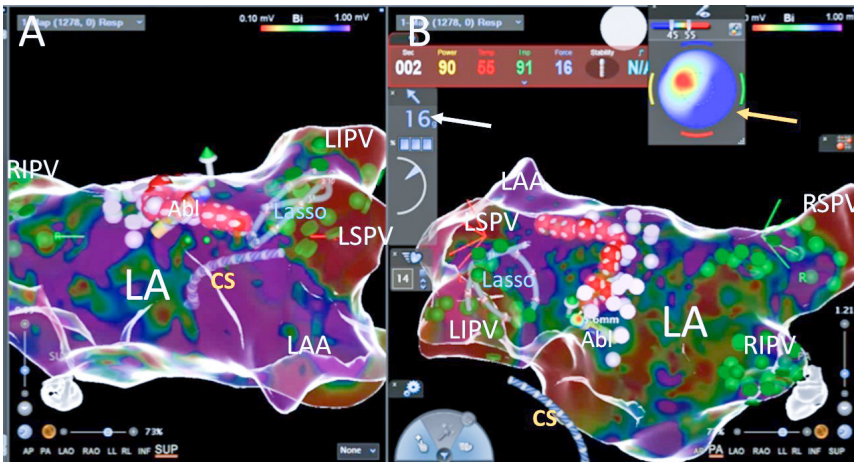

Fig. 1. One of the RF aplications during left pulmonary veins isolation; ressure (the white arrow) of the catheter on the posterior wall is 16g. Bulls' eye (the yellow arrow) indicate good pararell contact of the catheter tip with the left atrial tissue with optimal temperature increase at the second second of aplication (red zone in left superior quadrant); green dots – pulmonary vein potentials, pink dots – fragmented potentials, red dots – ablation points; Abl – ablation catheter, CS – catheter in the coronary sinus, LA – the left atrium, LAA – the left atrial appandage, Lasso – LassoNav catheter inside the left inferior pulmonary vein, LIPV – the left inferior pulmonary vein, LSPV – the left superior pulmonary vein, RIPV – the right inferior pulmonary vein, RSPV – the right superior pulmonary vein; panel A – superior view, panel B – posteriori view on the left atrium

At the beginning of the left superior pulmonary vein isolation, the sinus rhythm returned without pathological pause. After pulmonary veins' isolation the right superior ganglion plexus was mapped using 100 ms cycle length stimulation (mixed type reactions were observed). The pacing induced unsustained atypical atrial flutter. Ablation was performed at ganglionated plexi (GB) stimulation sites, however we did not observe significant acceleration of the sinus rhythm. Because of the small distance between the lines, the posterior segment was isolated (the line in the roof and in the lower part of the posterior wall) (Fig. 4-5). LassoNav catheter mapping was performed with accessory applications to obtain full pulmonary veins and the posterior segment isolation. The post- procedure and follow-up (on the following day) transthoracic echocardiography did not reveal any fluid in the pericardium.

Duration of the procedure was 140 min (puncture and catheter introduction 20 min, right atrial mapping – 10 min, transseptal access – 10 min, left atrial and pulmonary vein mapping – 20 min, left pulmonary vein isolation – 22 min, right pulmonary vein isolation – 33 min, mapping of the right superior GP and its ablation – 10 min, posterior segment isolation –10 min, control mapping of the pulmonary veins and posterior wall – 5 min). The procedure was performed without the use of fluoroscopy. The total time of 203 RF current applications was 13 min 32 s. After the procedure we observed sinus rhythm 75 bpm, BP 115/70 mmHg. There were no complications. Sinus rhythm was maintained during the postoperative monitoring. On the next day, the patient was discharged home in good general condition. Pharmacotherapy with dabigatran, rosuvastatin and a proton pump inhibitor was maintained. There were no palpitations or syncope during follow-up.

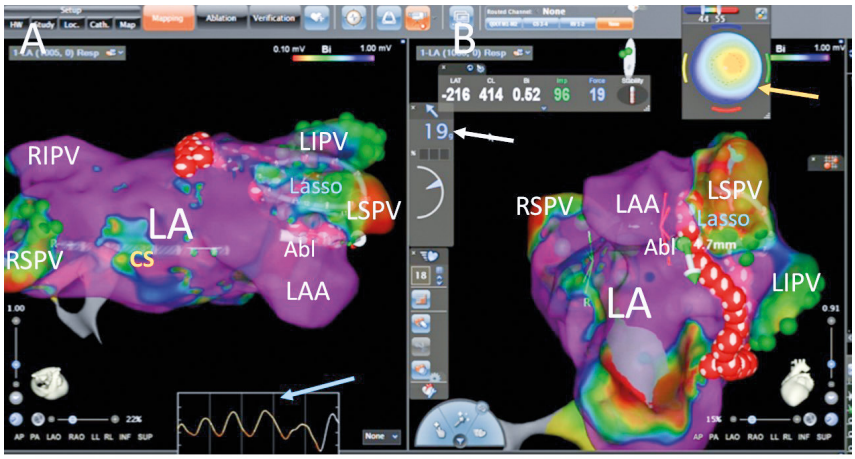

Fig. 2. Another RF aplications on the limbus between the left atrial appandage and the left superior pulmonary vein' pressure (the white arrow) of the catheter on the limbus is 19g. Bulls' eye (the yellow arrow) indicate good perpendicular contact of the catheter tip with the left atrial tissue with optimal temperaturę increase at the fourth second of aplication (red zone in central part); green dots – pulmonary vein potentials, red dots – ablation points; blue arrow – respiratory curve; Abl – ablation catheter, CS – catheter in the coronary sinus, LA – the left atrium, LAA – the left atrial appandage, Lasso – LassoNav catheter inside the left superior pulmonary vein, LIPV – the left inferior pulmonary vein, LSPV – the left superior pulmonary vein, RIPV – the right inferior pulmonary vein, RSPV – the right superior pulmonary vein; panel A – superior view, panel B – modyfied left anterior oblique view on the left atrium

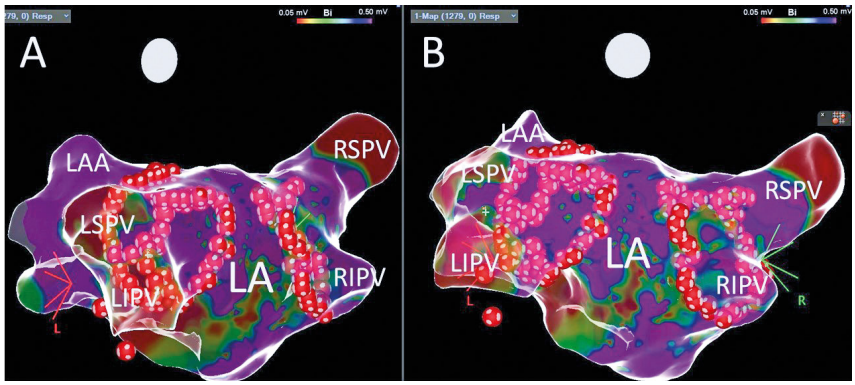

Fig. 3. The LAO projection (panel A) and posteriori view (panel B) on the left atrium after pulmonary vein isolation; red dots – ablation points; LA – the left atrium, LAA – the left atrial appandage, LIPV – the left inferior pulmonary vein, LSPV – the left superior pulmonary vein, RIPV – the right inferior pulmonary vein, RSPV – the right superior pulmonary vein

Discussion

The Qdot micro catheter is a new generation catheter that offers advantages of the recent technological developments. Below we present all advantages of this catheter.

Typical thermocool catheter with pressure control

The design of the Qdot Micro catheter allows it to be used as a typical ablation catheter with a cooled tip and with

pressure control. This is similar to the newest version of Thermocool Smarttouch SF catheter (used at present in centers not involved in the implementation program for the Qdot Micro catheter), employing a porous irrigation tip with the same contact force sensor to provide directional contact force information. This makes it possible to calculate the "ablation index", parameters which together with the "close protocol " increased the success rate and decreased the complication risk during left atrial ablations in recent years. This catheter may be used in two options: unidirectional and bidirectional with different curves (the second one is preferred in our center). This catheter also allows temperature measurement, however this will be discussed below. Therefore, the new Qdot Micro catheter can be used with the same mode as the Thermocool Smarttouch SF catheter.

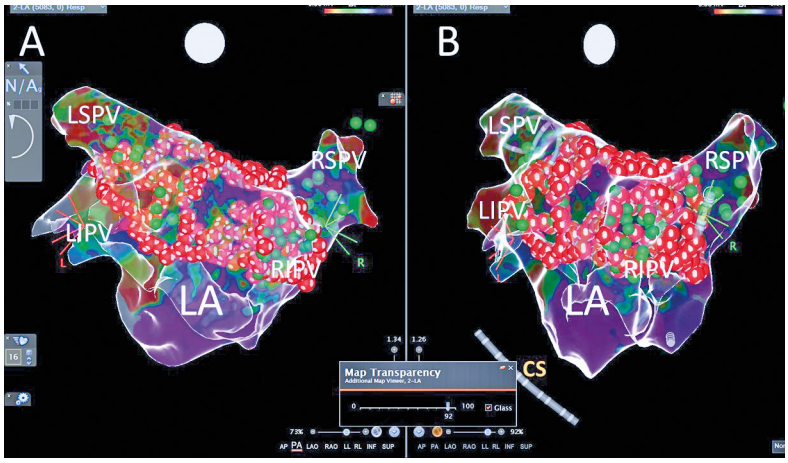

Fig. 4. The final bipolar voltage map after isolation of the pulmonary veins and posterior wall in PA projection (panel A) and the right posteriori oblique view (panel B); green dots – pulmonary vein potentials before ablation, red dots – ablation points; CS – decapolar catheter in the coronary sinus, LA – the left atrium, LIPV – the left inferior pulmonary vein, LSPV – the left superior pulmonary vein, RIPV – the right inferior pulmonary vein, RSPV – the right superior pulmonary vein

High-density mapping

High density mapping is one of the milestones of modern electrophysiology. It allows to increase the precision of arrhythmogenic substrate mapping and thus increase the success rate in complex arrhythmias [14]. There are different technical solutions for high density mapping. The resolution of this mapping varies with catheters from different brands. For example, the Orion catheter (Boston Scientific) is fold-out rosette consisting of 8 arms. There are 8 small electrodes with an area of 0.4 mm^2 spaced 2.5 mm apart (a total of 64 electrodes) on each arm. The electrode is compatible with the Rhythmia system (Boston Scientific) and enables automatic map-making from high density of points (annotation of signals is automatic as it would be impossible to conduct manually). Another example is the Advisor HD-grid (Abbot) high-density mapping catheter cooperating with the EnSite system (Abbott) consisting of 4 electrode strands (1 mm length/width; 3 mm apart) with 4 electrodes each. The distance between the electrodes in both directions is the same, thanks to what we obtain 24 pairs of electrodes for bipolar recordings and 16 electrodes for unipolar recordings). Due to the even distribution, it is possible to evaluate bipolar electrographs in both perpendicular planes. Another example of catheter for high density mapping is the Octarey catheter (Johnson & Johnson) cooperating with CARTO 3 system. This catheter consists of 8 diverging flexible arms containing 6 small electrodes (0.5 mm long) 2 mm apart (48 electrodes in total). Unlike the Qdot micro catheter, all of the above are only mapping catheters and require a separate ablation catheter.

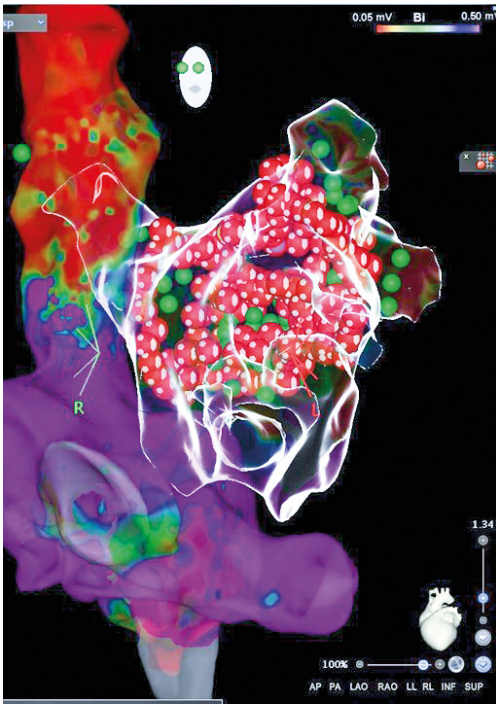

Fig. 5. Bipolar voltage map of the right atrium, on the basis of which the transseptal sheats with catheters were introduced into the left atrium via PFO together with the final left atrial anatomical glass map after pulmonary vein isolation and posterior segment isolation; LAO projection

In the Qdot micro catheter three microelectrodes are placed 1,5 mm apart around the distal end of the ablation tip (Fig. 6). The size of the electrodes is 0,32 mm in diameter with a surface area of 0,086 mm^2 each. Currently, these

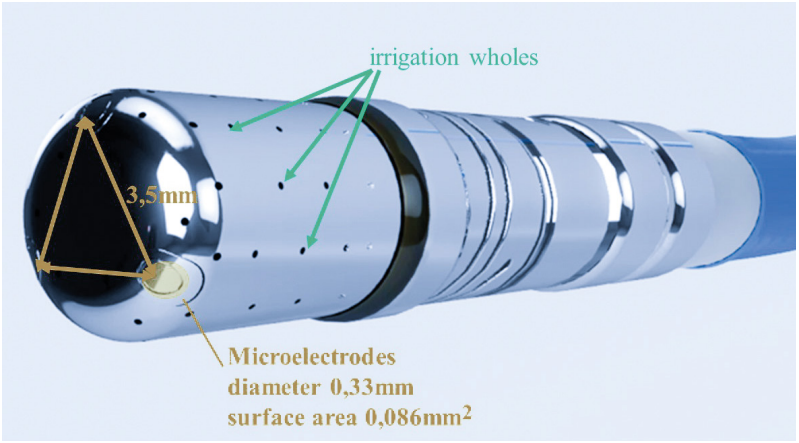

Fig. 6. Tip of the Qdot catheter; the microelectrodes are placed one and a half millimeters apart around the distal end of the ablation tip; the size of the electrodes is point three three millimeters in diameter with a surface area of point zero eight six millimeters each; the small surface area of the microelectrodes enables discrete, high resolution signals with less far-field information than the larger ablation tip affords

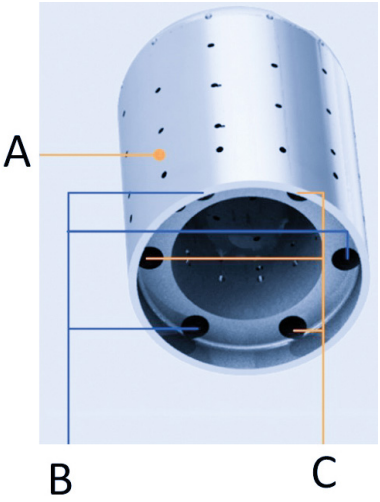

Fig. 7. The skeleton of the Qdot Micro™ Catheter tip presents channels within the wall of the tip itself, into which the thermocouples and microelectrodes are placed; as mentioned in the main text this designe places the thermocouples in close proximity to the tip--tissue interface, enabling temperature to be measured as the tissue is heated by the application of radiofrequency energy; A – thicker wall accomodates channels, B – thermocouple channels, C – microelectrode channels

are the smallest microelectrodes and the closest spacing between them among the available high-density mapping catheters. The small surface area of the microelectrodes enables discrete, high-resolution signals with less farfield information than the larger ablation tip affords. The microelectrode signals can be displayed as unipolar and bipolar electrograms. The highest voltage electrogram among three microelectrode pairs is used to apply color to the voltage map. The microelectrodes signals do not apply to LAT (local activation time) maps. The bipolar electrograms are displayed as microelectrode pairs: 1-2, 2-3, 3-1. The numbers of the microelectrodes may be displayed on the CARTO 3 map, which can help with the selection of microelectrodes pairs for pacing. Microelectrode pacing may facilitate very local capture and is routed between microelectrode pairs. Pacing is not routed between any microelectrode and a standard electrode. Microelectrodes are more sensitive to power line noise during the RF application than the larger electrodes. The CARTO 3 system employs advanced power line noise filters for exceptional signal quality. This could lead to damage of monitoring during RF current application. When using the Confidense module with continuous mapping feature, the stability filter must be used to prevent acquisition of points during catheter motion.

High density temperature control (including catheter orientation)

The predecessor catheter Smarttouch SF employs a single thermocouple in the center of the ablation tip, surrounded by cooling saline. Using this design, the temperature should be monitored only to confirm that the irrigation is adequate (and temperature rise during ablation may indicate blocked irrigation). In the Qdot catheter there are 6 thermocouples placed in the channels within the wall of the distal electrode itself around this electrode in close proximity to the tissue interface (Fig. 7). Three thermocouples are located distally around the catheter tip (0,075 mm from the real tip), and three thermocouples are located proximally around the catheter tip (3 mm from the real tip) (Fig. 6-7). The CARTO 3 system displays the temperature measured on the catheter, which helps to confirm energy delivery to the tissue and the tip-tissue contact stability (Fig. 1-2). The temperature color display aids in understanding the orientation of the catheter tip to the tissue during RF ablation(-Fig. 1-2). Red color centered on the "bulls' eye" indicates that the catheter is touching the tissue in perpendicular orientation (Fig. 2). Red color on one side of the display indicates heating on that side of the ablation tip, which signals that the ablation tip is connecting the tissue in a parallel orientation (Fig. 1).

Feedback temperature control

It has been shown that an increase of temperature >85°C is an important risk factor for the so-calle steam pop, which may be the cause of cardiac tamponade [15]. To increase the safety of the RF application, the temperature is measured every 33 ms and if it increases above programmed

value then the power is reduced or saline flow is increased [15]. There are two different modes of feedback control (Fig. 8-9).

When the maximum power of RF current is set for lower or equal 35 W, after activation of the generator, irrigation begins at four milliliters per minute for the pre-determined RF delay period (few seconds). After the onset of RF energy delivery, we begin to see a temperature rise. In this instance the temperature rise is steep and a burst of the higher flow rate (15 mL/min) is automatically applied. When the temperature drops, the irrigation is returned to the lower flow rate (4 mL/min). As the temperature reaches the target (65°C is recommended) there is another burst of the higher irrigation flow rate, until the temperature drops below the target. If the temperature exceeds the target temperature, another burst of the higher flow rate is applied, as well as decrease in the power delivered. When the temperature drops, the power increases again, and the flow rate decreases to the lower rate (Fig. 8).

When the maximum power of RF current is set for > 35 W, the irrigation flow rate starts at 15 mL/min. If the measured temperature does not rise, the irrigation rate drops to 4 mL/min until the temperature exceeds the preset max low flow temperature value. Once the temperature rises above this temperature, the irrigation rate is increased to 15 mL/min. The irrigation rate and the power are automatically adjusted to maintain the temperature between the max low flow and the target value, and the primary means of temperature control is the irrigation flow rate. Power is adjusted if the higher irrigation rate is insufficient to affect a temperature response. If the temperature reaches the predetermined cut-off temperature level, the RF application will be automatically terminated (Fig. 9).

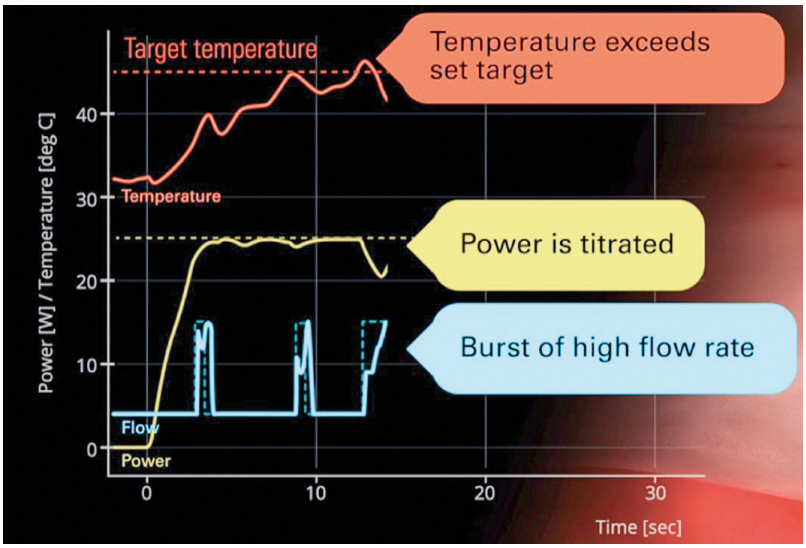

Fig. 8. Feedback temperature control when the maximum power of RF current is set for lower or equal 35W; description in the main text

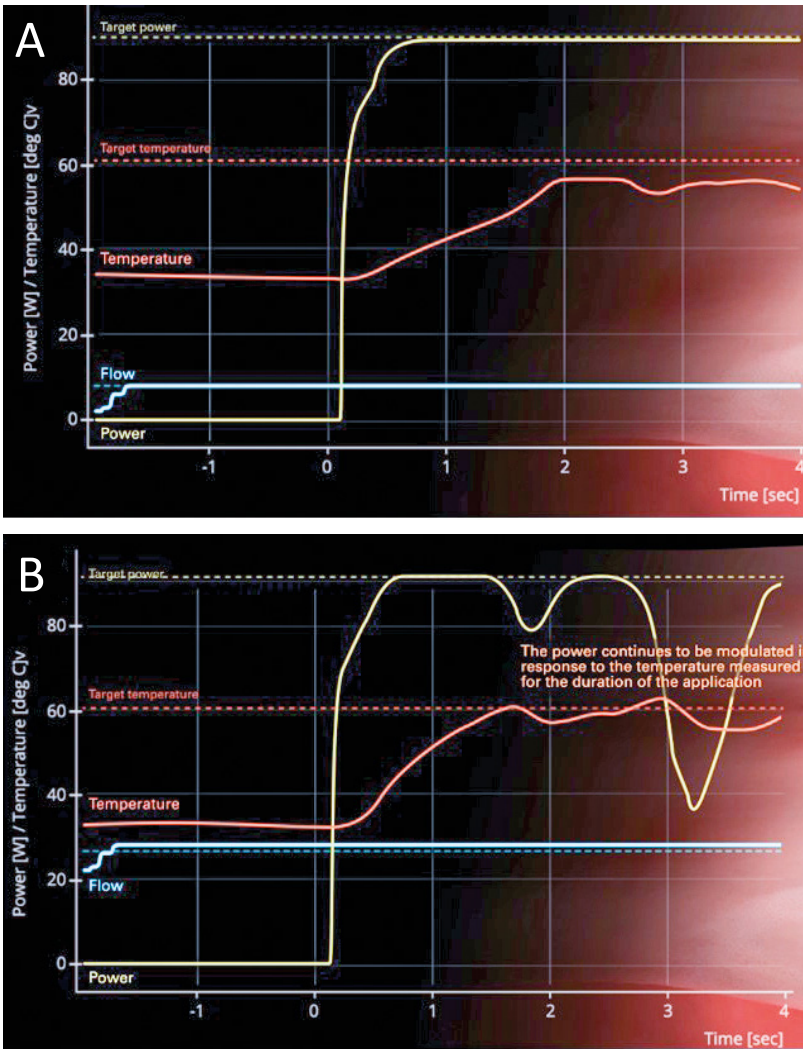

Fig. 9. Feedback temperature control when the maximum power of RF current is set for lower or equal 35W; description in the main text; panel A – temperature do not reach target level, panel B – temperature reach target level

High power, short duration ablation

The greatest advantage of the Qdot catheter is the ability to perform a high-energy short duration ablation [15-17, 19]. In RF ablation, the tissue damage occurs in two consecutive phases. The first is resistive phase, the second is the conductive phase. The first one depends directly on the amount of energy delivered at the catheter-tissue interface. The second one is mainly contingent on the time of application and results from the gradual passive heating extend to deeper tissue layers. When the temperature reaches 50ºC, an irreversible myocardial tissue injury occurs. At lower temperatures, the reversible damage often occurs with accompanying tissue edema [15]. In traditional RF ablations, the second effect dominates. It is difficult to predict when the application is transmural. There is a risk of underheating, which may be masked by the tissue edema (hence the acute effect may be effective and after the edema disappearance, conduction or automatism recurs). Alternatively, the overheating causes damage to neighboring organs. Hence, in order to increase the safety and effectiveness of the radiofrequency current ablation, the concept of high-power short-duration applications was developed [15-17, 19]. When using high power for a few seconds, we observe only the resistive effect. In animal experiments, it was shown that the best efficacy and safety rates was obtained with applications duration of 4 seconds [15-17]. Using the power of 90 W at this duration, the depth of lesion was 3,6+0,6 mm and the width was 10,4 + 1,2 mm [15]. The variances of these parameters were smaller than after classical applications, which resulted in more frequent continuous lines without the gap both in direct [15-17] observation and after 30 days follow-up [16]. As the thickness of the atrium muscle is comparable to the depth of the lesion [18], no damage to the adjacent organs was also observed [15].

The tissue overheating may result in steam pop which can cause cardiac tamponade. The steam pop was observed in animal models if temperature of the catheter-tissue interface was > 85°C or the catheter pressure was > 40g [15]. To avoid this phenomenon, a feedback temperature control was introduced. Without this protection, the phenomenon of steam pop was noticed during 3/174 applications (1,7%). After the introduction of this algorithm, this phenomenon was not observed during 233 applications. Shortening the time of a single application during the isolation of the right superior pulmonary vein resulted in a reduction in their duration compared to classic procedures in animal studies from 270 +60 s to 33 + 6 s [15].

Qdot procedure in our patient

There is limited experience with PVI isolation using Qdot catheter and high-power and short duration ablation. A single clinical study assessed its feasibility, safety and short-term effectiveness on 52 patients [19]. Direct isolation was achieved for all pulmonary veins including an adenosine/isoproterenol check. The study showed favorable mean procedure and fluoroscopy duration compared to classic ablation. There were 2 complications: a pseudoaneurysm and an asymptomatic cerebral thromboembolism. After 3 months of follow-up, 49 patients (94,2%) had sinus rhythm, whereas 2 had AF and one had atrial flutter. To the best of our knowledge, there is no data about GP ablation using high-power short duration applications. However, all the preclinical data suggest that when using this form of ablation, the damage is transmural. Thus, it should be effective in this kind of ablation.

Conclusion

The Qdot Micro catheter presented in this article offers some new advantages potentially increasing ablation safety and effectiveness. Specifically, the possibility to perform high-density mapping with the lowest available distance between points. Furthermore, the possibility to decrease the risk of collateral tissue damage and to improve atrial linear lesions contiguity, transmurality and durability due to the dominance of resistive heating supported by the feedback temperature control. Finally, the possibility to shorten the procedure and fluoroscopy duration due to the high shortening of application duration to 4 seconds only.

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RESEARCH ARTICLE

Risk factors for poor prognosis in heart failure with particular attention to the elderly population

Małgorzata Dobrowolska[1], Paweł Miękus[1], Michał Świątczak[2], Grzegorz Raczak[2], Ludmiła Daniłowicz-Szymanowicz[2]

[1]St.Vincent Hospital, Gdynia, Poland
[2]2nd Department of Cardiology and Electrotherapy, Medical University of Gdańsk, Poland

Abstract

Background: Heart failure (HF) is a leading cause of poor outcome. Age is considered one of the most critical risk factors for both the incidence and prognosis of HF. Therefore we aimed to assess the predictors of poor prognosis in HF patients with particular attention to the elderly population. **Material and methods:** We retrospectively enrolled patients hospitalized due to HF exacerbation during 2016-2017 (203 patients). The end-points were all-cause mortality and emergency rehospitalizations within a two-year follow-up period. A detailed analysis was performed in the subgroups of patients younger and older than 65 years old. **Results:** 121 (60%) patients experienced the end-points. Age, low systolic blood pressure, NYHA class IV, right ventricle HF symptoms, high C-reactive protein, troponin, NT-proBNP, hyponatremia, catecholamine therapy and mechanical ventilation during hospitalization independently predicted the end-points. The elderly were characterized by a higher incidence of concomitant diseases and HF with moderately reduced or preserved LVEF, worse laboratory parameters and pharmacological treatment, as well as worse prognosis. **Conclusion:** The prognosis of patients hospitalized due to HF, mainly the elderly, is poor. Simple clinical parameters could be useful in further decision-making regarding the intensification of their treatment.

Keywords: heart failure · risk factors · prognosis · elderly

Citation

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Corresponding author:
Małgorzata Dobrowolska, St. Vincent Hospital, Gdynia, Poland
e-mail: m.d.k.dobrowolska@domax.com

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Introduction

Heart failure (HF) is a continually growing global pandemic [1]. Despite significant advancements in the pharmacological and invasive treatment of HF, based on the widespread use of implantable cardioverter-defibrillators and resynchronization therapy to reduce mortality, the morbidity and mortality are still high [2-4]. HF exacerbation is the leading cause of hospital admissions [5], thus a considerable health burden for patients and the health care system [6-7]. Patients hospitalized due to HF exacerbation are known to have the worst prognosis [1, 4]. Therefore, assessing such patients and identifying predictors that affect the prognosis is critical from a clinical point of view. Age is considered one of the most important risk factors for HF incidence and prognosis [8-9]. The prevalence of HF increases rapidly with age: it doubles from 6% in people aged 60-79 years old to about 14% in those aged ≥ 80 years old; [10] respect 4-5% of individuals 45 years and older [9]. The clinical trials to date are quite poor in the elderly population [11-13]. However, recent data from the EuroHeart Failure Survey shows that older patients with HF have poor short-term survival and are not treated according to the HF guidelines [11, 14]. Given all of the above, we aimed to assess the predictors of poor prognosis in patients hospitalized with HF with particular attention to the elderly population.

Material and methods

Patients selection

This study is a sub-analysis of our retrospective project [15] of all patients hospitalized with a diagnosis of acutely decompensated chronic HF and new-onset acute HF [16] in at a single cardiology department (St. Vincent Hospital in Gdynia, Poland) during 2016-2017. Further analysis included: the patient's biometric parameters, the medical history available in the documentation (with particular emphasis on comorbidities, condition of the coronary vessels, implanted devices, the treatment so far), physical examination findings documented during hospitalization, laboratory results, electrocardiography and echocardiography [15]. The data was obtained from the hospital's medical records and electronic patient records. Exclusion criteria were as follows: patients < 18 years old, NYHA functional class I and II on admission, and no clinical signs of HF. All patients were followed up for two years. The *composite end-point* included: all-cause mortality and emergency rehospitalizations (including those due to HF exacerbation). Additional analyses were carried out differentiating between patients younger and older than 65 years of age. The study protocol was approved by the Local Ethics Committee of the Medical University of Gdańsk (decision number NKBBN/619/2018). All data we analyzed was collected during standard, routine clinical practice, therefore it was was not required to obtain the participants' written and informed consent for anonymized storage of information and its use for research.

Statistical analysis

Continuous data are presented as the median (25^{th}-75^{th} percentiles), whereas categorical variables as numbers (n) and percentages (%). Comparisons between the patients were made with U Mann-Whitney's test or the Fisher's exact test due to abnormally distributed variables. An association between the analyzed parameters and the end-point was assessed using the univariate and multivariate Cox hazard models. The accuracy of pre-specified cut-off values for the analyzed parameters and their association as potential predictors of the study end-point was determined by area under (AUC) the receiver-operating characteristic (ROC) curve. All the results were considered statistically significant with $p \leq 0.05$. The statistical analysis was performed using the Statistica 12.0 software (*StatSoft, Tulsa OK, USA*) and R 2.15.2 (R Project).

Results

We included 203 patients hospitalized due to HF exacerbation in the years 2016-2017. Within a two-year observation period, 121 instances of composite end-points (all-cause mortality and emergency rehospitalizations) were documented.

Clinical, laboratory, and echocardiographic findings

Most of the patients included in the study were men (Table 1). Patients who experienced end-points were older (median age 73 versus 68 years old in group without endpoints, $p = 0.046$), they more frequently had a history of atrial fibrillation/flutter (44% vs. 18%, $p < 0.001$) and diabetes (29% vs. 13%, $p = 0.010$), were also more likely to undergo coronary revascularization (47% vs. 30%, $p = 0.020$) and had lower systolic blood pressure (SBP) at discharge (median 120 vs. 128 mmHg, $p = 0.047$). In addition they had worse renal function indices, lower sodium values, higher troponin and natriuretic peptide levels (Table 1). In the echocardiographic assessment, these patients were characterized by more advanced left ventricular diastolic dysfunction and a trend towards worse systolic function (Table 1). There were no differences in pharmacotherapy; moreover, it should be emphasized that the majority of patients were treated according to the current guidelines [8]: > 90% of patients received a beta-blocker, > 80% were treated with an ACE-inhibitor or sartans; however the frequency of spironolactone/eplerenone use was lower than suggested in the guidelines (Table 1).

Table 1. Baseline clinical characteristics of the study patients according the end-point

Parameters	End-point (-) n = 82	End-point (+) n = 121	p
Male, n (%)	56 (68)	88 (73)	0.531
Age (years)	68 (61-79)	73 (64-81)	**0.046**
Medical history			
Coronary artery disease, n (%)	45 (56)	81 (68)	0.135
Revascularization (PCI/CABG), n (%)	25 (30)	57 (47)	**0.020**
ICD (including CRT-D)	14 (17)	30 (25)	0.212
Atrial fibrillation/flutter, n (%)	15 (18)	53 (44)	**< 0.001**
Hypertension, n (%)	28 (34)	47 (39)	0.554
Diabetes mellitus, n (%)	11 (13)	35 (29)	**0.010**
Cancer, n (%)	2 (5)	11 (26)	0,016
Cause of hospitalization			
Infections, n (%)	24 (29)	50 (41)	0.102
Acute coronary syndrome, n (%)	2 (2)	6 (5)	0.478
Tachyarrythmias, n (%)	20 (24)	30 (25)	1.000
Unknown reason, n (%)	38 (46)	40 (33)	0.077
Other, n (%)	9 (11)	12 (10)	0.818
The length of hospitalisation (days)	7 (5-9)	8 (5-12)	**0.041**
Clinical and diagnostic parameters			
Resting heart rate (beats /min)	83 (75-110)	90 (75-110)	0,225
SBP (mmHg)	128 (115-133)	120 (109-131)	**0.047**
NYHA class IV at admission, n (%)	27 (33)	55 (46)	0.080
Right ventricle HF symptoms at admission, n (%)	20 (24)	52 (43)	**0.007**
Hemoglobin (g/dl)	14 (12-15)	13 (12-15)	0.186
C-reactive protein (mg/l)	7 (3-16)	8 (4-22)	0.105

Sodium (mmol/l)	141 (139-142)	140 (137-142)	**0.030**
Glucose (mg/dl)	110 (98-141)	123 (105-163)	0.071
High-sensitivity troponin T (ng/ml)	0.042 (0.017-0.043)	0.105 (0.021-0.076)	**< 0.001**
Creatinine (mg/dl)	1.24 (0.81-1.32)	1.25 (0.91-1.51)	0.072
NT-proBNP (ng/l)	2432 (732-6115)	5170 (2474-10449)	**< 0.001**
Echocardiographic parameters			
LVEF (%)	37 (25-50)	33 (23-45)	0.061
LVEF < 40, n (%)	44 (54)	79 (66)	0.202
LVEF 40-49, n (%)	16 (20)	19 (16)	
LVEF ≥ 50, n (%)	22 (27)	22 (18)	
Diastolic dysfunction 2/3 °, n (%)	39 (64)	72 (82)	**0.032**
Left atrium diameter (cm)	4.84 (4.4-5.3)	5.04 (4.6-5.4)	0.085
Right ventricular systolic pressure increased, n (%)	64 (82)	115 (97)	**< 0.001**
In-hospital treatment			
Catecholamine, n (%)	1 (1)	8 (7)	0.087
Mechanical ventilation, n (%)	3 (4)	9 (7)	0.367
Treatment at discharge			
ACE-inhibitor/ Sartans, n (%)	73 (89)	95 (81)	0.166
Spironolactone / Eplerenone, n (%)	43 (52)	54 (48)	0.663
Beta-adrenolytics, n (%)	76 (93)	102 (91)	0.795
Statins, n (%)	50 (61)	80 (71)	0.164
Diuretics, n (%)	75 (91)	108 (96)	0.208
Potassium, n (%)	56 (68)	81 (72)	0.633
Antiplatelet therapy, n (%)	20 (24)	39 (35)	0.155
NOAC, VKA, LMWH, n (%)	58 (71)	79 (71)	1.000

Digoxin, n (%)	9 (11)	20 (18)	0.224
Amiodarone, n (%)	9 (11)	14 (13)	0.825

Categorical variables are presented as number and percentage. Continuous variables are presented as median and interquartile range. P values of < 0.05 are considered significant.

ACE-inhibitor – angiotensin converting enzyme inhibitor; CABG – coronary artery bypass grafting; CRT-D – cardiac resynchronization therapy defibrillator; ICD – implantable cardioverter-defibrillator; LMWH – low molecular weight heparin; LVEF– left ventricular ejection fraction; NOAC – novel oral anticoagulants; NT –proBNP-N-terminal pro brain natriuretic peptide; NYHA – New York Heart Association; PCI – percutaneous coronary intervention; SBP – systolic blood pressure; VKA – vitamin K antagonists.

Conversion factors to SI units are as follows: for creatinine mg/dl → umol/L: 0.6206; for CRP mg/l → nmol/L, 9.524; for glucose mg/dl → mmol/L, 0.05551, for hemoglobin g/dl → mmol/L, 88.42; for high-sensitivity troponin T ng/ml → µg/L,1,0; for NT-proBNP ng/l → pmol/L, 0.118

The predictors of the end-points

Table 2 presents the pre-specified on ROC curve analysis cut-off values for continuous variables with the highest discriminatory power in predicting the end-points (Table 2). In the Cox proportional hazard regression analysis, we revealed statistically significant clinical and laboratory variables, which predicted the end-points: age ≥ 73 years old, lower SBP (≤ 120 mmHg), NYHA class IV on admission, right-sided HF symptoms, C-reactive protein ≥ 31.4(mg/l), sodium ≤ 137 mmol/l, troponin level ≥ 0.044 ng/ml, NT-proBNP level ≥ 2453 ng/l, catecholamine use and mechanical ventilation during hospitalization (Table 3).

Table 2. Prognostic accuracy of the pre-specified cut-off values for analyzed parameters as predictors of composite end-points during the follow-up

Parameters	AUC	Characteristics (95% CI)		Predictive Value (95% CI)	
		Sensivity (%)	Specificity (%)	Positive (%)	Negative (%)
Age ≥ 73 years old	0.62	66	52	48	69
SBP ≤ 120 mmHg	0.58	70	50	47	73
C-reactive protein ≥ 31.4 mg/l	0.56	93	21	42	83
Sodium ≤ 137 mmol/l	0.59	95	23	45	87
High-sensitivity troponin T ≥ 0.044 ng/ml	0.64	79	45	49	76
Creatinine ≥ 1.15 mg/dl	0.58	70	44	46	68
NT-proBNP ≥ 2453 ng/l	0.67	51	75	58	69

Abbreviations: AUC – Area Under Curve; CI – confidence interval; other: see Table 1

Table 3. Univariate and multivariate Cox proportional hazard regression as a predictor of composite end-point)

Parameters	Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p
Age ≥ 73 years	1.49 (1.04-2.13)	0.028	1.89 (1.24-2.88)	**0.003**
Atrial fibrillation / flutter	1.65 (1.15-2.37)	0.007	-	**-**
Cancer	2.48 (1.24-4.96)	0.010	-	**-**
SBP ≤ 120 mmHg	1.52 (1.01-2.29)	0.044	1.65 (1.09-2.49)	**0.018**
NYHA class IV at admission	1.51 (1.05-2.17)	0.024	1.55 (1.06-2.25)	**0.023**
Right ventricle HF symptoms	1.62 (1.13-2.32)	0.009	2.72 (1.15-6.40)	**0.019**
C-reactive protein ≥ 31.4 mg/l	1.97 (1.25-3.10)	0.004	2.75 (1.16-6.53)	**0.022**
Sodium ≤ 137 mmol/l	1.97 (1.27-3.06)	0.003	3.42 (1.32-8.87)	**0.011**
High-sensitivity troponin T ≥ 0.044 ng/ml	2.0 (1.39-2.90)	< 0.001	1.67 (1.12-2.50)	**0.012**
Creatinine ≥ 1.15 mg/dl	1.47 (1.03-2.12)	0.039	-	**-**
NT-proBNP ≥ 2453 ng/l	2.25 (1.47-3.45)	< 0.001	3.21 (1.12-5.87)	**0.004**
Infection at hospitalization	2.74 (1.59-4.73)	< 0.001	-	**-**
Amine therapy	3.17 (1.54-6.50)	0.002	1.59 (1.10-2.29)	**0.014**
Mechanical ventilation during hospitalization	2.19 (1.11-4.31)	0.024	3.85 (1.35-11.03)	**0.012**

Abbreviations: HR – hazard ratio; other: see Table 1

Clinical characteristics and prognosis depending on age

Patients aged ≥ 65 years more frequently had a history of coronary artery disease (CAD) (72% vs. 45%, p < 0.001), and higher rate of revascularization (47% vs. 26%, p = 0.004); similarly, older patients more frequently had history of atrial fibrillation/flutter (39% vs. 24%, p = 0.040). Older patients who required longer hospitalization (median 8 vs. 7 days, p = 0.035) were characterized by lower hemoglobin and higher creatinine values and statistically significantly higher troponin levels (Table 4). It should be noted that the elderly group had a higher left ventricular ejection fraction (LVEF) and HF with LVEF > 40% was reported significantly more often (Table 4). Regarding the pharmacological treatment, the subgroup ≥ 65 years old was significantly less often treated with aldosterone antagonists (spironolactone and eplerenone) and more often with statins. Composite end-points were significantly more frequent in the elderly subgroup in comparison to younger: 65% vs 49% (p = 0.024), including a trend towards higher all-cause mortality (38% and 24% respectively, p = 0.057).

Table 4. Baseline clinical characteristics of the study patients in comparison between < 65 and ≥ 65-year old patients

Parameters	Age < 65 n = 68	Age ≥ 65 n = 135	p
Male, n (%)	55 (81)	89 (66)	0.033
Medical history			
Coronary artery disease, n (%)	30 (45)	96 (72)	**< 0.001**
Revascularization (PCI/CABG), n (%)	18 (26)	64 (47)	**0.004**
ICD (including CRT-D)	13 (19)	19 (14)	0.320
Atrial fibrillation/flutter, n (%)	16 (24)	52 (39)	**0.040**
Hypertension, n (%)	22 (32)	53 (39)	0.359
Diabetes mellitus, n (%)	13 (19)	33 (24)	0.478
Cancer, n (%)	2(9)	11(19)	0.331
Cause of hospitalization			
Infections, n (%)	23 (34)	51 (38)	0.644
Acute coronary syndrome, n (%)	1 (1)	7 (5)	0.272
Tachyarrythmias, n (%)	15 (22)	35 (26)	0.607
Unknown reason, n (%)	34 (50)	44 (33)	**0.022**
Other, n (%)	3 (4)	18 (13)	0.053
The length of hospitalisation (days)	7 (4-9)	8 (5-12)	**0.035**
Clinical and diagnostic parameters			
Resting heart rate (beats /min)	90 (75-115)	83 (70-110)	0.136
SBP (mmHg)	115 (106-130)	124 (115-135)	**0.007**
NYHA class IV at admission, n (%)	32 (47)	50 (37)	0.225
Right ventricle HF symptoms at admission, n (%)	20 (29)	52 (39)	0.217
Hemoglobin (g/dl)	14.2 (12.4-15.6)	13.4 (11.7-14.7)	**0.041**
C-reactive protein (mg/l)	7.9 (4.7-15.7)	6.9 (3.4-20.3)	0.360
Sodium (mmol/l)	140 (138-141)	140 (138-142)	0.076

Glucose (mg/dl)	113 (98-143)	120 (102-154)	0.221
High-sensitivity troponin T (ng/ml)	0.028 (0.018-0.05)	0.034 (0.019-0.069)	0.064
Creatinine (mg/dl)	0.95 (0.8-1.3)	1.1 (0.9-1.5)	**0.005**
NT-proBNP (ng/l)	3418 (863-7384)	4416 (1809-8557)	**0.027**
Echocardiographic parameters			
LVEF (%)	30 (20-39)	38 (27-50)	**0.002**
LVEF < 40, n (%)	51 (75)	72 (54)	**0.013**
LVEF 40-49, n (%)	8 (12)	27 (20)	
LVEF ≥ 50, n (%)	9 (13)	35 (26)	
Diastolic dysfunction 2/3°, n (%)	40 (80)	71 (72)	0.274
Left atrium diameter (cm)	5 (4.5-5.3)	4.9 (4.5-5.3)	0.166
Right ventricular systolic pressure increased, n (%)	59 (91)	120 (91)	1.000
In-hospital treatment			
Amine, n (%)	6 (9)	3 (2)	0.063
Mechanical ventilation, n (%)	7 (10)	4 (3)	**0.045**
Treatment at discharge			
ACE-inhibitor/ Sartans, n (%)	55 (83)	113 (85)	0.836
Spironolactone /Eplerenone, n (%)	41 (63)	56 (43)	**0.015**
Beta-adrenolytics, n (%)	59 (91)	119 (92)	0.784
Statins, n (%)	36 (55)	94 (73)	**0.016**
Diuretics, n (%)	62 (95)	121 (94)	0.754
Potassium, n (%)	41 (63)	96 (74)	0.132
Antiplatelet therapy, n (%)	18 (28)	41 (32)	0.622
NOAC, VKA, LMWH, n (%)	41 (63)	96 (74)	0.132
Digoxin, n (%)	11 (17)	18 (14)	0.670
Amiodarone, n (%)	8 (12)	15 (12)	1.000

Abbreviations: see Table 1

Discussion

Our single-center retrospective study analyzed risk factors for poor prognosis in HF with particular attention to the elderly population. Over the years, many important risk factors for worsened HF prognosis have been reported, including clinical, laboratory and echocardiographic parameters [17-19]. Among the well-known clinical parameters: age, male sex, high NYHA class, low SBP, arrhythmias with rapid ventricular rate, infections, and the presence of comorbidities (e.g. ischemic heart disease, diabetes, renal failure, pulmonary disease, cancer) are the essential risk factors of poor prognosis [20-23]. The results of our study are congruent with this data: age ≥ 73 years old, SBP at discharge ≤ 120 mmHg, NYHA class IV at admission, symptoms of right ventricle HF, infection as the cause of initial hospitalization and concomitant diseases (cancer or atrial fibrillation/flutter) were significant predictors of composite end-points (all-cause mortality and rehospitalizations due to emergency reasons) in the two-year follow-up period. The hospitalization time was significantly longer for patients with the end-points in the observation period (Table 1), which is in agreement with the previous data from the literature [6]. Mechanical ventilation or catecholamine therapy, which according to our data was used more frequently in patients with the end-points, became significant risk factors for poor prognosis in both univariate and multivariate Cox analysis. Among the laboratory parameters, the most robust predictors according to the literature are renal function, hyponatremia, increased troponins, and low hemoglobin [18-19, 24-25]. Our results confirm this data (Table 3).

The specific characteristics of the elderly population

It is well-known that elderly patients often present with complex comorbidities (hypertension, CAD, atrial fibrillation, peripheral vascular disease, kidney failure, or anemia) compared to the younger population [5, 26]. We confirmed this observation in our study (Table 4). Epidemiological data shows that CAD is the dominant comorbidity and the HF's main etiological unit [7]. In the presented project, almost 3 out of 4 patients from the elderly subgroup had CAD diagnosis. Additionally, these patients underwent revascularization more frequently, which is in agreement with the general trend observed in cardiology: the elderly may benefit more from invasive treatment, despite a higher periprocedural risk [27-28]. The general percentage of revascularized patients is surprisingly low in the study group with the diagnosis of CAD, although not every CAD patient requires invasive treatment. What is more, that could reflect the real condition of invasive treatment in a district hospital. The second most common comorbidity associated with HF, especially in the elderly, is atrial fibrillation. Data from the literature and large registries (EORP-AF, Framingham Heart Study [29-30]) confirm the increased rate of this arrhythmia with the aging of the population and the occurrence of HF exacerbations in the elderly [29, 31-32]. We also observed this correlation in our study: the number of patients with a history of atrial fibrillation was almost twice as high in the subgroup ≥ 65 years old, compared to subgroup < 65 years old: 39% vs. 24% (p = 0.040). According to the literature, arterial hypertension is another comorbidity increasing the number of hospitalizations in the elderly [17, 20] and although in our study these differences were not statistically significant, its incidence was higher (Table 4).

In the senior population laboratory tests typically show deterioration of kidney function and anemia [20, 24], both of which were noted in our study (Table 4). Particular attention should be paid to the role of the assessment of natriuretic peptides in older patients hospitalized due to exacerbation of HF. It is known that NT-proBNP predicts mortality among the older and very elderly patients with chronic HF [33]. In the elderly population with HF, the higher cut-off value for NT-proBNP should be considered. In our study, it was 2453 ng/l for the composite end-points prediction (Table 2), while in the study by Vergaro et al. for the prognosis of the annual death of people aged ≥ 77 years old, it was as high as 4188 ng / l [34].

During the last twenty years, it was established that a significant percentage of patients with HF, especially the elderly, have preserved left ventricular systolic function [5, 21, 26, 35]. The latest HF guidelines published by the European Society of Cardiology [8] distinguish HF with LVEF that is significantly reduced (< 40%), moderately reduced (LVEF 40-49%) and preserved (≥ 50%). A fascinating result of the presented study is a statistically significant higher incidence of patients with LVEF of ≥ 40% in the subgroup of older adults. Moreover, the median LVEF value in these patients was also higher (Table 4). It is of note that the diameters of the left atrium did not differ statistically between the groups, which could explain that the enlargement of the left atrium is not only consequence of the diastolic but systolic dysfunction as well.

Comparison of post-hospital recommendations according to the age

Data from the literature shows that older patients with HF are characterized by worse pharmacotherapy compared to younger patients [36]. This may be explained by numerous comorbidities and complications resulting from the use of certain groups of drugs or insufficient compliance [37]. The results of our single-center study present a therapy that can be considered optimal in comparison to the current guidelines [8, 38], because the percentage of people ≥ 65 years of age who use beta-blockers, ACE inhibitors/sartans remains high, which is consistent with the data from the Polish arm of the Heart Failure Pilot Survey

conducted in 26 Polish cardiological centers [12]. The exception is the percentage of patients using mineralocorticoid antagonists, which in the for the subgroup ≥ 65 years of age is 43%. This group is half the size of the subgroup of patients < 65 years of age who were taking mineralocorticoid antagonists. It can be associated with a more frequent occurrence of comorbidities, including chronic renal disease which can be exacerbated by these medications. This trend of lower adherence to current guidelines in the geriatric population, resulting from poorer renal function, is also confirmed by data from the literature [28, 37]. There are encouraging reports of a slight improvement in prognosis in this challenging group of patients taking spironolactone at GFR > 30 ml/min/1.73m2 [39], which may be an essential therapeutic direction in the future. Our results indicate an interesting trend towards greater use of anticoagulant and antiplatelet therapy in the elderly (Table 4), which is most likely due to both a higher proportion of CAD and atrial fibrillation in this subgroup. The above data is in agreement with the results from the European registers [29].

It should be noted that the percentage of patients enrolled in the study with implantable cardioverter-defibrillators (including cardiac resynchronization therapy) is disproportionately small concerning the number of patients with LVEF < 40 % and even lower in the elderly patients (Table 4). We precisely analyzed and discussed this problem in our previous study [15]. It also seems interesting that the subgroup with the composite end-points, was characterized by a higher rate of implanted ICD/CRT. This situation can be explained by the fact, that patients with implanted ICD and CRT devices usually have a worse prognosis. In addition, these devices protect mainly against life-threatening arrhythmic events and not against the other causes of cardiac death.

The prognosis of HF patients according to age

The latest data from the literature suggests that the overall mortality in the senior population is decreasing: the age-standardized death rate has decreased by 40%, and the mean age of death due to HF has increased from 80.0 to 82.7 years in seven European countries during the last two decades [37]. The survival of HF patients is low, amounting to 17% in the first year after diagnosis and approximately 40–50% in 5 years, which is a worse result than in the case of many cancers [14]. Data from the literature on patients with HF in the geriatric population shows that their mortality is slightly over 20% within one year of diagnosis [12, 37], increasing to 36.7% after two years [37]. In our study, the 2-year mortality in patients ≥ 65 years old was similar and amounted to 38%, which confirms the above-mentioned European trend of improving mortality in the geriatric population. However, this percentage is still higher than in the younger population.

As we mentioned in the introduction, hospitalizations due to HF exacerbation are the leading cause of hospital admissions, particularly in patients > 65 years old [5]. According to the ESC-HF Pilot registries, the general population's rehospitalization rates within one year amounted to 43.9% [14]. Korean studies conducted in patients > 65 years hospitalized for HF within 30 days showed a 34.6% rehospitalization rate [40]. Even more alarming results are reported by Tuppin et al. [22], who collected information from the national health insurance information system about hospitalizations in France in 2009, where the 2-year all-cause readmission rate was 69%. In our 2-year observation, as many as 65% of seniors experienced death or rehospitalization due to emergency cases, which was significantly greater than in the younger subgroup. That results seem to confirm that more extensive studies based on the elderly population are needed.

Study limitations

Our study has several limitations. First of all, it was a single-center, retrospective analysis and our results should thus be interpreted with caution. The study group is small and included only patients with HF exacerbation as the main diagnosis. In addition, some clinical data was not available in the documentation (e.g. the assessment of hypercholesterolemia, the width of the QRS complex, pre-admission drugs, smoking), hence the statistical evaluation of our data may raise some doubts.

Conclusions

Simple clinical parameters (e.g. age, systolic blood pressure, NYHA class IV, right ventricle HF symptoms, increased natriuretic peptide, C-reactive protein, troponin, hyponatremia) as well as catecholamine therapy and mechanical ventilation during hospitalization due to HF exacerbation, are the substantial, independent risk factors for poor prognosis. The elderly patients are characterized by more comorbidities, worse laboratory results, higher HF frequency with moderately reduced LVEF and worse prognosis. Our pilot study could help in decision-making on the intensification of outpatient and inpatient treatment to increase its effectiveness. In turn, this could translate into a reduction of the frequent end-point events, a reduction in the frequency of hospitalizations, improved prognosis and measurable economic benefits.

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RESERACH ARTICLE

Multiplexed Immunobead-Based Cytokine Profiling in patients with ovarian cancer

Aleksandra Wyciszkiewicz[1], Michał Lach[2], Alicja Kalinowska[1], Sławomir Michalak[1], Błażej Nowakowski[3]

[1] Department of Neurology, Division of Neurochemistry and Neuropathology, Poznań University of Medical Sciences, Poland
[2] Radiobiology Laboratory, Greater Poland Cancer Centre, Poznań, Poland
[3] Surgical, Oncology and Endoscopic Gynecology Department, Greater Poland Cancer Centre, Poznań, Poland

Abstract

Due to late diagnosis, ovarian cancer is the most deadly gynecologic malignancy. Inflammation is one of the risk factors of ovarian cancer and the inflammatory response implicates all stages of tumorigenesis. The purpose of this study was to analyze the concentration of molecules, which can take part in malignant processes. We analyzed patients with ovarian cancer, with endometriosis and healthy controls. Thirty-seven analytes were measured in serum using BioPlex Pro Human Inflammation Panel. We were able to detect 28 of the proteins among the studied groups. We found a significant increase in 22 of the tested molecules (BAFF, Chitinase3-like 1, IFN-alpha2, IFN-beta, IFN-gamma, IFN-lambda2, IFN-lambda1, gp130, IL-2, IL-12 (p40), IL-11, IL-32, IL-35, MMP3, Osteocalcin, Pantraxin-3, sCD163, TNFRSF8, sIL-6Ralpha, STNF-R1, STNF-R2, and TSLP) in the ovarian cancer group in comparison to the healthy controls. Two of them (IL-20, MMP1) did not show significant differences between groups. Moreover, we identified decreased concentrations of APRIL and osteopontin in ovarian cancer vs. healthy controls. While this study is a preliminary report, we hope this will encourage a further use of multiplex analysis in ovarian cancer biomarker research.

Keywords: ovarian cancer · cytokines · metalloproteinases · inflammation markers · endometriosis

Citation

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Corresponding author:
Aleksandra Wyciszkiewicz, Department of Neurology, Division of Neurochemistry and Neuropathology, Poznań, Poland
e-mail: aleksandra.wyciszkiewicz@gmail.com

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Introduction

Ovarian cancer (OvCa) is often an asymptomatic disease, without reliable specific biomarkers indicating the early forms of developing cancer, leading to clinical diagnosis at an advanced stage with metastases. For these reasons, it represents the third most common gynecologic malignancy and the fifth cause of death among cancers in women [1].

To date, only the serum cancer antigen 125 (CA-125) and human epididymis secretory protein E4 (HE4/WFDC2) are used widely in OvCa diagnosis. However, it has limited specificity and sensitivity. At an early stage of the disease, the detection of CA-125 is not always possible. Moreover, the elevated level of CA-125 is also observed during endometriosis and ovarian cysts. 50% of patients with stage I OvCa, where the disease is limited to the ovaries, have a normal preoperative CA125 level [2-4].

Another marker, namely HE4, has similar limitations in detecting early and asymptomatic cancers [5-6] and, as our team in concordance with others have reported previously, it has no added diagnostic value over ultrasound examination [7]. Therefore, two algorithms, such as Risk of Malignancy Index (RMI) and Risk of Ovarian Malignancy Algorithm (ROMA), were used to enhance the essential character of these biomarkers. The RMI was proposed by Jacobs et al. [8]. It is a validated clinical algorithm used for the risk stratification of OvCa lesions. In comparison to CA-125, RMI combines three features: serum CA-125, menopausal status, and ultrasound score. This enables to obtain higher sensitivity and specificity in detecting malignant cases than using HE4 or CA-125 alone [9]. Another clinical tool is ROMA, which was developed by combining CA-125 and HE4 serum levels with patients' menopausal status. Based on the ROMA score, a woman can be classed according to their risk of malignancy level, i.e. low or high.

Over recent decades, researchers have been evaluating additional serum proteins as potential biomarker candidates, which could support the early diagnosis of OvCa. The panels of biomarkers seem to be more accurate and provide the elevated sensitivity and specificity necessary for screening [10].

Inflammation is one of the hallmarks of many cancers, including ovarian cancer. Identification of specific serum markers related to this process could be a useful tool for screening patients with increased risk of malignant disease [11]. The local inflammatory response could be a double edge sword. On the one hand, cytokines can inflect an anti-tumoral response but on the other hand, chronic inflammation could also lead to malignancy. This phenomenon is related to the balance of pro-and anti-inflammatory cytokines and the activation state of the surrounding cells [12]. In this study, we aimed to identify serum concentration of the most common cytokines, which could be involved in malignant processes, and pinpoint the potential involvement in ovarian cancer pathobiology. Specifically, this study aimed to evaluate the screening panel of the inflammatory cytokines.

We hypothesized that the inclusion of inflammatory or immunosuppressive biomarkers present in serum may enhance or complement the identification of factors supporting ovarian cancer progression and early diagnosis aside from the current diagnostic methods including CA-125, HE4, and ROMA, and RMI. Moreover, the research on the milieu of cytokines and chemokines in patients with ovarian tumors can support the insight into pathomechanisms of such a complication as hypercoagulability.

Material and Methods

Study Group

The study involved the analysis of serum obtained from 24 subjects: 14 women diagnosed with distinct stages of ovarian cancer, 6 women with endometriosis, and 4 healthy women. Detailed patient characteristics are shown in Table 1. The serum samples were collected from the Surgical, Oncological, and Endoscopic Gynecology Department of The Greater Poland Cancer. Approximately 9 mL of blood was collected into EDTA plasma tubes (Sarstedt Ltd, Leicester UK) and then centrifuged at 1200×g at 4°C for 30 minutes to obtain serum. The serum samples were then stored at –80°C until analysis. The Bioethics Committee of the Poznań University of Medical Sciences approved the study protocol (decision no. 784/13 and 1126/16). Written informed consent was obtained from all the participants. All methods were performed following the relevant guidelines and regulations. The following exclusion criteria were defined in this study: another neoplastic disease, a history of any autoim-

Table 1: Study population including detailed diagnosis

Diagnosis	Grade	Patients, N
Ovarian Cancer	IA	4
	II B G3	1
	III C G3	5
	IVB G3	4
Endometriosis	bilateral ovarian cyst	6
Healthy volunteers	-	4

mune condition, treatment with immunomodulatory drugs, clinical manifestation or elevated markers of inflammation.

Analysis

The composition of cytokines was analysed using Bio-Plex 200 System (Bio-Rad Laboratories, Hercules CA, USA). This System allowed for the simultaneous analysis of 37 different molecules in a single well. The assay contained dyed beads conjugated with monoclonal antibodies specific for a target protein. In this study, we used Bio-Plex Pro Human Inflammation 37-plex (Bio-Rad Laboratories, Hercules CA, USA). The 50 ul of serum of each patient was used. The concentrations (pg/ml) of the analysed molecules are measured against the standard curve. The analysis was performed according to the manufacturer's instructions.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.01 (GraphPad Software, CA, USA). For the statistical analysis, the non-parametric Kruskal-Wallis test with Dunn's post hoc multiple comparison test was performed. The differences were considered statistically significant at $p < 0.05$—*; $p \leq 0.01$—**; $p \leq 0.001$—***; $p \leq 0.0001$—****.

Results

On the first view, the concentration pattern of analysed molecules in OvCa was distinct from the healthy volunteers. We found a significant difference among 22 tested molecules. The differences were also observed between patients with endometriosis and the healthy controls. The mean concentrations of detected analytes are presented in Table 2.

We found that the concentration of 22 analytes was significantly increased in patients with OvCa compared to the healthy controls, namely BAFF, Chitinase3-like 1, IFN-alpha2, IFN-beta, IFN-gamma, IFN-lambda2, IFN-lambda1, gp130, IL-2, IL-12 (p40), IL-11, IL-32, IL-35, MMP3, Osteocalcin, Pantraxin-3, sCD163, TNFRSF8, sIL-6Ralpha, STNF-R1, STNF-R2, and TSLP (see Figure 1 and Figure 3) In the endometriosis group, the concentration of 17 analytes was significantly higher than in the healthy controls group (see Figure 1, Figure 3, Table 2). On the other hand, both ovarian and endometriosis patients are characterized by decreased concentration of APRIL and osteopontin (see Figure 2 and Figure 3, Table 2).

The following proteins were all largely undetectable in sera of the studied groups: IL-12(p70), IL-19, IL-22, IL-27, IL-34, and TNFSF14. These analytes were omitted from further analysis.

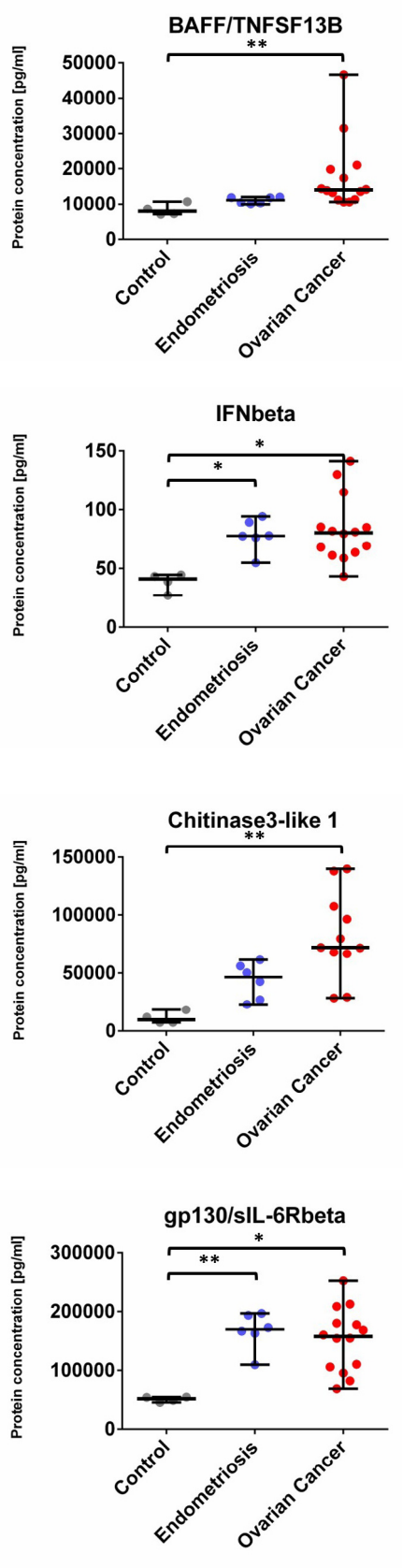
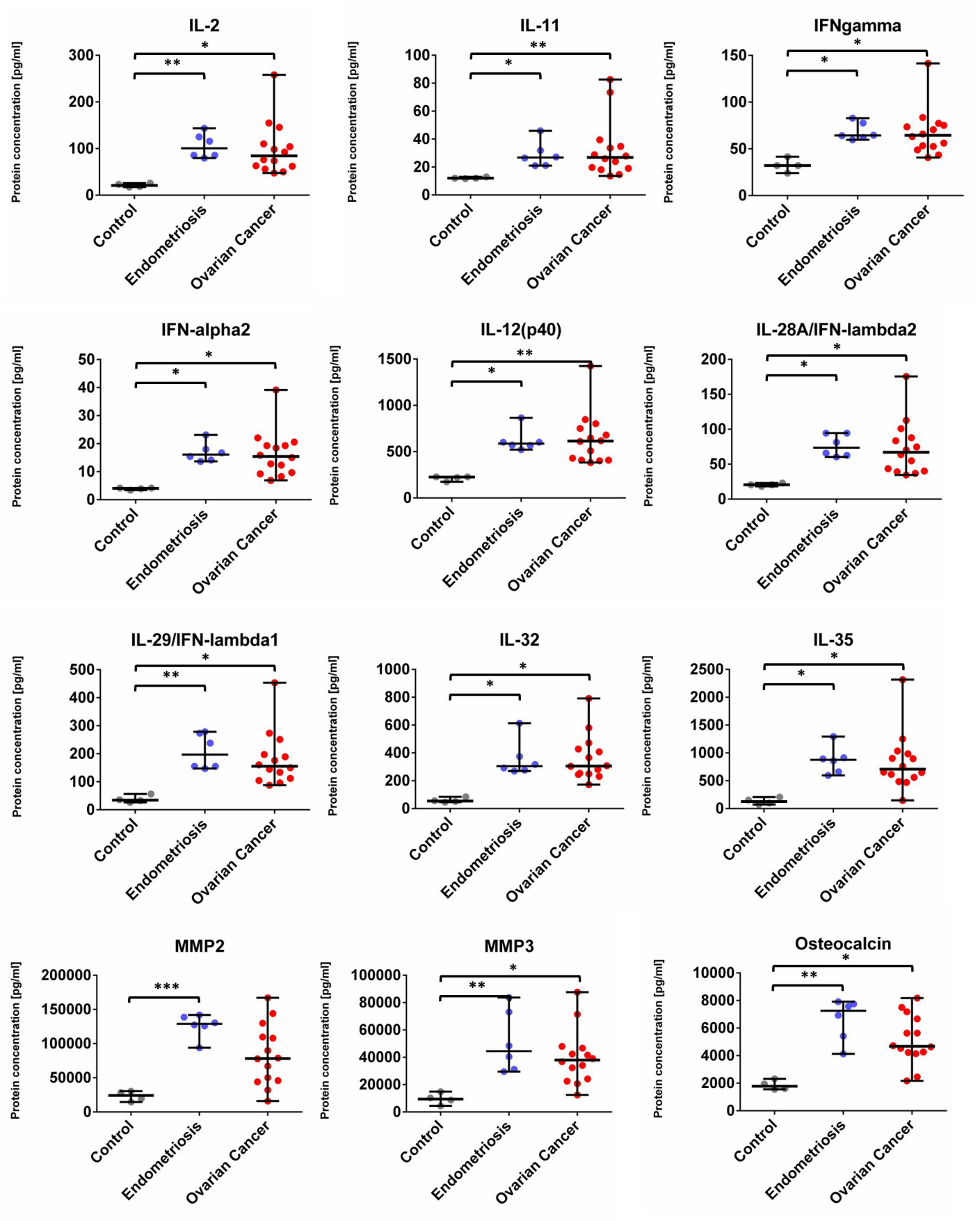


Figure 1. Dot plots (medians and interquartile range, IQR = Q1 – Q3) presenting analytes concentrations (pg/mL) within serum samples collected from patients with ovarian cancer, endometriosis, and healthy controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: based on Kruskal–Wallis test with Dunn's post hoc multiple comparison test.



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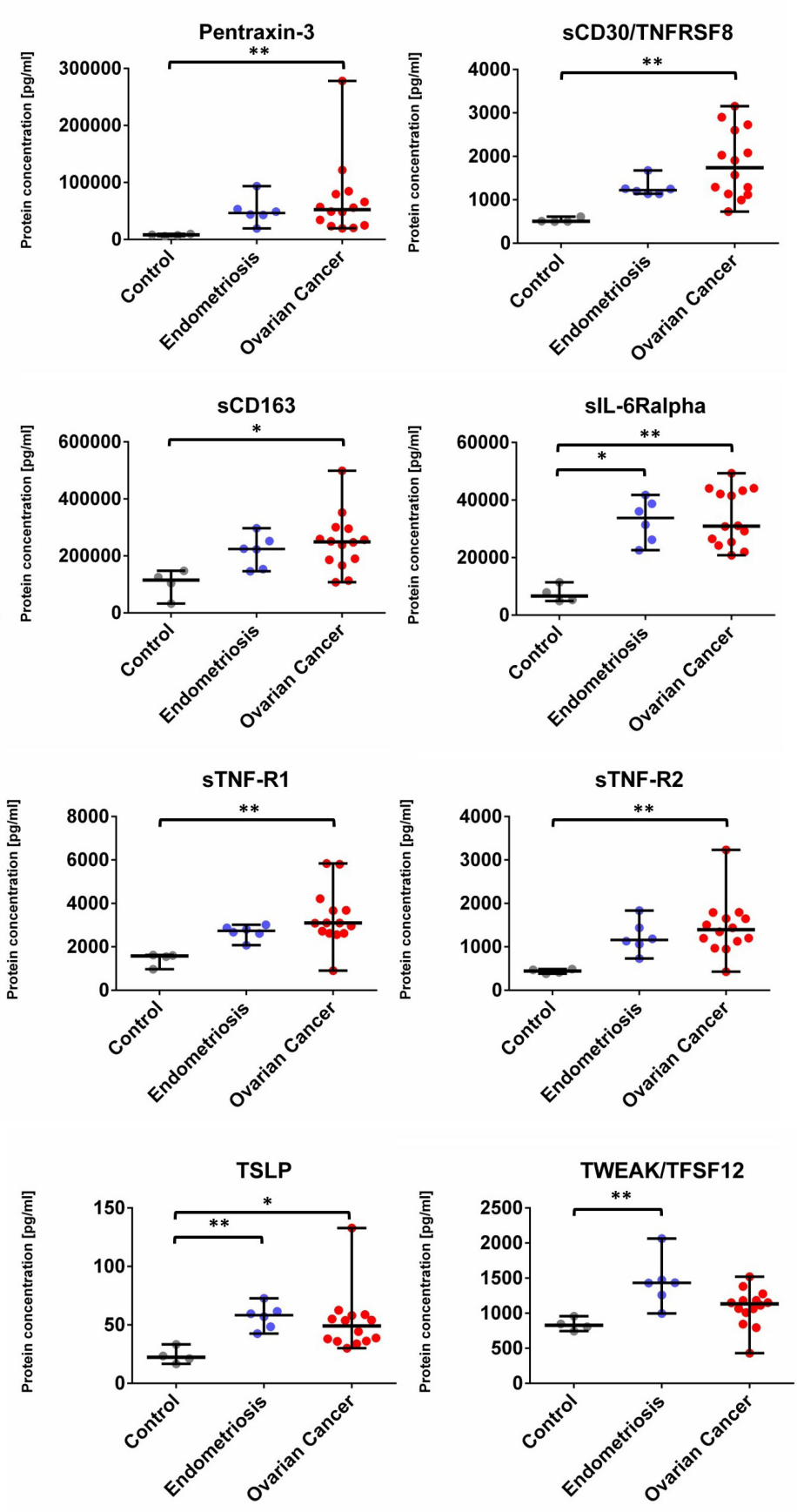

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Discussion

OvCa is typically diagnosed at the advanced stages of the disease, therefore successful treatment challenging. The survival of patients with stages I and II of OvCa ranges from 60% to 90% shows the higher success rate of treatment depends on earlier detection of the disease. Therefore, the development and/or identification of an assay or finding an appropriate biomarker could bring significant benefits to OvCa patients. In this small, preliminary and comparative study, we conducted a multiple, quantitative analysis of 37 cytokines, chemokines, and MMPs in OvCa patients and endometriosis patients compared to the healthy volunteers. Our research confirmed that serum concentrations of analysed molecules varied between the studied groups.

Ovarian Cancer vs. Healthy Control

We divided the results into two distinct patterns of differences in the mean concentrations of specific proteins in patients with OvCa. Specifically, BAFF, Chitinase3-like 1, IFN-alpha2, IFN-beta, IFN-gamma, IFN-lambda2, IFN-lambda1, gp130, IL-2, IL-12 (p40), IL-11, IL-32, IL-35, MMP3, Osteocalcin, Pantraxin-3, sCD163, TNFRSF8, sIL-6Ralpha, STNF-R1, STNF-R2, and TSLP were all increased in OvCa patients, whereas APRIL and osteopontin concentrations were found to be decreased in OvCa as compared with the healthy controls.

We are aware that these differences in the cytokine profiles may be due to the secretion by malignant or non-malignant types of cells i.e. immune or endothelial cells, which could influ-

ence immune-mediated anti-tumor responses. Our present results are following other studies of ovarian cancers. However, to our knowledge, not all the 22 molecules have so far been analyzed and published, including single-cytokines analysis and multiplex analysis.

Consistently with our findings, significantly elevated mean concentrations of the Tumor Necrosis Factor Superfamily (TNFSF) cytokines, namely TNFSF8 (sCD30) and soluble Tumor Necrosis Factor Receptor-1 (sTNFR1) and Receptor-2 (sTNFR2), were demonstrated by Mielczarek-Palacz et al. [13-14] and Dobrzycka et al. [15]. These proteins are responsible for the regulation of apoptosis and immune response. The above-mentioned studies suggested that these molecules could be used as early diagnostic indicators of OvCa [13-15]. Moreover, sTNFR1 and sTNFR2 are also independently related to poor prognosis [16]. Also, we found the elevated level of another member of the TNFSF family: Tumor necrosis factor-like, a weak inducer of apoptosis (TWEAK/TNFSF12). Similar data have only been published regarding prostate cancer [17] or colorectal cancer [18]. Although TWEAK was firstly identified as an inducer of apoptosis in tumor cells, it can also stimulate cell proliferation, angiogenesis, and inflammation [19-20].

Another interesting finding is the decrease of a proliferation-inducing ligand (APRIL), also known as TNFSF13, which is primarily involved in B-lymphocyte maturation, in sera OvCa patients compared to the healthy controls. According to recent studies, this cytokine is highly expressed in several tumor tissues, including breast cancer, which stimulates the growth of tumor cells [21-22]. However, we observed the opposite results, with the concentrations decreased in both, ovarian patients and endometriosis patients, compared to the healthy controls. Moreover, the retrospective studies indicated that the role of APRIL protein in ovarian, bladder, and head and neck carcinoma was not associated with the promotion of tumor development and its presence was not related to autocrine production by malignant cells, but rather derived from APRIL-producing neutrophils [23]. However, the role of neutrophils in OvCa is probably crucial in cancer progression and treatment, which was shown by the comparison of neutrophil to lymphocytes ratio (NLR), indicating the better outcome and survival of OvCa patients with decreased NLR [24]. Neutrophil extracellular traps (NETs) and their interplay with platelets contribute to paraneoplastic thrombophilia [25] thus the analysis of factors that orchestrate the dynamic interplay between tumor cells, immune system, and hemostasis seems to be crucial for monitoring and treatment decisions in OvCa patients.

The second molecule with a significantly lower level in both groups (OvCa and endometriosis vs. healthy control) was osteopontin. However, these findings are in contrast with our previous study where the concentration of osteopontin in serum was higher in OvCa patients than in benign OvCa [26], similar data showed also a team by Schorge [27]. One of the reasons for these differences might be the limited sample size in our study.

Chitinase-3-like-1 (YKL-40) is mainly responsible for tissue injury and repair,and inflammation [28]. Our results are consistent with others, which indicated the elevated level of YKL-40 in serum in patients with metastatic disease, and poor diagnosis in OvCa. High expression of YKL-40 is strongly associated with a high FIGO stage and histological type of tumor [29-30].

In our study, the concentration of two receptors of interleukin 6: siL-6R beta (Gp130) and sIL-6R alpha were also increased in ovarian patients. Both soluble receptors can bind to IL-6 and serve as the major IL-6 signalling pathways including cancer, thereby contributing to cancer progression [31-32].

The vitamin K-dependent protein, osteocalcin is a specific product of the osteoblast and is a biomarker for bone formation activity [33]. Previous studies have shown that osteocalcin is highly expressed in solid tumors, including osteosarcoma [34], breast [35], and prostate cancers [36-37]. Osteocalcin was also correlated with cancer cell transformation [38]. Our findings confirmed the elevated levels of osteocalcin in OvCa.

In terms of the metalloproteases (MMPs) family, we identified increase expression levels of two members: MMP2 and MMP3. According to previous studies, higher levels of MMP2 were significantly higher in advanced stages of OvCa compare to benign premalignant counterparts [39]. Also, it may contribute to the poor prognosis of OvCa patients [40]. Another reported role of MMP2 in OvCa is the involvement in the adhesion of OvCa cells to the peritoneal surface [41]. There are also studies showing no correlation between tumor-derived MMP2 and survival rate [42-43]. MMP3 as another member of the metalloproteases family together with miR200 can modulate OvCa invasiveness. It has been shown that overexpression of MMP3 can decrease the ability of miR200 to inhibit OvCa invasion [44]. The higher expression level of MMP3 in OvCa was also positively correlated with a poor survival rate [45-46].

In our analysis, interleukin 32 (IL-32) was overexpressed. The same findings were reported in previous studies including solid tumors, but not OvCa. As a pro-inflammatory cytokine, IL-32 is involved in the progression of different malignancies, including gastric [47], breast [48], and lung cancers [48]. IL-32 together with nuclear factor-κB (NF-κB)-mediated cytokines and metalloproteinase production support the tumor development [49-50]. In a study presented by Luo et. al. [51] on 147 patients and 337 healthy controls using the polymerase chain reaction-restriction fragment length polymorphism (RLPF), the association between IL-32 single nucleotide polymorphism (SNP) and the OvCa was found. This SNP in IL-32 gene indicates higher OvCa susceptibility and may play a crucial role as a progression marker.

Given its suppressive function, interleukin 35 (IL-35) is involved in tumor progression [52] by enhancing angiogenesis [53] and inhibition of CD8+ T cells via transforming growth factor-beta 1 (TGF-β) production [54]. This data supports our results, where we observed higher IL-35 levels in OvCa group compared to the healthy.

Table 2. The concentrations of detected analytes [pg/mL] in the studied groups. IQR= interquartile range.

Analyte	Helathy control (median, IQR, pg/mL)	Endometriosis (median, IQR, pg/mL)	Ovarian cancer (median, IQR, pg/mL)
APRIL	64 698 58 202 - 76 189	31 423 28 444 - 36 890	32 377 28 568 - 39 847
BAFF/TNFSF13B	7 955 7 087-10 230	11 133 10 148 - 11 937	14 030 11 332 - 20 115
Chitinase3-like1	9 634 7 303 - 16 726	46 408 25 976 - 57 424	72 086 66 733 - 107 383
Gp130/siL-6Rbeta	52 140 47 047 - 55 007	170 220 150 184 - 194 390	158 059 103 403 - 187 421
IFN alpha2	4.075 3.56 - 4.2	16.10 14.03 - 19.30	15.45 9.555 - 19.63
IFN beta	40.94 29.99 - 44.18	77.73 70.95 - 90.52	80.29 63.03 - 92,78
IFN gamma	32.08 26.09 - 39.23	64.28 61.67 - 78.99	64.50 51,69 - 75.73
IL-2	20.99 17.58 - 25.32	100.7 84.10 - 129.6	84.38 60.54 - 118.9
IL-11	12.01 11.76 - 12.75	26.82 21.07 - 35.38	26.90 18.82 - 36
IL-12 (p40)	225.1 186.2 - 228.7	586.6 557.0 - 667.4	614.4 408.7 - 763.3
IL-20	22.60 20.75 - 66.02	82.74 70.77 - 98.34	77.28 60.86 - 101.6
IL-28A (IFN-lambda2)	20.65 18.87 - 22.41	73.66 62.11 - 94.40	67.07 39.99 - 91.14
IL-29 (IFN-lambda1)	34.34 28.20 - 51.36	196.8 153.3 - 274.9	155.3 110.4 - 210.6
IL-32	53.49 47.51 - 77.17	304.6 274.9 - 434.5	305.8 248.4 - 439.3
IL-35	126.0 80.31 - 193.2	875.5 644.0 - 1009	707.6 542.2 - 997.0
MMP1	2 691 1 329 - 3 466	2 824 1 196 - 5 323	2 911 2143 - 6 463
MMP2	24 045 15 909 - 29 867	128 969 117 954 - 139 570	78 324 45 385 - 114 745
MMP3	9 498 5 551 - 13 727	44 464 30 828 - 75 763	37 928 23 829 - 47 008

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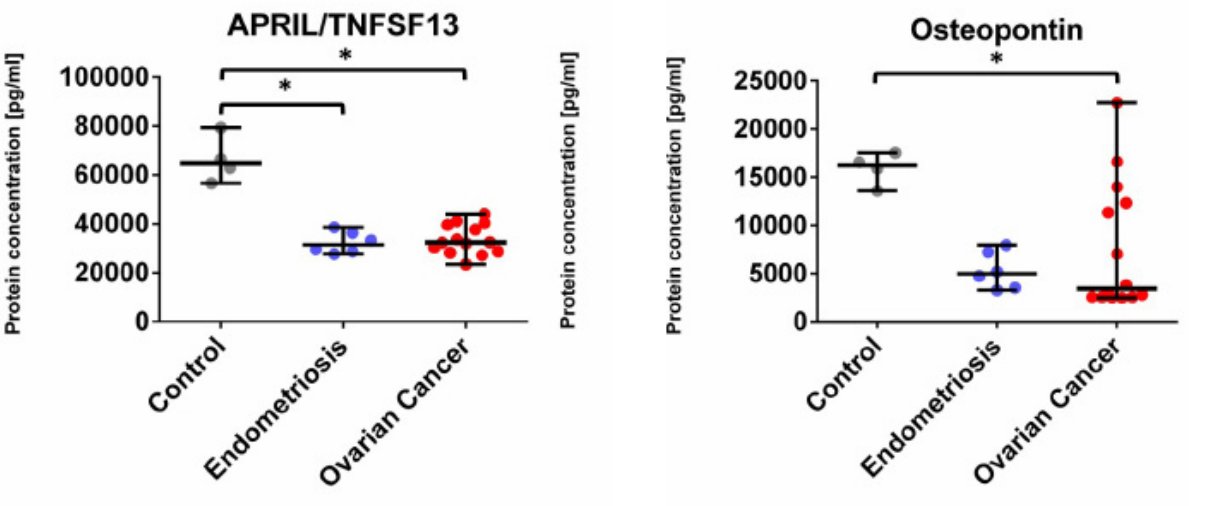
Analyte	Helathy control (median, IQR, pg/mL)	Endometriosis (median, IQR, pg/mL)	Ovarian cancer (median, IQR, pg/mL)
Pentraxin-3	8 017 6 251 - 9 505	46 450 37 184 - 63 533	52 447 24 567 - 80 868
Osteocalcin	1 778 1 566 - 2 225	7 254 5 100 - 7 793	4 677 4 207- 6 802
Ostepontin	16 234 14 170 - 17 291	4 985 3 465 - 7 433	3 418 2 550 -12 756
sCD30/TNFRSF8	504.8 499.1 - 584.6	1 223 1 138 - 1 360	1 739 1 131- 2 634
sCD163	115 060 50 613 - 142 274	224 350 151 750 - 263 420	249 445 81 542 - 297 032
sIL-6R alpha	6 614 5 033 - 10 546	33 759 25 329 - 39 500	30 934 25 115 - 43 489
sTNF-R1	1 573 1 112 - 1 620	2 739 2 483 - 2 911	3 094 2 625 - 3 816
sTNF-R2	441.8 388.2 - 483,5	1 159 979.8 - 1 536	1 394 1 088 - 1 687
TSLP	22.35 17.81 - 30.84	58.35 46.98 - 64.39	49.07 36.15 - 58.29
TWEAK/TFSF12	827.3 760.9 - 928.9	1 432 1 194 - 1 621	1 131 968.2 - 1 206



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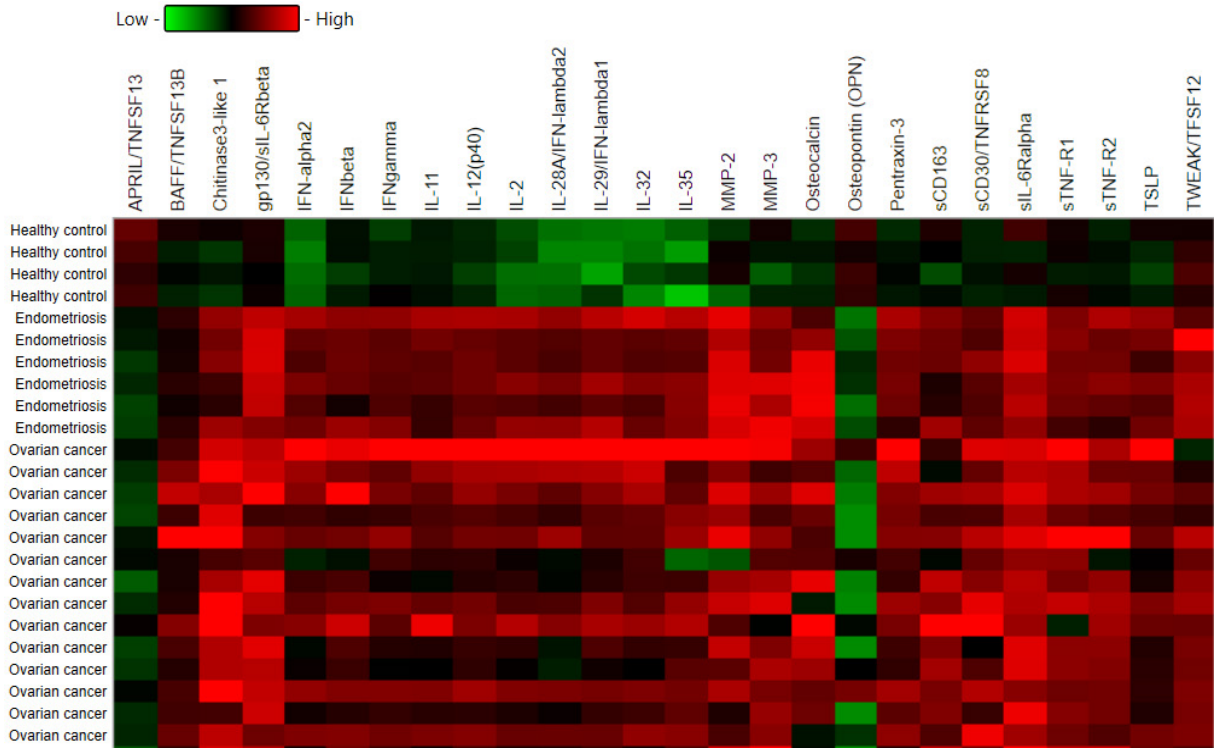

Figure 3. Heatmap representing a concentration of all analyzed molecules in patients with ovarian cancer and endometriosis group compared to healthy controls.

Interleukin-12 subunit beta (IL-12p40), which we found to be significantly overexpressed, is known as a component of the bioactive cytokines IL-12 and IL-23. No other studies have shown the presence of IL-12p40 in OvCa.

Interleukin-2 (IL-2) as a T-cell growth factor has an essential role in T cell-dependent immunity. Because of its properties, IL-2 is used for cancer therapy [55]. However, a study by Bosek et. al. [56] indicated the elevated level of IL-2 in patients with colon cancer and type 2 diabetes [56]. One of the functions of IL-2 is increased proliferation and activation of Treg Lymphocytes, which according to the study by Bosek et al. [56] could be associated with tumor progression. This may be the case in our study, as well, as we observed a significantly higher concentration of IL-2 in the OvCa group compared to the healthy controls. This was also shown in the Dutch-Wicherek group, where increased T-regulatory lymphocyte levels in peripheral blood were correlated with poorer prognosis in a serous ovarian adenocarcinoma [57].

Interferons (IFNs) are mostly described regarding viral infections. The main function of type I IFNs (IFNα, IFNβ) is the stimulation of the immune system, namely the control of dendritic cell maturation, growth of granzyme, and perforin expression in cytotoxic T-lymphocytes, which makes these cytokines essential in cancer immunosurveillance [58]. Recently, we have shown that the expression of granzyme in peripheral blood mononuclear cells (PBMC) was higher in ovarian cancer patients than in lung cancer patients [59]. Moreover, PBMCs granzyme expression was upregulated in patients with onconeural antibodies than in seronegative persons [59]. Such a phenomenon indicates the role of cytotoxicity on paraneoplastic neurological syndromes.

Type I interferons (IFNs) —IFNα and IFNβ — have been widely used for the treatment of several cancers [58]. However, IFNs are also known as inflammatory factors within the tumor microenvironment [60-61]. In our study, we observed a significant increase in both, type I and type II interferons. Interferon concentrations in OvCa and endometriosis groups are similar, which suggests that either this is a consequence of immune response to inflammation and cancer progression, or that immune response was damaged, and the high concentration could be an effect of this damage.

Endometriosis vs. Healthy Control

Aberrant production and secretion of immune mediators, including cytokines, prostaglandins, and metalloproteinases, are also observed in endometriosis patients. In our study, we analysed 6 patients with endometriosis, and we found a significant increase in 17 analytes compared to the healthy controls (Table 2 and Figure 1). Interestingly, there were three proteins (MMP2, Osteocalcin, and TWEAK), which levels were increased in the endometriosis group

compared to OvCa and the healthy group, however, these differences were non-significant.

The recent studies suggest that the use of single cytokines or only traditional proinflammatory molecules could underappreciate the potential benefit of using cytokines to identify patterns of response [62]. Enzyme-linked immunosorbent assay (ELISA) is one of the popular methods in the analysis of cytokines. However, to analyse a wide range of cytokines, chemokines, and MMPs simultaneously is expensive, time-consuming, and requires a large sample volume. Therefore, in our study, we decided to use multiplex analysis of serum samples. While this study is a preliminary report, we hope this will encourage further use of multiplex analysis in ovarian cancer biomarker research.

OvCa is a complicated and heterogeneous malignancy, with multiple histological subtypes, which still determine a challenge in diagnosis. We need to acknowledge the small sample size of our study groups, which limits the ability to accurately examine the relationships between the stage of cancer and cytokine levels. Further research will require an increase in the study sample.

In conclusion, our results suggest increased levels of analytes (cytokines, chemokines, MMPs, growth factors) in serum, most spectacularly in patients with ovarian cancer, but also in patients with endometriosis compared to the healthy controls. Importantly, a similarity between the two groups of distinct pathologies emphasizes diagnostic difficulties. More studies about the use of cytokines as biomarkers are crucial for getting the essential information about changes in signalling networks which may help to understand the tumorigenesis, complications related to cancer-induced thrombophilia, and immune-mediated paraneoplastic syndromes. Although this study was not powered to provide clinical prognostic utility, the results highlight the need for further research of the patterns of cytokines in patients with ovarian cancer.

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Conflicts of Interest: The authors declare no conflict of interest.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Bioethics Committee of the Poznan University of Medical Sciences (decision no. 784/13 and 1126/16).

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RESEARCH ARTICLE

Irrigated DiamondTemp catheter and return to ablation under temperature control. First Polish experience with DiamondTemp catheter in pulmonary vein isolation

Edward Koźluk[1], Agnieszka Piątkowska[2,1], Dariusz Rodkiewicz[1], Grzegorz Opolski[1]

[1] I Chair and Department of Cardiology, Medical University of Warsaw, Warsaw, Poland
[2] Department and Clinic of Emergency Medicine, Wrocław Medical University, Wrocław, Poland

Abstract

We present the first Polish experience with ablation performed using DiamondTemp catheter. The study was conducted with 3 male patients diagnosed with atrial fibrillation (AF). In the first 2 patients typical transseptal punctures were performed, followed by mapping with the Advisor catheter and EnSite-Precision system. One patient had a residual atrial septal leak, therefore ablation without fluoroscopy was attempted. High-power, short-duration ablation under temperature control was performed around pulmonary vein (PV) ostia. The power was 49-53 W, the temperature was 45-48˚C. Duration of procedures/fluoroscopy were: 146/8.9, 177/5.9, 132/0.0 min. In the reference group, 10 recent AF identical ablation procedures performed with traditional equipment resulted in 143.0±27.0/6.0±4.4 min. Duration of DiamondTemp applications were 14.7, 32.7, 30.8 min (reference group 37.3 ± 11.4 min). Procedural endpoints were achieved in all but one patient with incomplete isolation of the low segment of the right inferior PV. There were no procedural complications noted. In conclusion, the DiamondTemp saline-irrigated catheter is safe and effective for high-power short-duration ablation in patients with AF. Furthermore, this technology makes it possible to complete the procedure without fluoroscopy. However these findings must be confirmed in larger group of patients.

Keywords: atrial fibrillation · zero fluoroscopy · pulmonary vein isolation · high-power short-duration ablation · temperature control RF ablation

Citation

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Corresponding author:
Dariusz Rodkiewicz, I Chair and Department of Cardiology, Medical University of Warsaw, Poland
e-mail: elektrofizjologia@wum.edu.pl
Available online: www.ejtcm.gumed.edu.pl

Introduction

In the recent years there has been a technological breakthrough in the field of atrial fibrillation (AF) ablation due to the introduction of the ablation with high-power and short-duration [1-9]. In classical ablation, thermal injury occurs in two phases: resistive and conductive, with the second one being the dominant mechanism. In the resistive phase, RF current delivery leads to immediate heating of the superficial tissue layers approximately 1.0-2.0 mm depth. This phase creates a heat source that then extends passively to deeper layers as conductive heating [1-2]. Conductive heating injury is time dependent. Irreversible myocardial injury occurs at temperature ≥ 50°C [10]. At lower temperature the tissue injury often is reversible. When the power is increased the duration of current application is reduced and the resistive heating dominates the lesion creation [1-2]. Thus, the depth of permanent injury is more predictable, and the risk of collateral tissue damage is reduced. High-power ablations are classified as those performed with a power of ≥ 50 W [1-9].

Since temperature control during ablation is a better marker of effectiveness and safety in regard to tissue damage, it was favored overpower control for as long as until the end of the last century [11-15]. The gradual introduction of irrigated catheters that followed, resulted in loss of the possibility to accurately measure temperature at the electrode-tissue control interface, due to technological difficulties [15]. This impasse seems to be now solved by introduction diamond electrodes placed at the tip of catheter, which allows for efficient and fast heat removal [8-9].

In our previous article we presented a different catheter featuring very high-power and short-duration ablation technology [16]. This ablation method increases the proportion of resistive phase versus conductive phase of tissue damage, making RF application more predictable and safer. An additional benefit is the shorter application and treatment time.

In this technical report we aim to present a different solution: DiamondTemp Catheter (Medtronic) (Fig.1-3).

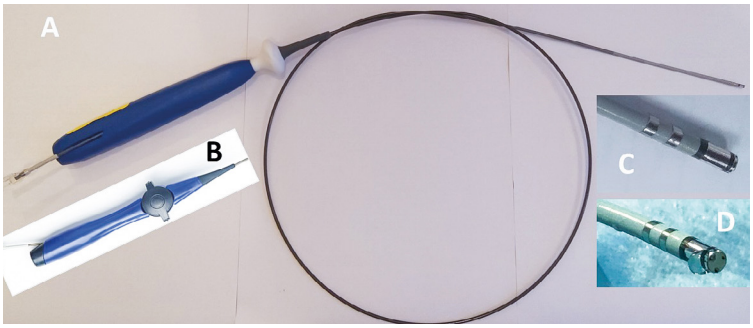

Figure 1. A - unidirectional version of the DiamondTemp catheter. B – bidirectional version of the catheter handle. C – close-up of the distal part of the DiamondTemp catheter. As shown, the ablation catheter tip consists of two electrodes: 0.6 and 3.0 mm with a 0.5 mm diamond in between. Proximal to the second electrode there is another diamond ring. Next, there are two diagnostic electrode rings. D – front of the catheter tip with its three thermocouple

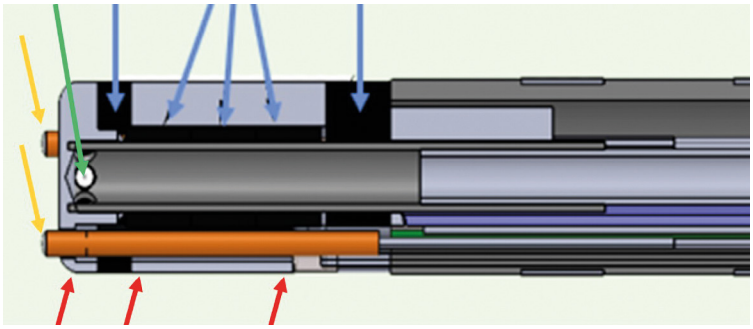

Figure 2. Longitudinal section of the DiamondTemp catheter. Yellow arrows indicate termocouples in front of the distal electrode. Red arrows – the RF platinum-iridium rings. The left one indicates the distal 0.6 mm electrode, the next two indicate the beginning and the end of the proximal RF electrode. Green arrow indicates one of its six irrigation ports. Blue arrows indicate positioning of the diamond. The left one indicates the distal diamond, the next three indicate the central diamond, the right one indicates the proximal diamond.

Figure 3. DiamondTemp RF generator.

Specifically, we described the practical use of a new, diamond-tipped catheter which allows for high-power short-duration ablation, where the amount of energy delivered depends on the temperature in the tissue adjacent to the catheter tip. Therefore, this method can also be defined as delivering the right power for the right duration. Our aim was to present a new technology related to the DiamontTemp catheter and its first use in Poland in patients undergoing pulmonary vein isolation.

Materials and methods

First we performed wet-lab tests with DiamondTemp catheter (Fig. 4-5) to visualize lesion formation and to evaluate the risk of steam-pop during high-power ablation. The next day we performed our first 3 clinical procedures (Fig. 6-10).

The first patient was a 46-year-old male with 4-year history of lone long-term persistent AF. The second patient was 26-year-old male with 2-year history of paroxysmal AF and previous ablation of the slow pathway, because of paroxysmal atrioventricular nodal reentrant tachycardia. The third patient was 61-year-old male with arterial hypertension, hyperlipidemia and coronary artery disease (NSTEMI treated with PCI 7 years ago) and paroxysmal AF diagnosed 7 years ago. All 3 patients were highly symptomatic (EHRA score 3). In the first 2 patients, typical transseptal punctures were performed followed by left atrial mapping with the Advisor catheter (Abbott) using EnSite-Precision system (Abbott) (Fig. 6). The third patient had a residual atrial septal leak, therefore we attempted to ablate without fluoroscopy (Fig. 7). Anatomical mapping of the right atrium was performed. Based on the images obtained, both catheters (the DiamondTemp and Advisor) were introduced into the left atrium through the leak in the septum using technique presented in previous publication [17]. In all 3 patients pulmonary vein isolation was performed using the "close protocol" (the distance between neighboring ablation points was < 6 mm) [18] using DiamondTemp catheter (Medtronic) with nominal high-power (50 W) under temperature control (Fig. 8-10).

Figure 4. Screen presenting the entire progress of RF application in wet lab. The first two seconds show the saline infusion (8 mm/min), after which the RF application started. 50W were reached within 1s. After 2 seconds of 50 W application, the maximal temperature of 60°C was reached and the power was then reduced. From that point, the power was kept at an optimal level to stabilize the temperature at 60°C. In the last second the power was reduced to 0 W. From 25 seconds shown on the diagram, 23 seconds depict the achievement of optimal power. Red line – temperature, yellow line – power, green line – impedance.

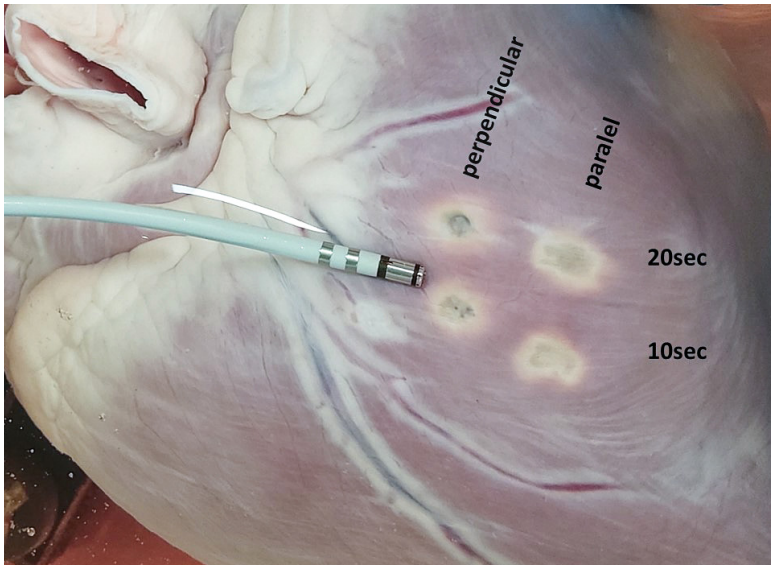

Figure 5. Final results of four applications of DiamondTemp RF, performed with different settings. The photo presents the surface area of a pig's heart post-application.

Results

The procedure (and fluoroscopy) duration were, respectively: 146.0 (8.9), 177.0 (5.9), 132.0 (0.0) min. The dose area product (DAP) were: 218.5, 68.1 and 0.0 mGy. To further illustrate the presented values, we compare them to the parameters recorded in the reference group of 10 patients which underwent point-by-point similar procedures using the classical technique. The values for this group were respectively: 143.0 ± 27.0 min (6.0 ± 4.4 min), DAP 82.1 ± 54.4 mGy.

The number of RF application was 73, 203 and 117 (in the reference group it was 122 ± 20), duration application was 14.7, 32.7, 30.8 min (reference group 37.3 ± 11.4). Power average (range) was: 50 (49-53), 49 (30-55), 50 (45-55) W, temperature 47 (45-52), 48 (40-60), 45 (40-60)°C.

Procedural endpoints were achieved in all but one patient with incomplete isolation of the low segment of the right inferior pulmonary vein. In this patient the "close protocol" was ended, and few accessory applications were performed in lower region of this vein, but with no effect. During pacing from the pulmonary vein a new map was obtained and the earliest atrial activation was confirmed at lower part of the right inferior pulmonary vein ostium. Serial application in this region did not terminate atrial capture during pacing from the vein. There were no procedural complications noted.

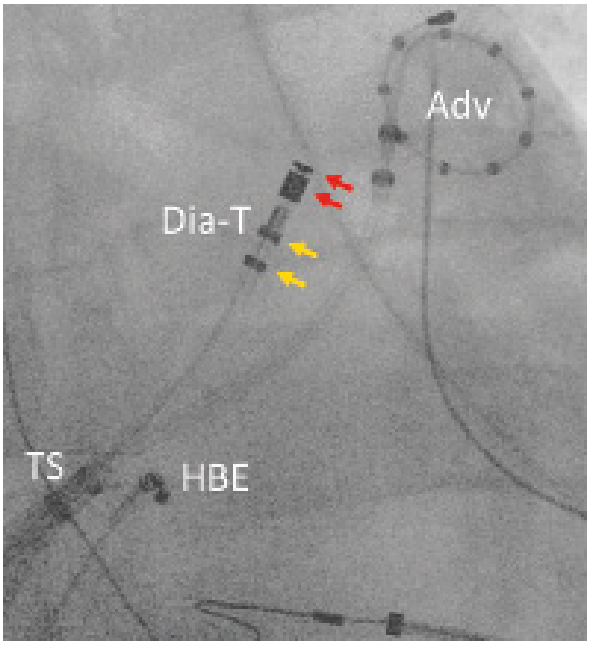

Figure 6. X-ray from the first clinical procedure using DiamondTemp ablation system. Adv – Advisor circular multipolar mapping catheter in the left superior pulmonary vein. Dia-T – the DiamondTemp catheter in the left atrial roof proximal to the left superior pulmonary vein ostium. Red arrows – two distal RF electrodes, yellow arrows – proximal diagnostic ring electrodes, HBE – the quadripolar catheter in His bundle position, TS – transseptal sheath.

Discussion

The DiamondTemp catheter is a new generation 7.5 F saline-irrigated catheter with a real time power modulation in the temperature control mode. Distal RF electrode tip is 4.1 mm and comprised of platinum/iridium (90%/10%) and chemical vapor deposition diamond network. This tip has two components separated by a 0.5 mm ring of chemical vapor deposition diamond. The distal electrode segment is 0.6 mm and contains 3 thermally insulated external thermocouples and 6 saline irrigation ports. Proximal electrode segment is 3.0 mm and also contains 3 thermocouples spaced equally around the proximal edge of the RF electrode and ring of chemical vapor deposition diamond at the proximal edge of the RF electrode for additional cooling. Heat and cooling

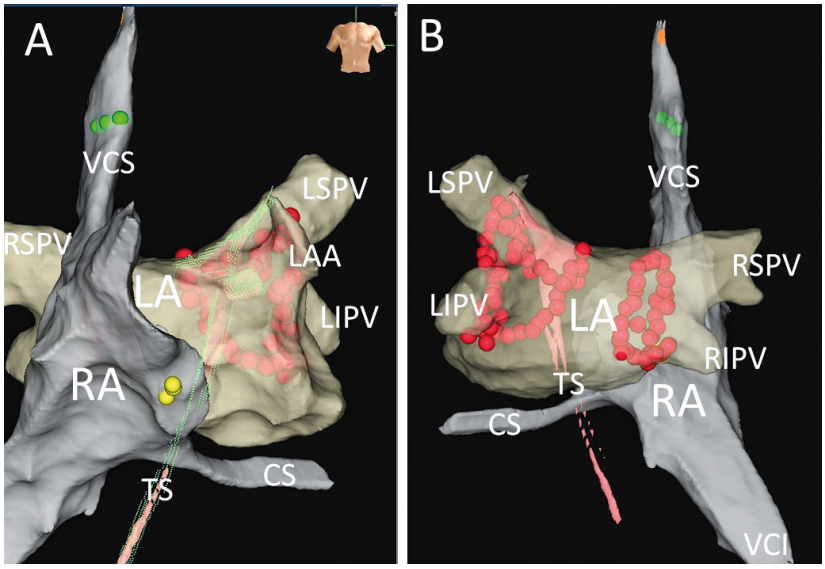

Figure 7. Final results presented with EnSite system's anatomical maps, exhibiting lesions resulting from pulmonary vein isolation, performed with no fluoroscopy using DiamondTemp catheter. Panel A – AP view, panel B – PA view. LA – the left atrial map, RA – the right atrial map, TS – map presenting the path of catheter through the PFO. CS – the coronary sinus, LAA – the left atrial appandage, LIPV – the left inferior pulmonary vein, LSPV – the left superior pulmonary vein, RIPV – the right inferior pulmonary vein, RSPV – the right superior pulmonary vein, VCI – the inferior vena cava, VCS – the superior vena cava. Yellow dots – His bundle position, red dots – ablation points. Both lines performed with the „close protocol" (the distance between points <6 mm). Green points – points of successful pacing of the phrenic nerve.

transfer are 200-400 times faster with diamond than with platinum-iridium. Extremely high thermal diffusivity allows to quick conduction of thermal energy through the diamond shunt network. Little to no heat is retained at the catheter tip to be cooled, therefore allowing a lower irrigation flow rate (8 ml/min) for all power delivery. This combined effect of rapid catheter tip cooling driven by the diamond shunt network and a low-flow irrigation rate allows for safe, effective and efficient lesion formation.

Temperature from each of the 6 thermocouples is sampled every 20 msec (50 times per second) and RF generator modulates power delivery based on the highest sensor temperature of all thermocouples. RF generator delivers 50 W to achieve set-point temperature (optimal value is 60°C). When temperature set-point is achieved the generator modulates power to maintain set temperature.

Because temperature provides direct feedback about lesion formation [10-12], in this model we do not need catheter-tissue pressure control. If the pressure is higher and temperature is going to reach set-point, the power was reduced (Fig. 9). Whereas when the contact was poor, we did not observe enough temperature rise (Fig. 8). In high power ablation the best method for the assessment of lesion efficacy is the change in electrogram (ECG) voltage. The duration of the application should be about 3.0-5.0 sec longer than potential disappearances or is significantly reduced (at least 75-80%) [8, 19] (Fig. 10). To reduce the influence of far-field potentials on the local signal amplitude, the high-resolution ECG is crucial. For this purpose, the distal RF electrode was divided into two segments, as described above. If the reduction of the local potential is inconclusive (e.g. during atrial fibrillation, RF current artifacts), the evaluation of the effectiveness of the application can be carried out based on changes in impedance [20].

Fig.8. RF power generator monitor during one of the applications. The last second of a 20 second RF application presents insufficient pressure of the catheter against cardiac tissue. A relatively low temperature despite the constant 50 W and the lack of a sufficient decrease in impedance suggests ineffective application. Time of application: 20 sec, Impedance:114 ohms (decrease of 3 ohms compared to the start of application), Power: 50 W, Temperature: 44˚C . Yellow line – power, red line – temperature, green line – impedance. Saline infusion via DiamondTemp catheter – 8 ml/min. Marker W104 – system suggests loss of contact between the catheter and tissue.

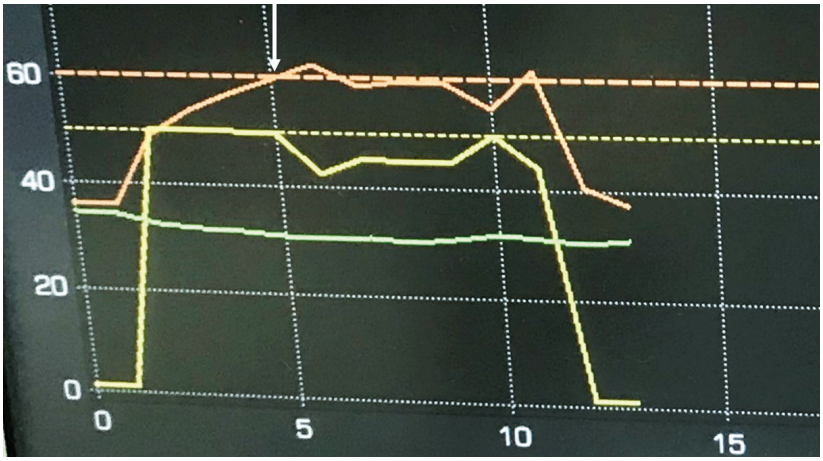

Fig.9. DiamondTemp RF Generator monitor after a successful application is completed. Power of 50W, Temperature achieved: 60˚C after 5sec. When temperature set-point is achieved (the white arrow) the generator modulated power to maintain set temperature (in this case between 41 and 50W). Due to optimal tissue contact and early dissappearence of local signal, the application was terminated after 10 seconds. Yellow line – power, red line – temperature, green line – impedance.

Significant tissue heating is associated with a predictable fall in impedance. Impedance drop of 5-10% can offer an independent means of assessing the true outcome [20].

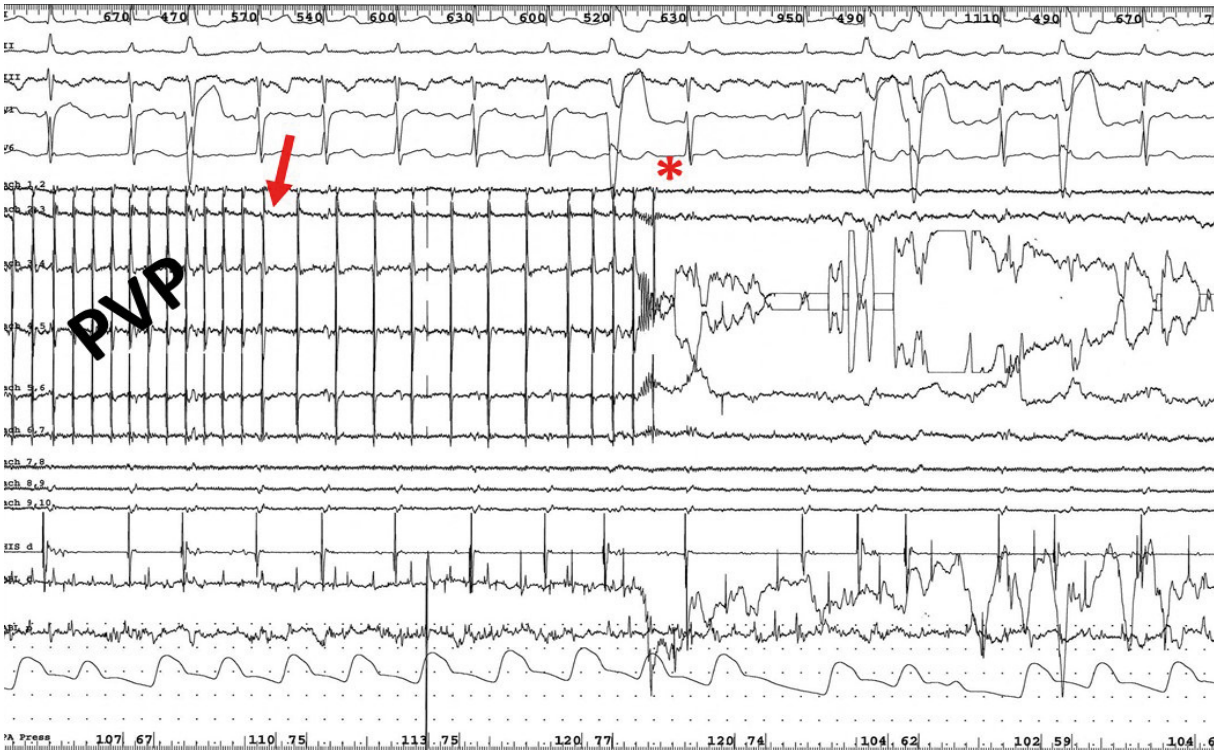

Fig.10. Disappearance of the pulmonary vein potentials during RF current application from DiamondTemp ablation system, preceded by their gradual slowing down in the first patient diagnosed with persistent atrial fibrillation. Arrow – the beginning of the pulmonary vein potentials slow down, star – disappearance of the pulmonary vein potentials. PVP – pulmonary vein potentials

In the TRAC-AF study [8] on porcine model, the investigators presented lesion transmurality created by the DiamondTemp catheter in 51 of 55 lesions (92,7%) performed in 6 pigs. In the second part of the study, the DiamondTemp catheter was used in 35 patients at a single center. The ablation was performed in temperature-control mode (60°C/50 W) with the goal of achieving 80% reduction in high-resolution electrogram (EGM) amplitude. All pulmonary veins were successfully isolated during the procedure. Compared with the retrospective control group (standard force-sensing ablation catheter), the study cohort had significantly shorter mean RF application duration (average difference 33.0 min), fluoroscopy time and lower acute dormant pulmonary veins reconduction. At 3 months, 23 patients underwent remapping. In 17 of 23 patients (73.9%) and 39 of 46 pulmonary veins pairs (84.8%) remained durably isolated.

The largest clinical evaluation of the DiamondTemp catheter was DIAMOND-AF study [9]. It was FDA-regulated, prospective, multicenter (n=23), single-blind, noninferiority, randomized (1:1) controlled trial designed to compare the safety and effectiveness of the DiamondTemp ablation system with a contact force sensing ablation system in patients with drug-refractory symptomatic paroxysmal AF. A total of 482 patients were randomized (239 DiamondTemp catheter ablation, 243 control). Acute PVI was confirmed in 239 subjects (100%) in the DiamondTemp ablation group and 241 subjects (99.2%) in the control group. Total procedure, fluoroscopy, and left atrial dwell duration were similar between arms. Total RF time and individual RF ablation duration were significantly shorter in the DiamondTemp group and significantly less fluid was infused through the DiamondTemp catheter. Complications occurred in 8 (3.3%) patients treated with DiamondTemp catheter and 16 (6.6%) patients in control group. In the DiamondTemp group there were 2 cardiac tamponades, 2 TIA episodes, 1 permanent phrenic nerve paralysis, 1 vagal nerve injury and 2 vascular complications. In control group there were 2 cardiac tamponades, 1 pericarditis, 1 pulmonary edema, 1 stroke, 1 TIA and 4 vascular complications. There was no deaths. After 1 year follow up (with 3-month blanking period) free of atrial arrhythmias lasting ≥ 30.0 sec were 189 (79.1%) patients after ablation with the DiamondTemp catheter and 184 (75.7%) patients in the control group.

Conclusions

The DiamondTemp saline-irrigated catheter seems to be safe and effective for high-power short-duration ablation in patients with atrial fibrillation. This must be confirmed in a larger group of patients. This technique makes it possible to complete the procedure with zero fluoroscopy exposure.

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RESEARCH ARTICLE

Do parents properly assess children's physical activity level? Study of over 20 thousand children and parents dyads

Paulina Metelska[1,2], Michał Brzeziński[3], Marek Jankowski[2,4], Aleksandra Niedzielska[2,4], Adam Szarszewski[5]

[1] Department of Public Health & Social Medicine, Medical University of Gdańsk, Poland
[2] *6-10-14 for Health* Programme, University Medical Center, Gdańsk, Poland
[3] Department of Paediatrics, Gastroenterology, Allergology and Paediatric Nutrition, Medical University of Gdańsk, Poland
[4] Gdańsk Center for Health Promotion and Addiction Prevention, Gdańsk, Poland
[5] Department of History and Philosophy of Medical Sciences, Medical University of Gdańsk, Poland

Abstract

Background: From the public health perspective, it is particularly important to establish links between a persons' perception of their own health and their predispositions to undertake pro-health behaviours that enable them to maintain proper functioning of their body. In the case of the paediatric population, it is crucial to emphasise that adults are responsible for the adoption and development of appropriate health-enhancing behaviours by children. The aim of this study is to verify parents' perception of the level of children's physical activity (PA) in comparison with objective measures describing children's physical fitness. **Material and methods:** Material for analysis consists of the results of anthropometric tests and cardiopulmonary exercise capacity of children as well as questionnaires concerning 28,891 children completed by parents. **Results:** In more than 40% of the sample, the subjectively assessed level of PA was not consistent with the cardiorespiratory fitness level. It was observed that the correct assessment of the level of PA of children is more common for girls, increases with the age of the child and with the level of parental education.

Keywords: assessment · physical activity · parent view · children physical activity

Citation

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Corresponding author:
Paulina Metelska, Department of Public Health & Social Medicine, Medical University of Gdańsk, Poland
6-10-14 for Health Programme, University Medical Center, Poland
e-mail: metelska.paulina@gumed.edu.pl
Available online: www.ejtcm.gumed.edu.pl

Introduction

Over the last few decades, significant changes related to the level of physical activity among adults and children took place in most of developed societies. About 30 years ago in Poland, after the collapse of the communist government, the socio-economic conditions of the citizens significantly improved. Easier access to high-energy food and lifestyle changes were observed [1]. For several different reasons (e.g. safety, distance, time) the broadly understood mobility patterns have changed, with walking giving way to driving and using public transport, both of which reduce energy expenditure on daily basis [2]. Physical activity limited to the school environment alone does not allow the child to have the minimum recommended amount of daily exercise [3]. These changes also relate to children and young people spending more time in front of the television or computer, which is one of the risk factors for the decreasing physical activity level [4].

Health Behaviour In School-Aged Children (HBSC) research carried out between 2002 and 2010 in more than 30 countries showed that 77% of boys and 86% of girls in the countries included in the study are at risk of ill health due to insufficient physical activity [5]. The results of the Polish edition of the WHO (World Health Organisation) European Childhood Obesity Surveillance Initiative (COSI) study indicate that for more than half of 8-year-olds their journey to school does not involve any physical activity. At the same time, the authors report that parents declared in the survey that 82.9% of children followed the WHO recommendations and accumulated 60 minutes or more physical activity during the school day [6]. From the practical perspective, it is particularly important to establish links between person's perception of their own health and their predispositions to undertake pro-health behaviors that enable them to maintain best available health status [7]. In the case of the pediatric population, it is crucial to emphasize that adults are responsible for the adoption and development of appropriate health-enhancing behaviors by children and adolescents [7].

Interviewing parents/guardians either via a personal conversation or using surveys on health status and health behavior is the basis for a system of health statistics in all European countries [7]. It is generally accepted that survey responses are provided by parents/legal guardians who make the assessment. Therefore the data concerning health assessment of children are provided from the perspective of their parents and/or guardians [8]. Proper assessment by parents is often essential to ensure that they take appropriate and necessary measures to maintain the health of their child. However, questions arise as to the objectivity of such an assessment.

The aim of this study is to verify parents' perception of the level of children's physical activity in comparison with objective measures describing children's physical fitness. The analysis reported in this article was carried out within the scope of a population-based health program "6-10-14 for Health" designed to manage and reduce the risk factors of civilization diseases in children and adolescents, carried out by the University Clinical Centre in Gdańsk and funded by the Gdańsk City Hall since 2011.

Materials and Methods

Material for analysis consists of the results of anthropometric tests and cardiopulmonary exercise capacity of children as well as 28,891 questionnaires completed by parents/legal guardians concerning children and adolescents between 6 and 14 years of age from the City of Gdańsk. The results were obtained between 05/09/2011 and 14/02/2017 from children and parents participating in screening tests carried out within the "6-10-14 for Health" program [9]. The sample population represents over 65% of all population of all the children available for screening living in Gdansk aged 6, 9-11, 14 in the years 2011-2017. As the program "6-10-14 for Health" is designed to screen all children from the City of Gdańsk at the above-mentioned intervals and invites children with overweight and obesity to a one-year-long lifestyle modification intervention. In this article we present the data we obtained only from the screening.

The analysis included only the fully completed questionnaires from the parents of children who underwent full screening (Supplementary material). The parents chose the suggested answers, which in their opinion best described their child's situation. The respondents were also asked to provide estimates of each parent's body weight, height and to indicate highest education level.

The assessment of cardiorespiratory fitness (physical capacity) was performed using the Kasch Pulse Recovery Test (KPRT), a simple 3-minute step test that assesses the cardiorespiratory fitness of an individual based on changes in pulse within the first minute after performing the test [10]. The results were compared to dedicated reference systems [10]. The interpretation and evaluation of the responses to the questionnaires was based on the current WHO guidelines for physical activity [11], which indicate that a minimum of 60 minutes of physical activity each day is necessary for a child's normal development, ideally an aerobic effort of medium to moderate intensity.

Our study was conducted with the approval of an independent bioethics committee at the Medical University of Gdańsk (NKBBN/228/2012), in accordance with the requirements of the Helsinki Declaration. Each parent/legal guardian had to express written consent to the child's participation in the program. *6-10-14 for Health* program is retrospectively registered in clinical trials database: clinicaltrials.gov no. NCT04143074

Normality of quantitative variables was assessed using the Shapiro-Wilk test, and descriptive statistics included were presented as arithmetic means and upper and lower limits of the 95% confidence interval for the mean (95% CI). Mann-Whitney U test and Kruskal-Wallis test with Dunn's post-hoc test were used for between-groups comparisons of quantitative variables. The strength and direction of the relationship between pairs of quantitative variables were evaluated on the basis of the Spearman's Rank Correlation Factor (R). Pearson's chi-quadrant test was used for between-groups comparisons of distributions of qualitative variables. All calculations were performed using Statistica 10 software (StatSoft, United States), with the value of $p \leq 0.05$ as the threshold of statistical significance. The effects of variables, which turned out to differ significantly between the compared groups, were evaluated using one- and multi-dimensional logistic regression analysis.

Results

The analysis of the results carried out for the purposes of this study includes the results of objective and subjective assessments, as well as objectivization of subjective assessments. 18% of the questionnaires were rejected due to incompleteness. The study took into account results from of 24981 children aged 6, 9-11 (for the sake of simplicity, in the results we labeled this group the term “10 years old”) and 14 years old. Physical fitness of children was assessed using the Kasch Pulse Recovery Test (KPRT) method, with 20519 children (82.14% of the sample) taking part in t he test. A detailed distribution of Kasch Pulse Recovery Test results by age group is presented in Table 1.

Table 1. KPRT results by age

KPRT result	6 yo		10 yo		14 yo	
	n	%	n	%	n	%
excellent	274	4.09	401	4.81	357	6.52
very good	1644	24.52	1647	19.75	731	13.35
good	2198	32.79	2102	25.2	885	16.16
satisfactory	1560	23.27	2117	25.38	1207	22.05
poor	866	12.92	1749	20.97	1609	29.39
very poor	162	2.42	324	3.88	686	12.53

The group of 10-year-olds was the most numerous and constituted 42.25% of the sample. 6-year-olds and 14-year-olds constituted 33.39% and 24.36%, respectively. Girls and boys constituted respectively 50.03% and 49.97% of the studied group, sex distribution in the sample is presented in Table 2.

Table 2. KPRT results by sex

KPRT result	boys		girls	
	n	%	n	%
excellent	505	4.85	527	5.21
very good	2113	20.31	1909	18.87
good	2796	26.88	2389	23.61
satisfactory	2354	22.63	2530	25.01
poor	1963	18.87	2261	22.35
very poor	671	6.45	501	4.95

The studies of cardiopulmonary efficiency show that low (poor and very poor) cardiopulmonary efficiency is similarly common in boys and girls. The analysis did not reveal any significant differences between groups (Pearson's chi- squared test, $p = 0.098$) and percentage of children with poor and very poor cardiorespiratory (Pearson's square chi test, $p < 0.001$).

Regarding the physical activity status, 73.79% of parents indicated that in their opinion the child had enough exercise during the day, while 20.71% of parents noticed deficiencies in this area and 5.5% responded "I don't know.”

In order to facilitate the comparison of KPRT results with the parental subjective assessment of their children’s activity levels, the KPRT results were re-classified as either “normal fitness” (evaluation of cardiopulmonary efficiency: excellent, very good and good) or “abnormal fitness” (evaluation of cardiopulmonary efficiency: satisfactory, poor and very poor) [10]. The comparison demonstrated that the subjective assessments were not very consistent with the fitness evaluation based on the KPRT (Kendall's Tau-b coefficient

= 0.170). In more than 40% of the sample, the subjectively assessed level of physical activity was not consistent with the cardiorespiratory fitness level measured by KPRT, with 33.67% of children assessed as having enough exercise yet performing sub-normally on KPRT and another 9% of the children performing normally despite their parents assessing their activity level as insufficient. It was observed that in 33.67% of the children the level of physical activity considered by their parents as sufficient did not translate into a normal result of the cardiopulmonary efficiency test as presented in Table 3.

Table 3. Consistency of the assessment of cardiopulmonary efficiency of the examined children with the level of their physical activity subjectively assessed by the parent

KPRT result	subjective parental assessment			
	sufficient		not sufficient	
	n	%	n	%
normal	2113	20.31	1909	18.87
sub-normal	2796	26.88	2389	23.61

In order to assess the physical fitness and behaviors related to physical activity of children and adolescents, two additional questions about physical activity-related behavior were included in the questionnaire for parents/guardians.

In response to a question about screen time, 15.59% of parents estimated the time spent by their children in front of a screen as < 1 hour a day, 54.8% of parents indicated that their child spent 1-3 hours in front of a screen and 29.61% of parents indicated that screen time exceeded 3 hours a day.

The second question concerned the number of hours spent by the child on physical activity during the week. Parents responded as follows: minimum 5 hours (31.14%), 3-5 hours (27.09%), 1-3 hours (31.51%), < 1 hour (10.26%).

Based on the WHO recommendations [11] on physical activity of children and adolescents, the parents'/guardians' responses parents were classified into two categories indicating that the child has or has not developed desirable physical activity behaviors. In order for the behavior to qualify as desirable, parents/legal guardians had to indicate that the child spends <1 hour in front of a television/computer per day and that the child spends at least 5 hours per week actively playing/doing sport (excluding PE classes). Any other answer to either of the questions led to classifying the child into a group with undesirable behaviors. This is how the category of objectivized parental assessment was created. Desirable behavior was demonstrated in 5.72% of children. The remaining answers fell in the category of undesirable behaviors.

This objectivized assessment of physical activity behavior was compared with the assessment of the KPRT results as shown in Table 4.

Table 4. Assessment of cardiopulmonary efficiency in the examined children versus the objectivized assessment of physical activity-related behaviours

Cardiopulmonary efficiency	objectivized assessment of physical activity-related behaviours			
	desirable		undesirable	
	n	%	n	%
normal	811	67.14	9.274	48.73
sub-normal	397	32.86	9.758	51.27

Based on objectivizing the parents'/guardians' answers to the relevant question, sub-normal results in cardiorespiratory fitness test were observed more frequently among children categorised as having undesirable physical activity patterns (Pearson's chi-quadrant test, $p < 0.001$). The objectivized assessment of physical activity-related behavior was also compared with the parents' answer to the question "In your opinion, does your child have enough exercise during the day?" as shown in Table 5.

Table 5. Comparison of the child's physical activity subjectively assessed by the parent with the objectivized assessment of physical activity-related behaviours

Objectivized assessment of physical activity-related behaviour	subjective parental assessment of sufficiency of exercise			
	sufficient		not sufficient	
	n	%	n	%
desirable	1.336	5.46	64	0.26
undesirable	16.704	68.3	6.352	25.97

It was analysed whether the child's age, sex or parental education level influenced the consistency of the subjective assessment with behavioral estimates. It was observed that the correct assessment of the level of physical activity of children is more common for girls (Table 6), increases with the age of the child (Table 7) and with the level of parental education (Table 8).

More consistent assessments were observed among parents of girls than among parents of boys (Pearson's chi-quadratic test, p = 0.008).

Table 6. Parental assessments of sufficient amount of exercise versus the objectivized physical-activity-related behaviour by sex

Objectivized assessment of physical activity-related behaviour	child's sex			
	boy		girl	
	n	%	n	%
correct/consistent	3,743	30.65	3,945	32.22
incorrect/inconsistent	8,469	69.35	8,299	67.78

Table 7. Parental assessments of sufficient amount of exercise versus the objectivized physical-activity-related behaviour by age

Objectivized assessment of physical activity-related behaviour	child's age					
	6 yo		10 yo		14 yo	
	n	%	n	%	n	%
normal	1.705	21.15	3.422	33.19	2.561	42.08
abnormal	6.356	78.85	6.887	66.81	3.525	57.92

Discussion

The participation of parents/legal guardians can be observed at different stages in the diagnostic processes of children and adolescents, starting with the consent to perform tests, providing information necessary to interpret the results, to making the diagnosis and planning the appropriate course of action. The parents' knowledge of health behaviors and factors influencing their child's health is an invaluable addition to the physical examination [12]. It should be noted, however, that there are several factors that may distort or falsify information about the health condition of the child, e.g.:

- parents' misunderstanding of the behavior typical for a given phase of a child's development;
- incompatibility between parental expectations and the child's characteristics;
- parental characteristics (e.g. depression, lack of interest in the child, rejection, overprotection);
- stress (resulting from family or material situation, etc.) [13].

A reliable assessment of physical activity levels in children and adolescents is a key element of health assessment. It is also the starting point for therapeutic recommendations from specialists and their implementation by children and adolescents under the supervision of their parents/legal guardians.

This article presents an attempt to evaluate the reliability of assess-

Table 8. Parental assessments of sufficient amount of exercise versus the objectivized physical-activity-related behaviour by parental education

Objectivized assessment of physical activity-related behaviour	child's age											
	6 yo		10 yo		14 yo		6 yo		10 yo		14 yo	
	n	%	n	%	n	%	n	%	n	%	n	%
normal	1.705	21.15	3.422	33.19	2.561	42.08	1.705	21.15	21.15	33.19	2.561	42.08
abnormal	6.356	78.85	6.887	66.81	3.525	57.92	6.356	78.85	78.85	66.81	3.525	57.92

ments made by parents/guardians, comparing them with objective and objectivized measures. The reliability and accuracy of the assessments was verified on the basis of objective test results but also by asking parents questions that verified or providing more details concerning the areas of interest. The child's or parent's characteristics that influenced parental assessments were also identified.

The study showed that making a correct assessment of lifestyle-related behaviors is difficult for parents. It was observed that in 33.67% of children the level of physical activity considered by their parents as sufficient did not translate into the normal result of the cardiopulmonary efficiency test. Moreover, 68.30% of parents positively assessed their child's level of activity although their reported actual level of physical activity was less than desirable. Similar results can be found in British studies, where 80% of parents of inactive children wrongly assessed their children as sufficiently active [14]. Another British study showed that parents perceived physical activity itself as beneficial, but did not have knowledge about the recommendations, and assumed that organizing physical activity for children is primarily the responsibility of the school [15].

The parents' perspective may also be distorted by the emotional context. A study comparing evaluations of parents' own physical activity during their childhood with the current activity of their children showed that parents perceived their own activity as free and joyful. Despite this positive image, because of their concern for their children's safety, parents limit their children's free play outdoors. Some parents see the opportunities for structured physical activity in children participating in group activities, but perceive them as a "less joyful" than the free play they recall from their own childhood [16]. Concerns about child safety, lack of financial resources and difficulties in motivating children to engage in physical activity are the most common barriers to introducing physical activity reported by parents [15].

Correct assessment of the level of physical activity in children is more common for girls and increases with the age of the child and with the level of parental education. Our study indirectly revealed a deficit in the correct understanding of the levels of physical activity recommended for children and adolescents. These results also demonstrate that specialists taking history on physical activity cannot limit their investigation to asking simple questions: additional verification questions are necessary or, if possible, objective tests should be carried out.

In the recent decades, physical fitness of both adolescents and adults has deteriorated, which is demonstrated by the results of the CINDI project (Countrywide Integrated Noncommunicable Disease Intervention) carried out in several European countries within the framework of WHO activities. This is particularly worrying because these recommendations of global experts are not new and should have long become a part of the general public's awareness. In 2005, a team of experts from the American Heart Association developed a position, subsequently endorsed by the American Academy of Pediatrics, that each child should devote a minimum of 60 minutes a day to moderate to high-intensity motor activity [17]. The research reported in this paper did not assess knowledge in this area. Rather, it aimed to diagnose the current situation and assess the correctness of the parents' assessments in this area.

Moreover, the analysis of the results of the cardiorespiratory fitness test shows that poor fitness affects more boys than girls and that the percentage of children with poor and very poor fitness increases with age. This is also reflected in many studies conducted in Poland and abroad, which indicate that with age, adolescents become less active due to a greater number of mainly sedentary, activities, both at school and during leisure [18-21]. Restrictions in physical activity of children and young people do not result only from the progressing urbanization and automation, but mainly from the high attractiveness of media and internet content. Moreover, parents' low physical activity translates into lower physical activity in their children. Research indicates that children and adolescents who perceive their parents as physically active are more often active than their peers who do not have such a parental example [22].

Conclusions

Parental assessments of lifestyle-related behaviors show very low sensitivity and specificity. The results obtained make it possible to question the reliability of data obtained only through questionnaires without using any additional questions or an objective method. All collected data should be assessed with great care as it is based on the parental perceptions. This conclusion should also be reflected in practice: when planning programs to promote healthy lifestyles, there is a need to objectivize parental opinions through the use of verification methods (e.g. verification questions or, if possible, the use of objective measurement tools).

Supplementary material

Questionnaire completed by parents/guardians
→ **tu będzie link z materiałem uzupełniającym**

Conflicts of Interest

The authors declare no conflicts of interest.

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RESEARCH ARTICLE

Simultaneous determination of lopinavir, saquinavir and ritonavirin in human plasma using liquid chromatography – ion trap mass spectrometry

Marcin Lipiński[1], Krzysztof P. Bielawski[2], Ewa M. Słomińska[3], Ryszard T. Smoleński[3]

[1] Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Poland
[2] Laboratory of Molecular Diagnostics, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Poland
[3] Department of Biochemistry, Medical University of Gdańsk, Poland

Abstract

Background: Lopinavir, saquinavir, and ritonavir are viral protease inhibitors (PIs) developed for and widely used in the therapy of human immunodeficiency virus (HIV)-related disease. These compounds are also active *in vitro* against the pathogens causing tuberculosis, malaria and coronavirus infections. PIs have been regarded as a platform for the design of inhibitors targeting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-encoded proteases. This study aimed to develop a liquid chromatography/mass spectrometry (LC/MS) procedure for accurate simultaneous determination of concentrations of these three PIs in the plasma. **Methods:** Samples of human plasma were protein precipitated with 0.3 M zinc sulfate in a water/methanol solution (30:70, v/v). The extracts were analyzed with reversed-phase chromatography coupled with the electrospray ionization (ESI) source of the ion trap mass detector operating in mEass spectrometry (MS) and tandem mass spectrometry (MS/MS) modes. **Results:** Calibration curves demonstrated good linearity from 0.01 to 10 µg/mL and acceptable reproducibilities and recoveries. **Conclusions:** The described procedure proves that a very basic ion-trap LC/MS system could be applied for selective, rapid, and precise determination of antiviral protease inhibitors.

Keywords: antiviral therapy · lopinavir · saquinavir · ritonavir · LC/MS

Citation

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Corresponding author:
Marcin Lipiński, Department of Pharmaceutical Biochemistry, Medical University of Gdańsk, Gdańsk, Poland
e-mail: marlip@gumed.edu.pl
Available online: www.ejtcm.gumed.edu.pl

Abbreviations

- ESI – electrospray ionization,
- HAART – highly active antiretroviral therapy,
- HIV – human immunodeficiency virus,
- LC/MS – liquid chromatography/mass spectrometry,
- MS/MS – tandem mass spectrometry,
- PIs – protease inhibitors,
- SARS-CoV-2 – severe acute respiratory syndrome coronavirus 2,
- TMD – therapeutic drug monitoring

Introduction

Lopinavir, saquinavir, and ritonavir are peptidomimetic inhibitors of human immunodeficiency (HIV) protease and belong to the protease inhibitors (PIs) group of drugs. They were developed to treat HIV/AIDS (acquired immunodeficiency syndrome) and are currently used in clinical practice in combinations with nucleoside inhibitors of HIV reverse transcriptase in the highly active antiretroviral therapy (HAART)[1-2]. Lopinavir and saquinavir (or other new generation PIs) are routinely combined with ritonavir in the so-called boosted protease inhibitor regimens because ritonavir administered in low doses can additionally block the cytochrome P450 (CYP3A4), thus increasing the bioavailability of the two first compounds [3].

In addition to its anti-retroviral properties, PIs exert anti-malarial activity targeting aspartyl proteases in the *Plasmodium* species and may be associated with a reduced incidence of malaria in HAART receivers [4-5]. Saquinavir has also appeared as a potential agent for tuberculosis, based on an *in vitro* study of a *Mycobacterium tuberculosis* infection model [6].

The policy of repurposing existing drugs has brought the three PIs to light as strong candidates for anti-coronavirus disease 2019 (COVID-19) drugs, specifically as potential inhibitors of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-encoded proteases. Saquinavir could block the proteolytic activity of SARS-CoV-2 3CLpro protease *in vitro* and the *in silico* predictions demonstrated its ability to bind the dimeric SARS-CoV-2 Mpro protease [7-8]. Lopinavir/ritonavir was previously demonstrated to have *in vitro* activity against both severe acquired respiratory syndrome coronavirus 1 (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), with some efficacy in animal and clinical studies [9-10]. Despite the poor performance of PIs, when compared to the standard of care, in clinical studies with COVID-19 patients, these drugs can be still regarded as a platform to design new and more potent inhibitors of SARS-CoV proteases [8, 11-12].

In addition, the clinical data from HIV-infected patients indicate individual variations in responses to treatment, with some non-responders that demonstrate no phenotype- or genotype-related resistance [3]. This insufficient response could result from specific pharmacokinetics in the individual subjects and the extent to which the patients follow the prescribed therapy. Information about the drug kinetics profiles of patients can be very important in clinical practice [3, 13]. An optimal way to address it is to evaluate the PIs concentrations in the patient's plasma and to perform therapeutic drug monitoring. This study aimed to develop a liquid chromatography/mass spectrometry (LC/MS) procedure for accurate simultaneous determination of concentrations of these three PIs in the plasma.

Materials and methods

Preparation of standards and a calibration curve

The standards of lopinavir, saquinavir, and ritonavir (Fig. 1) were provided by Roche (Basel, Switzerland). Diazepam for use as an internal standard (IS) was from WZF Polfa (Warsaw, Poland). Stock solutions of drug standards were prepared at 1 mg/mL concentration by dissolving 5 mg of ritonavir in

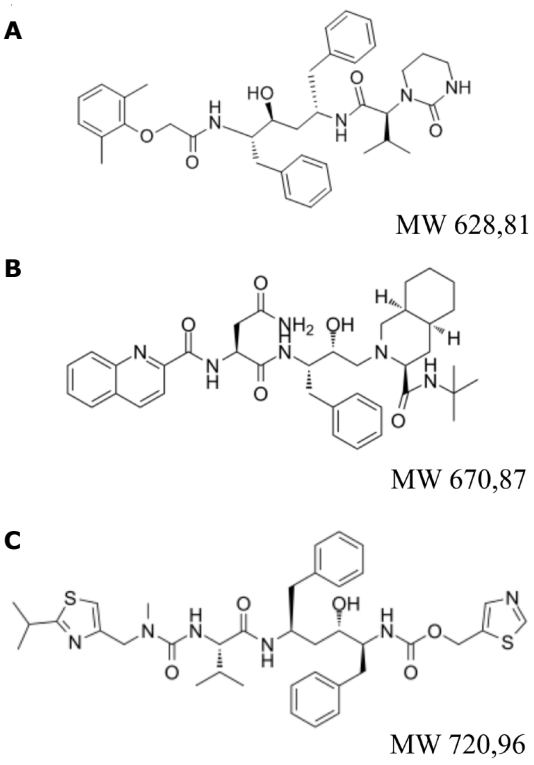

Figure 1. Chemical structures of three drugs: A. lopinavir, B. saquinavir, C. ritonavir.

5 mL of methanol, 5 mg of saquinavir in 5 mL methanol/water (50:50, v/v), and 5 mg lopinavir in 5 mL of methanol/water (50:50, v/v). To assess the linearity of the assay, seven-point calibration curves were obtained by diluting the stock solutions in acetonitrile/water solution (AcN/W, 4:6, v/v), obtaining finally 0.01, 0.03, 0.1, 0.3, 1, 3, 10 µg/mL of each drug in 200 µl. Diazepam was added to the solutions as an internal standard (IS) at the final concentration of 5 µg/mL.

Plasma sample preparation

0.3 M zinc sulfate (Sigma/Merck, Darmstadt, Germany) in water/methanol (30:70, v/v) was used as an extracting solution for protein precipitation. Diazepam was added to the solutions on the day of extraction as an internal standard (IS) at the final concentration of 5 µg/mL. To extract drugs from the plasma, 200 µL of human plasma samples were mixed with 600 µl of the extracting solution. Sample tubes were vortexed for 5 min and centrifuged at 13000 rpm for 3 min. Supernatants were transferred into autosampler injection vials and analyzed with LC/MS.

LC/MS analysis

The system applied for the analysis was the LCQ Advantage ion trap mass detector equipped with the electrospray ionization (ESI) source connected to the Surveyor autosampler and the Surveyor quaternary gradient pump (Thermo-Finnigan, San Jose, USA). Separation was carried out on a BDS Hypersil 5 µm column (2 mm/50 mm) protected with a 2x2 mm C18 Security Guard cartridge (Phenomenex, Torrance, USA). Buffer A was 10 mM ammonium acetate, whereas buffer B was acetonitrile. Starting conditions were 60% A/40% B which changed linearly to 1% A/99% B within 3 min. 1%A/99% B was maintained for 3 min and conditions returned to the initial values within the next 0.5 min. The total injection cycle was 6 min. The flow was maintained at 0.25 mL/min. The column temperature was maintained at 25ºC. The injection volume was 20 µL. The mass detector was operated in a positive ionization mode. Ion source parameters were optimized during direct drug infusion into the detector. The sheath gas flow rate was 35 arb. (instrument arbitrary units) and the spray voltage was 5.4 kV. The capillary temperature was set at 300ºC. Direct infusion allowed also to establish a fragmentation pattern of all three drugs and to optimize the collision energy for analysis in a fragmentation (tandem mass spectrometry, MS/MS) mode. Under those conditions, saquinavir formed a parent ion at m/z = 671.6 with some sodium adduct at m/z = 693.6, at quantities below 10%. The isolated ion 671.6 when subjected to a collision energy of 45% resulted in the formation of a major fragment at m/z = 570.2 which was used for detection. Ritonavir formed an ion at m/z = 721.3 with about 30% of sodium adduct (m/z = 743.3). Isolated ion 721.3 when subjected to a collision energy of 25% resulted in the formation of two major fragments at m/z = 295.1 and 426.1 which were used for detection. Lopinavir formed a parent ion at m/z = 629.1. The isolated ion 629.1 when subjected to a collision energy of 28% resulted in the formation of a major fragment at m/z = 447.1 which was used for detection. During chromatographic separation, MS detector was set to operate in alternate full MS (100-1000 m/z)/selective ion or reaction monitoring modes (m/z: 286, 671.6 > 570.2 (45%), 721.3 > 295.2 & 426.1 (25%), and 629.1 > 447.1 (28%)).

Determination of reproducibility and recovery

To determine reproducibility and recovery, we continued analyses on separate days with five injections of calibration standards and a quality control sample each day. Fresh samples were prepared each day. The data is presented in Table 1.

Table 1: Reproducibility and recovery data from patients plasma extracts analysis at three different concentrations of each drug (3, 0.3, and 0.03 µg/mL). Av: average, SD: standard deviation, CV: coefficient of variation, Rec: percentage of the recovery

3mg/L	lopinavir	saquinavir	ritonavir
Av	288.2	295.7	280.1
SD	16.79	15.32	24.35
CV	0.058	0.051	0.086
Rec	96%	98%	93%
0.3 mg/mL	**lopinavir**	**saquinavir**	**ritonavir**
Av	28.14	29.02	27.24
SD	0.78	2.1	1.46
CV	0.027	0.071	0.05
Rec	93%	96%	90%
0.03 mg/mL	**lopinavir**	**saquinavir**	**ritonavir**
Av	1.78	2.15	2.22
SD	0.45	0.32	0.59
CV	0.25	0.14	0.26
Rec	59%	71%	74%

We also used a negative control subject (treated with neither of the target compounds) to determine recovery at three different concentrations of each drug (3, 0.3, and 0.03 µg/mL).

Results and Discussion

As demonstrated in the chromatograms/LC reports in Figure 2, the internal standard (diazepam), lopinavir, saquinavir and ritonavir separated well chromatographically as standards, although with the possibility of construction of separate chromatograms for selected ions such a separation is not an absolute requirement. However, the problem with carryover occurred during several primary injections of the standards. We confirmed the presence of traces of analyzed compounds in compound-free samples following injections of higher concentrations of the standards. We solved this problem, using acetonitrile/water as a wash solution. The response of chromatographic peak areas for chromatograms extracted for selected ions was linear within the concentration range (between 0.01 and 10 µg/mL) for ritonavir, saquinavir, and lopinavir. Recoveries of drug standards added to the4 plasma were > 90% (Table 1). The chromatograms/reports for the calibration curve are attached to demonstrate the signal-to-noise ratio (Fig. 2). Figure 3 represents chromatograms obtained in the analysis of the control plasma extract from an individual non-treated with the analyzed therapeutics, for comparison. Figure 4 with representative chromatograms/reports from the analyses of the treated patients' plasma extracts demonstrates that the analyzed drugs, if present, form clear peaks.

Our method, although applied for monitoring of just three compounds, can be suitable for monitoring other drugs in the same sample. Low sensitivity preliminary data could be obtained from a full MS trace of already recorded chromatograms, while more accurate determination would require extending the selective ion monitoring/fragmentation mode and re-run of the separation.

Several previously described analytical methods using high-performance liquid chromatography (HPLC) for evalu-

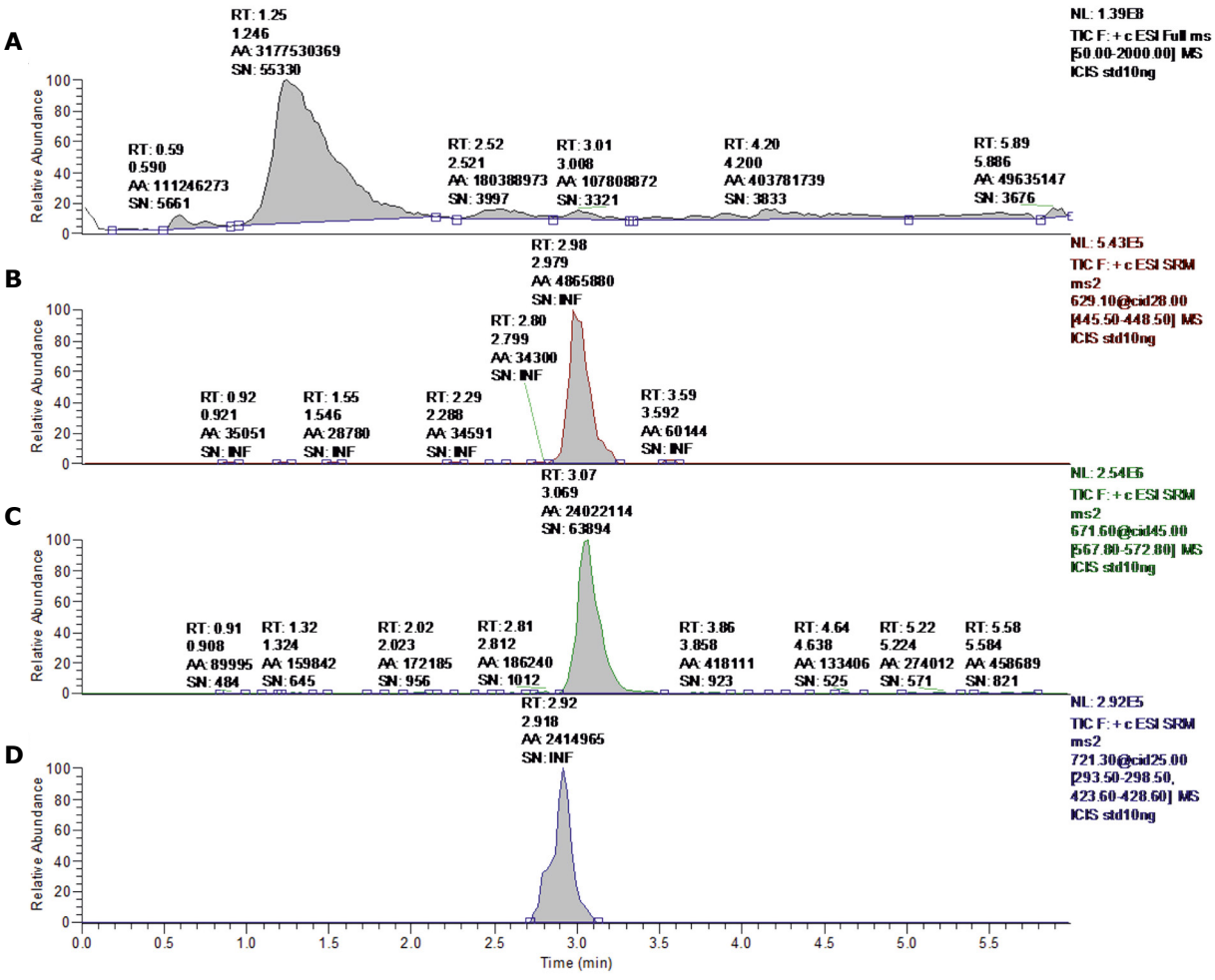

Figure 2. LC/MS chromatograms for standards of protease inhibitors in MS/MS mode: A. Total Ion Current. B. lopinavir, C. saquinavir, D. ritonavir

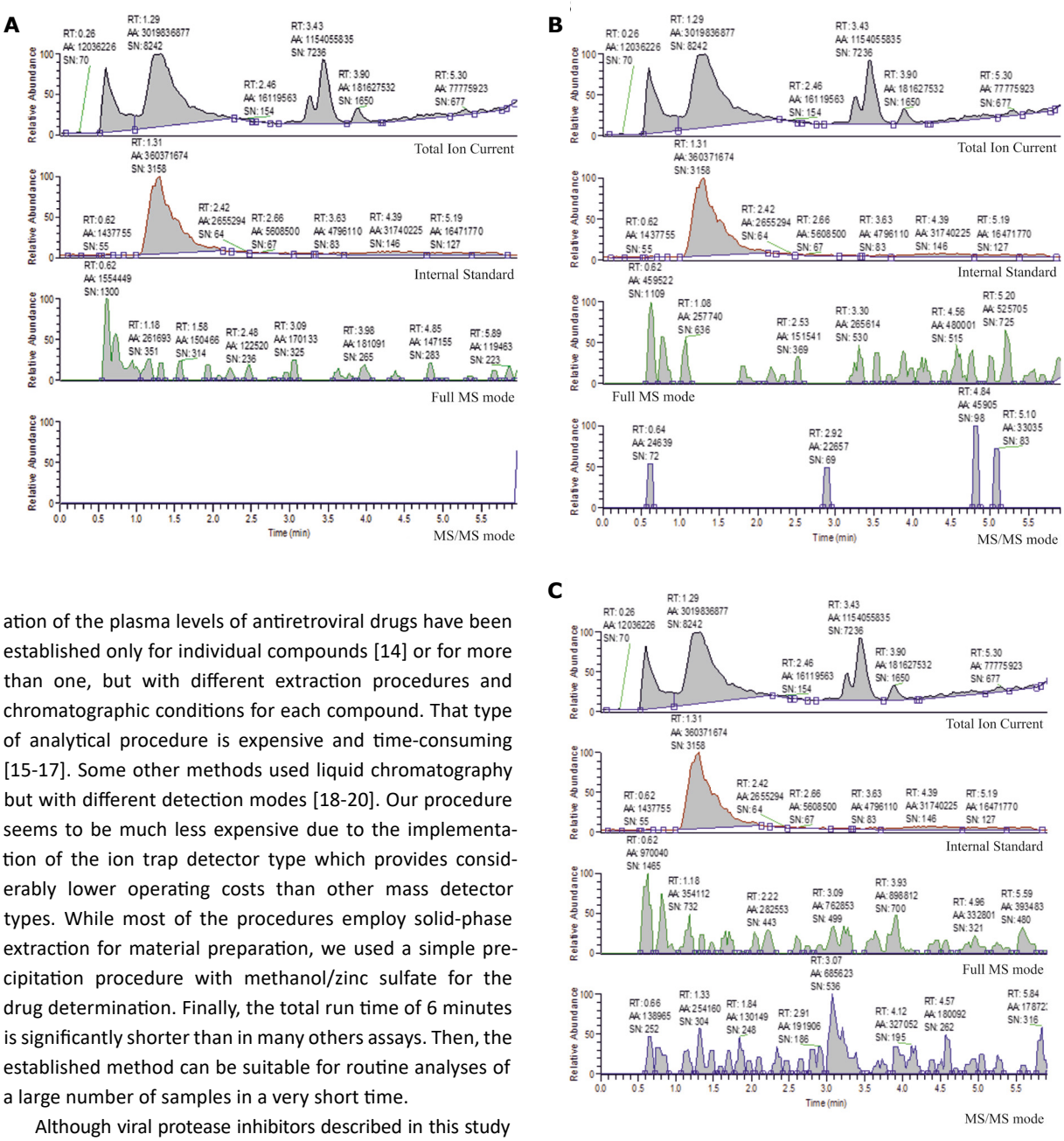

Figure 3. LC/MS chromatograms for the plasma extract of a control subject. Ion chromatograms for: A. lopinavir, B. saquinavir, and C. ritonavir. Each chromatogram consists of: Total Ion Current, Internal standard, Full MS mode, and MS/MS mode

ation of the plasma levels of antiretroviral drugs have been established only for individual compounds [14] or for more than one, but with different extraction procedures and chromatographic conditions for each compound. That type of analytical procedure is expensive and time-consuming [15-17]. Some other methods used liquid chromatography but with different detection modes [18-20]. Our procedure seems to be much less expensive due to the implementation of the ion trap detector type which provides considerably lower operating costs than other mass detector types. While most of the procedures employ solid-phase extraction for material preparation, we used a simple precipitation procedure with methanol/zinc sulfate for the drug determination. Finally, the total run time of 6 minutes is significantly shorter than in many others assays. Then, the established method can be suitable for routine analyses of a large number of samples in a very short time.

Although viral protease inhibitors described in this study take their origin from HIV treatment, their future use in the therapies of other diseases (e.g. malaria) is possible. There is still an urgent need for repurposed or new anti-SARS-CoV-2 therapeutics with sufficient clinical outcomes. So far four COVID-19 vaccines have been conditionally approved by the European Medicines Agency: 2 are based on the mRNA technology (Moderna and Pfizer/BioNTech) and 2 use the adenovirus platforms (AstraZeneca and Janssen/Johnson and Johnson) [21] and the number of vaccinated individuals has been increasing. However, fast-acting and efficient antiviral therapy based on the clinically well-defined compounds seems to be a necessary approach for the still recorded severely-ill COVID-19 patients, particularly in the parts of the world where the progress in vaccination is limited. The plasma drug concentrations of lopinavir/ritonavir administered in typical doses do not reach the levels that may be needed to inhibit SARS-CoV-2 proteases [22] and their use for COVID-19 treatment is currently not recommended. However, the data predicting saquinavir activity have appeared only recently [7-8], therefore if this drug is used in clinical tests, a method to control the concentration of this drug in the plasma with an inexpensive LC/MS system could be very useful for optimization and monitoring of such treatment.

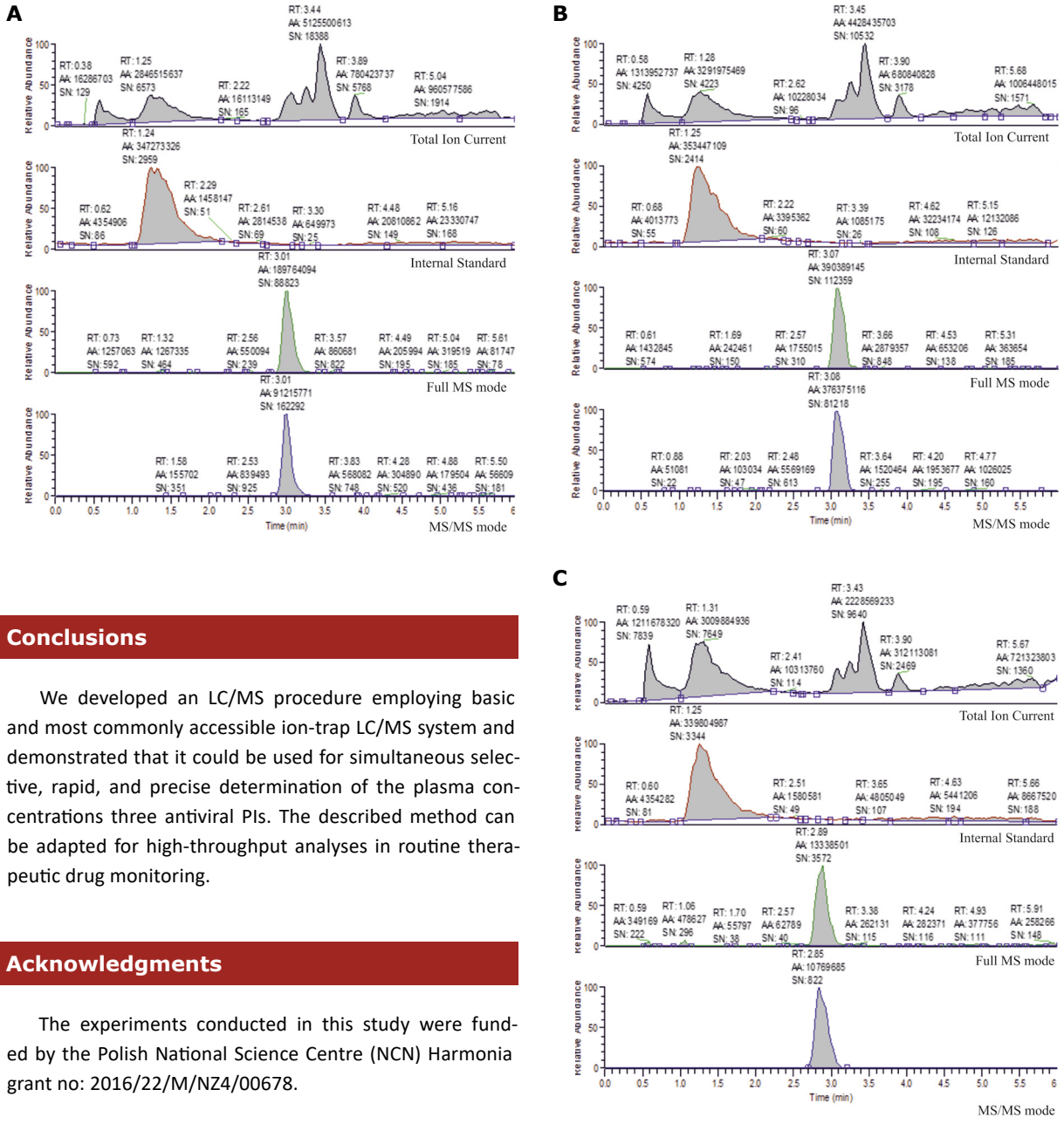

Figure 4. LC/MS chromatograms for plasma extract from a patient treated with: A. lopinavir, B. saquinavir, and C. ritonavir. Each chromatogram consists of: Total Ion Current, Internal standard, Full MS mode, and MS/MS mode

Conclusions

We developed an LC/MS procedure employing basic and most commonly accessible ion-trap LC/MS system and demonstrated that it could be used for simultaneous selective, rapid, and precise determination of the plasma concentrations three antiviral PIs. The described method can be adapted for high-throughput analyses in routine therapeutic drug monitoring.

Acknowledgments

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RESEARCH ARTICLE

Is there any variability in the level of cortisol, corticosterone and cortisone of healthy volunteers versus women and men with elevated cholesterol?

Lucyna Konieczna[1], Aleksandra Puch-Walczak[1], Tomasz Zdrojewski[2], Tomasz Bączek[1]

[1] Department of Pharmaceutical Chemistry, Medical University of Gdańsk, Gdańsk, Poland
[2] Department of Prevention and Didactics, Medical University of Gdańsk, Gdańsk, Poland

Abstract

Background: Cardiovascular diseases with the accompanied elevated level of total cholesterol have been a major problem in society for the last several decades. They belong to the diseases of civilization which affect people at an increasingly young age. For this reason, our aim was to investigate whether the concentrations of selected steroids are related to elevated total cholesterol in people without diagnosed cardiovascular diseases. **Material and methods:** The study involved 71 plasma samples. 19 of them were obtained from women and men with elevated cholesterol levels, whereas 52 samples were from healthy volunteers (control group). Liquid chromatography coupled with mass spectrometry (LC-MS) validated method followed by solid-phase extraction procedure were applied to measure the plasma concentrations of the three endogenous glucocorticosteroids (cortisol, corticosterone and cortisone). **Results:** Statistically significant differences between the concentration of cortisol were noted among healthy women and women with elevated cholesterol. The measured concentrations of cortisol in healthy women and men are comparable, 111.19 ng/mL and 112.22 ng/mL. respectively. However, the concentrations of cortisol in the elevated cholesterol group was significantly lower among women with elevated cholesterol than in healthy women (74.13 ng/mL and 111.19 ng/mL respectively). The concentration of cortisol for men with elevated cholesterol was 38.60 ng/mL. Hence, it is much higher than in women with elevated cholesterol and higher than in the case of healthy men. Distinctive changes can be observed also for corticosterone measured for both women and men. **Conclusions:** The observed differences on the level of steroids between healthy control group and patients with elevated cholesterol can be considered as worthy of further investigation from both biochemical as well as clinical points of view.

Keywords: Cardiovascular diseases · Elevated cholesterol · Steroids · Women and men

Citation

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Corresponding author:
Tomasz Bączek, Department of Pharmaceutical Chemistry, Medical University of Gdańsk, Gdańsk, Poland
e-mail: tbaczek@amg.gda.pl
Available online: www.ejtcm.gumed.edu.pl

Introduction

According to the data from World Health Organization, cardiovascular diseases are one of the most common causes of death worldwide and accounts for more than 50% of all deaths annually [1]. Biomarkers of cardiovascular disease play a vital role in accurate risk stratification. The biomarkers of cardiovascular disease include lipid panel [2], cardiac troponin [3], C-reactive protein [4], interleukin 6 [5], natriuretic peptides [6], homocysteine [7] or creatine kinase [8].

Steroid hormones are relatively easily accessible in biological materials and serve as a valuable source of information on patient's state of health and can also facilitate making the correct diagnosis. Of all steroid hormones, aldosterone has the greatest share in the pathogenesis of cardiovascular disease. Apart from its role in increasing in blood pressure (via increased potassium loss and the retention of sodium and water in the kidneys), its proinflammatory activity is also conducive to fibrosis of the heart muscle [9]. Endogenous steroids were of interest in clinical and preclinical studies as biological indicators (biomarkers) of various diseases resulting from steroid disturbances e.g. bladder and renal cancers [10-11]. Data concerning hypothesis that hormone-related factors can be associated with stress (e.g. measuring urinary cortisol, cortisone and corticosterone in parachutists and depressed patients) were also documented as well as it was suggested that steroid hormones influence the growth of neuroendocrine tumors [12-13].

A number of analytical methods can be developed to determine endogenous steroid hormone in different biological fluids, including gas chromatography, liquid chromatography and capillary electrophoresis. Liquid chromatography considered as reversed phase liquid chromatography (RP-LC) [14], especially in combination with mass spectrometry is commonly used for biomedical and pharmaceutical purposes. Attempts to find the alternatives are also considered, like the use of micellar liquid chromatography (MLC) [15]. However, liquid chromatography coupled with mass spectrometry (LC-MS) is yet considered to be the most effective method for biomedical goals with respect to steroid analysis due to its specificity and versatility [16-17].

The aim of the study was to investigate whether the concentrations of selected steroids (corticosterone, cortisol and cortisone) are related to elevated total cholesterol in people without diagnosed cardiovascular diseases.

Material and methods

A Chemicals and reagents

Standard substances (with high performance liquid chromatography purity) of cortisol, corticosterone and cortisone

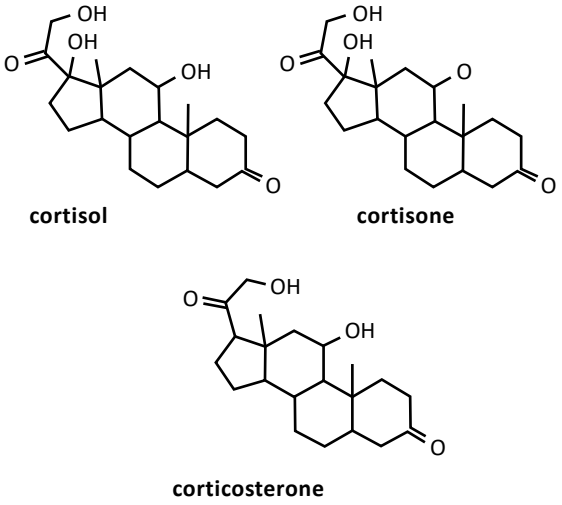

Figure 1. Chemical structures of three glucocorticosteroids considered in the study: cortisol, corticosterone and cortisone

(Figure 1) were obtained from Sigma (St. Louis, USA). Formic acid, methanol, acetonitrile and chloroform with HPLC purity were purchased from Sigma (St. Louis, USA). Ultrapure water was obtained from the Milli-Q purification system (Millipore, Bedford, USA).

Standard solutions of all analytical steroid standards: corticosterone, cortisol, cortisone were prepared in the following concentrations: 1 mg/mL, 10 µg/mL, 1 µg/mL and 100 ng/mL. Acetonitrile was used as a solvent to prepare 1 mg/mL reference solutions. The detection and quantification limits were determined based on a low concentration liquid chromatography coupled with mass spectrometry (LC-MS) analysis (0.05-10 ng/mL) of standard steroid hormone solutions. No interference between the steroids, the internal patterns and endogenous compounds in the matrix was demonstrated.

LC-MS equipment

A reversed phase liquid chromatography system (RP-LC) from Agilent Technologies, Model 1260 Infinity (Agilent Technologies, Santa Clara, USA) was used, with the 1260 Bin Pump G1312B, 1260 Degasser G1322A, 1260 Autosampler G1329A, 1290 Thermostat G1330B, 1260 VWD G1314F, and a Poroshell 120 EC-C18 chromatography column (Agilent Technologies, St. Clara, USA) measuring 3.0 × 100 mm; 2.7 µm.

The assays were performed using combined techniques: a liquid chromatograph coupled with a mass spectrometer (Agilent Technologies, Santa Clara, USA), equipped with an electrospray ion source and a single quadrupole analyzer (Single Quadrupole 6120 LC-MS, G6120B). Chemstation Rev. B.04.02 SP1 software was used to collect and process the chromatographic data. (Agilent Technologies, Santa Clara,

USA). The mobile phase consisted of 0.1% formic acid in Millipore grade water (eluent A) and 0.1% formic acid in LC-MS grade methanol (eluent B).

The analytical method was validated with respect to linearity, accuracy, precision and specificity. Based on the obtained results, the standard deviation and the limit of detection (LOD) and limit of quantitation (LOQ) values were calculated, which were respectively 0,06 ng/mL and 0,2 ng/mL. The analytical method used had a linear relationship between the amount of analyte present in the sample and the area of the resulting chromatographic peak.

Solid-phase extraction equipment

Plasma samples were prepared based on solid-phase extraction method. For solid-phase extraction we used Supelco Discovery DSC-18, 500 mg 6 ml standard polypropylene (PP) – tubes (Sigma, St. Louis, USA), Supelco Supel – Select HLB, 500 mg 6 ml standard PP – tubes (Sigma, St. Louis, USA), LiChrolut RP-18, 500 mg 6 ml standard PP – tubes (Merck, Darmstadt, Germany) and Sep-Pak C-18, 500 mg 6 ml standard PP – tubes (Waters, Milford, USA), along with the Vacuum Extraction Kit – VacElut SPS 24 Manifold (Agilent Technologies, Santa Clara, USA). The Vacuum Concentrator was from CentriVap Aqueous System (Labconco, Kansas City, USA). The corresponding optimization of sample preparation involved the choice of the appropriate extraction sorbent.

In the case of the Supelco Supel – Select HLB, LiChrolut RP-18 and Sep-Pak C-18, the preliminary analysis of certified blank plasma samples indicated that the matrix was not cleaned sufficiently well. Additionally, the analysis of plasma sample spiked with known concentration of analytes confirmed that their measured concentration levels were lower using mentioned the above columns in comparison to the Supelco Discovery DSC-18. Hence, the latter choice of the extraction sorbent was Supelco Discovery DSC-18 due to higher back-concentration achieved and closer to the real spiked one.

Sample preparation

The plasma samples were centrifuged (10,000 rpm, 7 min). Then, 250 µL of each sample was put into the described rotor tube. Next, 1 µg/mL of betamethasone standard solution was added as internal standards. The tubes were mixed on a Vortex laboratory shaker (IKA Werke GmbH & Co.KG, Staufen, Germany) for 30 seconds, then each of them was measured with 3 mL of deionized water and remixed on the Vortex shaker (30 s). The tubes were transferred to a shaker (amplitude 300, 10 min). At the end of shaking the tubes were centrifuged (10,000 rpm, 7 min).

Supelco Discovery DSC-18 columns were used for the extraction of plasma samples, which were activated with 2×2 mL methanol and 2×2 mL deionized water, not allowing the bed to dry. Then the plasma specimens were prepared on the columns. The samples were purified with 2×2 mL deionized water. In the next stage, the bed was dried (15 min). After this time, the analytes were eluted from the bed using 2×1 mL of methanol. The contents of the eluate tubes were then evaporated to dryness in a vacuum concentrator at 50 °C.

The dry evaporation residue was dissolved in 100 µL of 80% methanol and mixed on a Vortex laboratory shaker (30 s), then transferred to Eppendorf tubes and centrifuged (10,000 rpm, 7 min). The contents of the tubes were then placed in the inserts and analyzed by LC-MS.

Study population and sample collection

71 plasma samples were collected, including 19 from women and men with elevated cholesterol (total cholesterol levels more than 180 milligrams per deciliter, identified on the basis of the provided biochemical parameters within the diagnostic analysis) and 52 from healthy volunteers. Women and men with elevated cholesterol were not yet diagnosed with any cardiovascular diseases (e.g. ischemic heart disease, coronary artery disease, myocardial infarction, hypertension, atrial fibrillation). All participants were diagnosed at the Department of Hypertension and Diabetology of the Medical University of Gdańsk.

All statistical analyses were conducted using STATISTICA 10.0 software (TIBCO Software Inc, Palo Alto, USA). The hormone levels were log-transformed prior to statistical analyses. Using log-transformation we obtained normal distribution of individual concentration data. The statistical significance of intergroup differences was verified with the parametric tests with $p < 0.05$. The Student's t-test for independent samples was used as the inference tests, according to assumptions of normal distribution – checked with Shapiro-Wilk test and Kolmogorov-Smirnov test. Homoscedasticity (homogeneity of variance) was checked with Levene's test and Brown-Forsythe test. If the assumption of homogeneity of variance was not fulfilled, Welch's t-test (unequal variances t-test) was applied. If the assumption of normal distribution was not fulfilled, Mann-Whitney U test was applied.

The samples were prepared to determine the calibration curves, which in turn allowed for the calculation of mean concentrations of individual steroid hormones and standard deviations by the least squares method.

Results

The obtained results expressed as mean concentrations of individual glucocorticosteroids measured in ng/mL are collected in Table 1. As it can be seen, The mean concentration of cortisol in healthy women was 111.19 ng/mL and it was highly similar to the mean concentration of that glucocorticosteroid measured in men (112.22 ng/mL). However, a different trend can be observed for the concentrations of cortisol in the elevated cholesterol group: the mean concentration

Table 1. Collected data for three tested glucocorticosteroids in healthy volunteers and patients with elevated cholesterol

	Women		Men	
Glucocorticosteroid	Healthy volunteers (N = 28)	Elevated cholesterol (N = 9)	Healthy volunteers (N = 23)	Elevated cholesterol (N = 10)
Cortisol [ng/m]	Mean = 111.19 SD = 57.42	Mean = 74.13 SD = 42.95	Mean = 112.22 SD = 47.75	Mean = 138.60 SD = 106.26
Corticosterone [ng/mL]	Mean = 6.50 SD = 5.07	Mean = 4.48 SD = 3.48	Mean = 13.30 SD = 32.52	Mean = 6.72 SD = 8.42
Cortisone [ng/mL]	Mean = 21.2905 SD = 9.1669	Mean = 22.3572 SD = 13.1252	Mean = 21.4927 SD = 9.9549	Mean = 23.1119 SD = 13.5998

of cortisol was much lower among women with elevated cholesterol than in healthy women (74.13 ng/mL and 111.19 ng/mL respectively). On the contrary, for men with elevated cholesterol the concentration of cortisol was 38.60 ng/mL. Hence, it was much higher than in women with elevated cholesterol and higher than in the case of healthy men.

Considering corticosterone, generally lower mean concentrations of that glucocorticosteroid were noted in comparison to the mean concentrations of cortisol (Table 1). The mean concentration of corticosterone assessed in healthy women was 6.50 ng/mL and was higher than the mean concentration of that hormone measured in women with elevated cholesterol (4.48 ng/mL). Similar trend was observed for the group of men. Here also the mean concentration of corticosterone was higher in healthy volunteers in comparison to men with elevated cholesterol (13.30 ng/mL vs. 6.72 ng/mL). It is noteworthy that this difference the concentrations of corticosterone was greater between the studied groups of men than groups of women.

The most stable concentration results were obtained for cortisone (Table 1). In all four tested groups of participants, the mean concentrations of cortisone were in the range of 21.29-23.11 ng/mL. Specifically, for healthy women the mean concentration of cortisone was 21.29 ng/mL, and for women with elevated cholesterol was 22.35 ng/mL. Hence, only slightly higher mean concentration of cortisone was found for women with elevated cholesterol. Similar situation was noted among men: the mean concentration of cortisone was 21.49 ng/mL for healthy men and 23.11 ng/mL in men with elevated cholesterol. Once again, as in the case of women, only a slightly higher mean concentration of cortisone was found in men with elevated cholesterol.

The most distinctive differences were noted in cortisol level for tested groups of women. A statistically significant decrease in the concentration of cortisol in women with elevated cholesterol in contrary to healthy women was noted ($p < 0.03$; Student's t-test). It is not a very strong significance, as confirmed additionally by a box plot (see Figure 2). Nevertheless, it is a trend indicating a potential influence of variable cortisol concentration in both studied groups. Although the mean concentration of cortisol evaluated in men with elevated cholesterol is much higher in comparison to the concentration of cortisol in women with elevated cholesterol, there were no statistically significant differences noted between the healthy men and men with elevated cholesterol ($p < 0.96$; Welch's t-test).

No statistically significant differences in corticosterone concentrations were found in the two groups of interest. Hence, no statistically significant differences were noted between healthy volunteers and women and men with elevated cholesterol in the case of women ($p < 0.26$; Student's t-test). Although the absolute difference between mean concentrations of corticosterone measured for men is almost twice higher for the group of healthy volunteers in comparison to women and men with elevated cholesterol, again no statistically significant differences are found ($p < 0.91$; Mann-Whitney U test).

Finally, the results for cortisone are similar for all tested groups, as confirmed via statistical analysis. No statistically significant differences for the mean concentrations of cortisone can be observed between healthy volunteers and women and men with elevated cholesterol in the case of women ($p < 0.92$; Student's t-test). Also, no statistically significant

differences for the mean levels of cortisone were found analyzing healthy volunteers and women and men with elevated cholesterol in the case of men ($p < 0.96$ Student's t-test).

Discussion

In our study, we demonstrated that cortisol level is higher in healthy female volunteers than in those with elevated cholesterol. There were no other significant differences in cortisol levels between men. Corticosterone and cortisone level was similar in any studied subgroup.

Cortisol, an essential glucocorticoid, is regulated by two izoenzymes that affect its metabolism. However, individual daily changes of cortisol level could alter diagnostic studies, including our findings [18]. It has been demonstrated that hair or plasma cortisol level correlates with stress, obesity, depression, cardiac diseases, stroke and diabetes [19-20]. Owing to the importance of glucocorticosteroids on human health, we showed that the elevated cholesterol level correlates with decreased cortisol level in women. The current dogma is that when cortisol is released, it elevates the cholesterol level. The described physiological reaction to stress has short duration, although long-term effects such as diseases are well-known consequences. On the cellular level, only a few studies directly explain the possible connection between cholesterolsynthesis, release and its influence on cortisol [21]. Oxysterol-related-binding-protein, a lipid binding protein, was found to influence both cholesterol homeostasis and cortisol synthesis [21]. Interestingly, this protein contributed to the reduction of cellular level of oxysterols and binds 11-deoxycholesterol. In another study, ATR-101 in adrenocortical cells was investigated to control of cholesterol level [22]. Based on our results, we assume that plasma cortisol level is subject to daily changes. Furthermore, a significantly lowered cortisol level in females while the cholesterol was elevated, although an interesting finding, could be in fact accidental. the adrenal cortex prevents accumulation of toxic level of cholesterol and is therefore unique in its multifunctional role. To date, this relation remains poorly investigated.

On the other hand, we demonstrated that cholesterol level is not altered by corticosterone and cortisone levels. Our findings conflict with studies on the cellular level using laboratory animals. Corticosterone administration for two weeks dysregulated cholesterol levels in chickens, in whom cholesterol was accumulated in muscle cells [23]. The administration of cortisone influenced total cholesterol level in rabbits [24]. More studies on humans are yet to be published. One study clearly demonstrated that cortisone-to-cortisol ratio in females did not correlate with type 2 diabetes mellitus [26]. On the contrary, in our study corticosterone level was reduced in both men and women.

On a population level, hypercholesterolemia remains a clinical challenge. According to large cohort observational studies, an increased cholesterol level (≥ 190 mg/dl) was diagnosed in one-half of the screened population [25]. It seems that public awareness campaigns and further research developing better cholesterol control and finding its relations with glucocorticosteoids are needed.

Conclusions

We noted statistically significant differences in the case of tested levels of cortisol between healthy women volun-

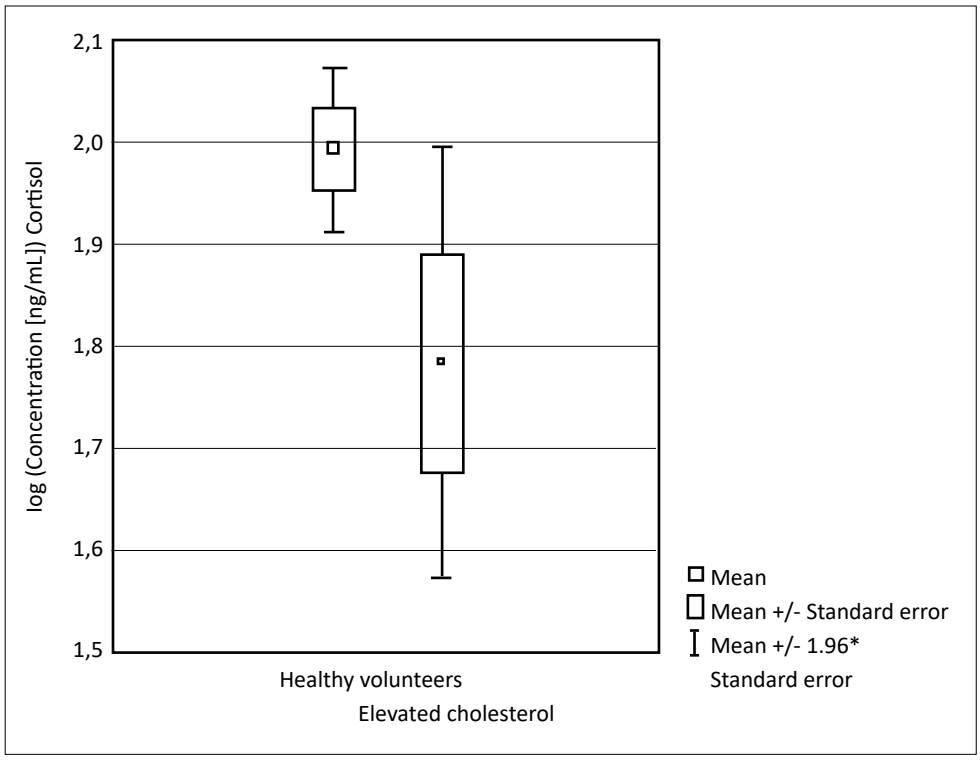

Figure 2. Box plot showing the statistical significance of the cortisol levels in healthy women vs. women with elevated cholesterol

teers and women with elevated cholesterol. In other groups evaluated within one gender (women or men), no statistically significant differences were found. The found differences in the level of steroids between healthy volunteers and people with elevated cholesterol can be considered worthy of further investigation from both biochemical as well as clinical points of view.

Conflicts of interest

Conflict of interest: none declared.

The authors declare that the study complies with the Declaration of Helsinki. A locally-appointed ethics committee approved the research protocol and informed consent was obtained from the subjects.

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REVIEW ARTICLE

The effect of probiotic supplementation as alternative therapy for NAFLD: a literature review

Muhammad Luthfi Adnan

[1] Universitas Islam Indonesia

Abstract

Background: Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver diseases, influenced by various risk factors associated with metabolic disorders. Currently there is no specific effective treatment for NAFLD. Probiotics have been extensively researched for their health benefit Probiotic Supplementation for NAFLD. **Methods:** A simple literature review was performed based on searches via PubMed, ScienceDirect and Google Scholar, using the keywords "probiotic," "microbiota," "non-alcoholic fatty liver disease," "metabolic disorder," and "therapy." **Results:** Research on the use of probiotics for NAFLD demonstrated improvement in liver function and histology. However, the literature is inconsistent regarding the probiotics' influence on the NAFLD risk factors. Probiotics can be an alternative therapy for NAFLD through the ability to modulate the microbiota of the gastrointestinal tract. **Conclusion:** Probiotics can be an alternative therapy in NAFLD patients, however more extensive studies are needed to ensure the safety of this approach.

Keywords: Non-alcoholic fatty liver disease · NAFLD · probiotic · therapy

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease in the world [1]. NAFLD is characterized as ≥ 5% fat accumulation from hepatocytes with no other secondary diagnostic cause having similar histological characteristics such as viral hepatitis infection, excessive alcohol consumption, other chronic liver diseases (e.g. Wilson's disease, Celiac disease) and drug therapy (e.g. corticosteroids, methotrexate, isoniazid and anti-retrovirals [2-3]. The prevalence of NAFLD has increased in the last 20 years to reach 25.54% worldwide, with the largest number of cases in the Middle East and South America [4]. NAFLD can cover a wide clinical spectrum in the course of disease severity from non-alcoholic steatohepatitis (NASH), fibrosis, cirrhosis to hepatocellular carcinoma [5].

Corresponding author:
Muhammad Luthfi Adnan, Univesritas Islam Indonesia, Yogyakarta, Indonesia
e-mail: luthfiadnan35@yahoo.co.id
Available online: www.ejtcm.gumed.edu.pl

The main risk factors for NAFLD are type 2 diabetes mellitus and the components of metabolic syndrome (e.g. central obesity, dyslipidemia, insulin resistance), while genetic factors, age and alcohol consumption can worsen the progression of NAFLD to fibrosis and cirrhosis [6]. Metabolic syndrome can be caused by a sedentary lifestyle, a diet high in fat and calories and infrequent exercise. NAFLD is also more common in men than women, although post-menopausal women have the same risk of developing NAFLD as men [2]. With the increasing trend of obesity and diabetes, there is a need for improvements in effective management and prevention efforts to address risk factors and reduce the incidence of NAFLD [7].

Currently, there is no definitive pharmacological therapy to treat NAFLD. Instead, patients are offered therapies to reduce the risk of NAFLD, e.g. by increasing insulin sensitivity and decreasing inflammation [8]. Current findings suggest that the microbiota in the gut may influence the course of NAFLD disease [9]. This condition is influenced by an unhealthy diet that affects the development of the gut microbiota and affects the metabolism of nutrients in the gut [10]. Probiotics are one of the supplements that can be used to modify the diet of NAFLD patients [11]. Several studies have shown the effect of probiotics on the gut microbiota in patients with diabetes, obesity, cardiovascular disease and chronic liver disease [12]. This review aims to discuss the effect of the gut microbiota on NAFLD and the role of probiotics on NAFLD.

Material and methods

A simple literature review was performed. Literature search was carried out from December 2019 to March 2020 using the PubMed, ScienceDirect and Google Scholar search engines and the keywords "probiotic," "microbiota," "non-alcoholic fatty liver disease," "metabolic disorder" and "therapy." The inclusion criteria used were: full-text in English or Indonesian, article published within the last 10 years. Articles in other languages or published earlier than 10 years ago were excluded. The articles included in the analysis were: randomized controlled trials (RCTs), meta-analyses, literature reviews and systematic reviews.

Results

A total of 13 articles were included in the analysis (see Table 1).

Table 1. Studies on the effects of probiotics in NAFLD patients

Author (year of publish)	Type of probiotic	Control	Duration	Result
Aller *et al* (2011) [47]	500 million *Lactobacillus bulgaricus* and *Streptococcus thermophilus*	Placebo	3 months	↓ liver enzymes, ↑ cholesterol levels. There is no significant change in anthropometrics
Vajro *et al* (2011) [48]	*Lactobacillus rhamnosus* strain GG	Placebo	8 weeks	↓ ALT, ↓ BMI and TNF-α were not significant
Malaguarnera *et al* (2012) [52]	*Bifidobacterium longum* with fructooligosacchaarides	Lifestyle modification	24 weeks	↓ ALT, AST, total cholesterol, HOMA-IR, pro-inflammatory cytokines, steatosis and NASH activity index
Alisi *et al* (2014) [45]	VSL#3 (four strain of *Lactobacillus* (*Lactobacillus acidophilus*, *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus delbrueckii subspecies bulgaricus*), three strains of *Bifidobacterium* (*Bifidobacterium breve*, *Bifidobacterium longum* and *Bifidobacterium infantis*), and a strain of *Streptococcus* (*Streptococcus salivarius subspecies thermophilus*))	Placebo	4 months	↓ NAFLD severity, ↓ BMI, ↑ *glucagon-like peptide* 1 (GLP-1) and *activated* GLP-1 (aGLP-1). There is no significant change in ALT and triglyceride

Author (year of publish)	Type of probiotic	Control	Duration	Result
Nabavi *et al* (2014) [50]	Probiotic yogurt (*Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *Bifidobacterium lactis* Bb12 and *Lactobacillus acidophilus* La5)	Convetional yogurt (*Lactobacillus bulgaricus* and *Streptococcus thermophilus*)	8 weeks	↓ ALT, AST, total cholesterol, and LDL. Changes in serum glucose, triglycerides, and HDL were not significant
Sepideh *et al* (2015) [54]	*Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium longum,* dan *Streptococcusthermophilus*	Placebo	2 months	↓ Fasting blood glucose, insulin, HOMA-IR and IL-6 between groups with no significant change in TNF- α level.
Manzhalii *et al* (2017) [46]	*Lactobacillus casei, L. rhamnosus, L. bulgaricus, Bifidobacterium longum,Streptococcus thermophilus* and fructooligosaccharides with low calorie diet	low calorie diet	12 weeks	↓ BMI, ALT, AST, total cholesterol. There is no significant change in triglycerides, glucose, and gamma-glutamyl transferase (GGT)
Famouri *et al* (2017) [49]	*Lactobacillus acidophilus* ATCC B3208, *Bifidobacterium lactis* DSMZ 32269, *Bifidobacterium bifidum* ATCC SD6576, *Lactobacillus rhamnosus* DSMZ 21690	Placebo	12 weeks	↓ ALT, AST, LDL, triglycerides and waist circumference. There is no significant change in BMI and body weight
Asgharian *et al* (2017) [51]	*Lactobacillus casei, Lactobacillus acidopholus, Lactobacillus rhamnosus, Lactobacillus bulgaricus, Bifidobacterium breve, Bifidobacterium longum, Streptococcus thermophiles* and fructooligosaccharides	Placebo	5 months	↓ body weight, body fat, and total cholesterol. There is no significant change in triglycerides, fasting blood glucose and HDL-c
Behrouz *et al* (2017) [53]	*Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus acidophilus, Bifidobacterium* longum, *dan Bifidobacterium brev*	Placebo and prebiotic	12 weeks	↓ leptin serum concentrations, insulin and HOMA-IR, ↑ *Quantitative insulin-sensitivity check index* (QUICKI)
Kobyliak *et al* (2018) [59]	*Bifidobacterium, Lactobacillus, Lactococcus,* dan *Propionibacterium*	Placebo	8 weeks	↓ liver enzyme, fat liver, IL-6 and TNF-α. There is no significant change in lipid profile and anthropometrics

Author (year of publish)	Type of probiotic	Control	Duration	Result
Ahn *et al* (2019) [56]	*L. acidophilus* CBT LA1, *L. rhamnosus* CBT LR5, *L. paracasei* CBT LPC5, *P. pentosaceus* CBT SL4, *B. lactis* CBT BL3, dan *B. breve* CBT BR3	Placebo	12 weeks	↓ intrahepatic fat fractions and visceral fat. There is no significant change in insulin HOMA-IR, IL-6, and TNF-α
Duseja *et al* (2019) [55]	*Lactobacillus paracasei* DSM 24733, *Lactobacillus plantarum* DSM 24730, *Lactobacillus acidophilus* DSM 24735 and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 24734, *Bifidobacterium longum* DSM 24736, *Bifidobacterium infantis* DSM 24737, *Bifidobacterium breve* DSM 24732, and *Streptococcus thermophilus* DSM 24731	Placebo	1 year	↓ hepatocyte ballooning, lobular inflammation, fibrosis, NAFLD *activity score* (NAS), pro-inflammatory cytokines, liver enzyme and leptin

Discussion

Pathophysiology of NAFLD and Effect on Gut Microbiota

NAFLD is influenced by various metabolic disorders [13]. NAFLD is associated with metabolic disorders resulting from worsening metabolic syndrome, e.g. obesity and insulin resistance [14]. The condition of obesity caused by unhealthy consumption patterns with a diet high in fat and calories plays a role in an increase in insulin resistance in the periphery and an increase in the proliferation of adipose cells in the tissue [15]. In obesity there is an increase in the release of free fatty acids (FFA) and cholesterol due to insulin resistance and causes the liver to increased *de novo* lipogenesis activity [13]. Increased lipogenesis in the liver then increases fat storage in hepatocyte cells, which in turn causes the liver to undergo steatosis [16].

Obesity can also lead to insulin resistance through increased release of pro-inflammatory tumour necrosis factor (TNF)-α, C-reactive protein (CRP), interleukin (IL)-6 cytokines, plasminogen activator inhibitor-1 (PAI-1) and many inflammatory mediators in obese patients [17]. The release of proinflammatory cytokines is caused by hyperplasia and hypertrophy of adipose cells due to excess fat accumulation in adipose cells [18]. Inflammation in obesity interferes with insulin activity through several mechanisms, namely interfering with insulin receptor substrate-1 (IRS-1) signaling which functions as an insulin receptor and peroxisome proliferator-activated receptor γ (PPARγ) function which plays a role in directing fat storage and lipid synthesis and FFA levels. through stimulation of lipolysis and an increase in triglycerides [18-20]. Obesity condition also results in increased insulin secretion from β cells in the pancreas which is stimulated by increased levels of fatty acids and glucose in the blood [21]. Inflammation and increased insulin production in the pancreas lead to the decrease in insulin receptor sensitivity and hyperinsulinemia [22]. This condition then leads to insulin resistance which in turn results in increased lipogenesis of fat in the liver and increases the risk of NAFLD [16].

Ma *et al* demonstrated the influence of the gut microbiota in the course of NAFLD disease [9]. In the gut, four main phyla bacteria are consisting of *Firmicutes, Bacteroidetes, Actinobacteria*, and *Proteobacteria* with 90% of the bacterial population consisting of *Firmicutes* and *Bacteroidetes* [23]. In the condition of obesity, there is a change in the composition of the intestinal microbiota with changes in the ratio of Firmicutes/Bacteroidetes (F/B ratio) found in NAFLD conditions [24]. Such dysbiosis of the intestinal microbiota can damage the tight junctions in the intestinal epithelium and increase the permeability of the gastrointestinal tract [25]. Increased permeability then results in lipopolysaccharide (LPS) produced from bacteria and parts of bacteria such as DNA and RNA in the intestine to enter the body and liver circulation through the gut-liver axis in the portal vein and activate inflammatory reactions systemically through activation of Toll-like receptors (TLRs) [26]. One of the TLRs that influence the pathophysiology of NAFLD is TLR4 which stimulates the release of proinflammatory cytokines TNF-α and IL-1β through the nuclear factor κβ (NF-κβ) pathway and activates the body's innate immune system [27].

Activation of the innate immune system against NAFLD is influenced by Kupffer cells found in the sinusoid liver and stimulated by LPS from bacteria in the gut [28]. Kupffer cells will produce various types of chemokines and proinflammatory cytokines such as IL-1β, IL-6, IL-12 IL-18 and TNF-α which play a role in the formation of reactive oxygen species (ROS) and can cause oxidative stress in hepatocyte cells [15]. Increased production of ROS will then result in mitochondrial dysfunction due to interference with antioxidants in cells and excess lipid peroxidation due to high levels of FFA in the liver so that liver cells are damaged [29]. Also, injury to liver cells due to oxidative stress due to the production of ROS can result in increased disease progression through stimulation of fibrotic cells to liver cells resulting in fibrosis [30].

Dysbiosis in the intestinal microbiota plays an important role in increasing the absorption of monosaccharides in the intestine, accelerating the activity of lipogenesis de novo activity in the liver and increased triglycerides [31]. Intestinal microbiota dysbiosis affects the production of short-chain fatty acids (SCFAs) consisting of acetate, propionate and butyrate [32]. In patients with NAFLD, butyrate levels were lower than acetate and propionate level [32]. Butyrate acts as a source of energy for colonocytes in the intestines that maintain intestinal integrity and improve insulin sensitivity of β cells, thereby reducing the number of adipose cells in the body [33]. Meanwhile, acetate and propionate affect gluconeogenesis and lipogenesis which lead to NAFLD [32]. These SCFAs can increase absorption, can stimulate G-protein coupled receptors (GPRs) to increase fat and sugar intake from the intestine and increase storage in the adipose tissue [34]. Also, SCFA can activate carbohydrate response element-binding protein (ChREBP) in the liver and increase lipogenesis [35].

Dysbiosis in the gut microbiota can also result in disorders of choline metabolism [32]. Choline is a type of membrane-forming phospholipid and a precursor to the neurotransmitter acetylcholine [32]. Choline also plays a role in the synthesis of VLDL and the transport of lipids to the liver to prevent fat accumulation in the liver [32]. In the dysbiosis of microbiota, choline obtained from the consumption of animal products can be converted into trimethylamine (TMA), which in the liver is converted into trimethylamine-N-oxide (TMAO) and triggers choline deficiency [36]. In conditions of choline deficiency and elevated TMAO, hepatocyte damage occurs and liver steatosis increases [37]. Choline deficiency also affects the permeability of the intestinal barrier which can increase intestinal bacterial infiltration to the liver via the gut-lung-axis which triggers lipid accumulation and steatosis in the liver [21]. With their findings on an important role in NAFLD gut microbiota modulation via the intestinal microbiota has become a potential therapeutic target for treating NAFLD.

Effect of Probiotics on NAFLD

Probiotics are defined as "live microorganisms when consumed can provide health benefits to the host" [38]. Probiotics have been widely used in the fermentation of foods such as cheese and yoghurt [39]. Several types of probiotic bacteria from the *Lactobacillus, Bifidobacterium, Lactococcus, Streptococcus* and *Saccharomyces* groups have been widely studied for their usefulness in the treatment of diarrhoea-associated with *Clostridium difficile*, gastroenteritis, irritable bowel syndrome (IBS), *Helicobacter pylori* infection, and respiratory tract infections [40]. Also, probiotics can be used in metabolic disorder due to their anti-obesity, anti-diabetic, and anti-hyperlipidemic activity [41]. With the benefits of probiotics in the improvement of conditions of metabolic disorders, many studies have shown the benefits of probiotics for NAFLD therapy [42].

The role of probiotics in reducing the risk factors associated with NAFLD has been widely assessed [43]. Obesity is one of the main risk factors for NAFLD [44]. Several human studies have shown probiotics to have anti-obesity effects that are associated with a reduced risk of NAFLD, although other studies have shown weight loss does not correlate with NAFLD improvement [43]. In a study by Alisi *et al*, administration of VSL #3 consisting of 8 probiotic strains has a weight loss effect in obese children with NAFLD after probiotic administration along with a reduced risk of progressive severity of NAFLD and liver steatosis [45]. The study by Manzhalii *et al* also showed decreased BMI accompanied by liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes and decreased liver stiffness [46]. However, the relationship between weight loss and improved course of NAFLD does not always have a significant effect. In the studies by Aller *et al,* Famouri *et al* and Vajro *et al*, improvement of hepatic function through decreased ALT and AST enzymes in the probiotic consumption group was not influenced by significant weight loss and cardiovascular risk in NAFLD patients [47-49].

Probiotics also have the benefit of improving lipid profiles in NAFLD patients. A study by Nabavi et al showed an improvement in total cholesterol and low-density lipoprotein cholesterol (LDL) followed by a decrease in liver enzyme levels after 8 weeks, although not followed by changes in triglycerides, serum glucose, and high-density lipoprotein cholesterol (HDL) compared to the control group [50]. The study by Asgharian et al and Manzhalii *et al* also showed a decrease in total cholesterol, body weight and total body fat in NAFLD patients although there were no significant changes in the levels of HDL, triglycerides, and fasting blood glucose [46, 51]. Also, Asgharian *et al* noted that in the control group there was an increase in total cholesterol, waist circumference and LDL, thus demonstrating the effect of probiotics to prevent worsening of the disease in NAFLD patients

[51]. Another study by Famouri et al also showed improvements in the lipid profiles of NAFLD patients although without any significant changes in body weight.[49] However, in the study by Alisi *et al*, the consumption of probiotics led to insignificant results on triglycerides despite an improvement in the condition of NAFLD patients and a reduction of disease progression [45]. Aller *et al* also showed no significant improvement in cholesterol levels and other cardiovascular risks after probiotic administration, although there was the improvement in liver function in NAFLD patients [47].

Probiotics can also influence insulin resistance in NAFLD patients. A study by Malaguarnera *et al* showed that giving probiotics with the addition of fructooligosaccharides can improve insulin resistance with a decrease in the homeostasis model assessment of insulin resistance (HOMA-IR) accompanied by improved levels of liver enzymes, lipids and proinflammatory cytokines in NAFLD patients [52]. The study by Behrouz *et al* also demonstrated improvement of insulin resistance in NAFLD patients with decreased insulin and HOMA-IR levels and increased insulin sensitivity [53]. The study by Sepideh *et al* also showed an improvement in the glycemic index accompanied by a decrease in fasting blood glucose and proinflammatory cytokine levels after the administration of probiotics [54]. However, the effects of insulin repair were inconsistent. This was shown in a study by Duseja et al where improvement of hepatic function with decreased hepatocyte ballooning, lobular inflammation, and NAFLD activity score (NAS) did not affect HOMA-IR improvement [55]. The study by Ahn *et al* and Alisi *et al* also showed no significant effect of improved HOMA-IR and decreased insulin levels on the improvement of hepatic function and intrahepatic fat [45, 56]. Although studies on NAFLD-model rats have shown the effect of improving insulin resistance on liver repair in NAFLD, further studies are needed regarding the relationship between NAFLD improvement and insulin resistance in humans [57].

Probiotics also have anti-inflammatory effects in NAFLD patients who have improved liver condition and reduce the risk of disease progression [58]. The study by Malaguarnera *et al* and Sepideh *et al* showed the anti-inflammatory effect of probiotics on the improvement of liver function in NAFLD patients, although in the study of Sepideh *et al*, the decrease in levels of proinflammatory cytokine TNF-α between groups was not significant in the intervention group there was a significant decrease compared to before the intervention. Studies by Kobyliak *et al* also demonstrated the effect of lowering pro-inflammatory cytokines on reducing levels of liver enzymes and fat accumulation [59]. However, the results were inconsistent in other studies, e.g. by Vajro *et al* and Ahn *et al* in which the anti-inflammatory effect was not significant despite the improvement in liver function and histological features in NAFLD patients [48, 56]. Although studies in animal models have shown an anti-inflammatory effect related to the course of NAFLD disease, further studies in humans are needed to determine the effectiveness of probiotics in NAFLD.

Effect of Probiotics on NAFLD with Modulation of Gut Microbiota

Although probiotics improve the liver function in NAFLD patients, the specific mechanism of this effect is unclear [43]. Several studies have shown the effect of probiotics via the repair of tight junctions and the integrity of the barrier in the gut [60-62]. Improvement of barrier integrity by probiotic bacteria in animal studies model NAFLD can reduce the level of liver steatosis by improving choline levels from the gut microbiota which correlates with decreased levels of fat accumulation in the liver and decreased steatosis [61-63]. Intestinal barrier repair can also improve the immune response in intestine to inhibit the growth and translocation of harmful bacteria to the liver and reduce the level of inflammation in the liver [36, 64-66].

Improvements in gut barrier integrity also reduce LPS from the gastrointestinal tract [67] which can reduce the level of inflammation in the NAFLD group [64]. Probiotics can reduce the levels of LPS that enter the liver through the portal vein and reduce TLR4 activation which results in activated Kupffer cells and result in hepatocyte cell damage [65]. Decreased levels of LPS and levels of inflammation can also affect the repair of microbiota in the gastrointestinal tract thereby inhibiting lipid disposition and chronic inflammation due to NAFLD [68].

The ability of probiotics to modulate the gastrointestinal microbiota also has anti-obesity effects on NAFLD through weight loss activities, inhibition of lipogenesis and decreased levels of inflammation [69]. Probiotic-mediated weight loss is influenced by the ability of probiotics to change the composition of the gut microbiota by increasing the number of *Bacteroides, Bifidobacteria* and *Lactobacilli* bacteria and decreasing the number of *Firmicutes* bacteria which correlates with weight loss [70–72]. Changes in the composition of the gut microbiota have the effect of decreasing fat accumulation in the liver and decreasing the expression of proteins in the liver that affects lipid and glucose metabolism such as PPARγ, PPARα, fatty acids synthase (FAS) and glycogen synthase kinase-3 (GSK-3) [72]. Apart from inhibiting lipogenesis, the antiobesity effect of probiotics also reduces leptin and resistin levels which play a role in fat disposition in the body and affect food intake to lose weight [64, 73-74]. The reduction in lipogenesis and fat accumulation in the body by probiotics also contributes to a decrease in the level of inflammation, which correlates with an increase in insulin sensitivity [68, 74].

Several studies have shown that the ability of probiotic bacteria to produce SCFA plays a role in improving the condi-

tion of patients with NAFLD and of all the SCFAs butyrate has the greatest effect [75]. Butyrate has an anti-inflammatory effect through the regulation of anti-inflammatory cytokines in T cells via GPRs receptors, namely GPR41 and GPR43 so that it can maintain the integrity of the gastrointestinal mucosa and prevent bacterial translocation to blood vessels [33, 68, 75-76]. Several studies have shown the effect of butyrate on decreasing TLR4 expression to inhibit NF-κβ and release of proinflammatory cytokines in the liver, thereby reducing oxidative stress in the liver and preventing damage to the liver. Butyrate also affects the improvement of function and histology in the liver by increasing insulin sensitivity through the activation of glucagon-like peptide-1 (GLP-1), so that it can reduce the risk factors associated with NAFLD such as obesity and insulin resistance [77].

Although probiotic bacteria have beneficial effects on NAFLD, there are still some limitations to the use of probiotic bacteria as NAFLD therapy [43]. One limitation is that at this time there is no known, specific mechanism by which probiotics can modulate liver repair [43]. Further studies are also needed to ascertain the safety and side effects of using probiotics as NAFLD therapy [78]. Furthermore, although several studies showed improvement in the condition of NAFLD patients, their small sample sizes make interpretation of results a challenge [79]. Therefore, larger studies with more heterogeneous sample sizes are needed to determine the effectiveness of probiotics as part of NAFLD therapy.

Conclusion

Probiotics have been extensively researched for their wide-ranging health benefits. Several studies have shown the effect of probiotics on improved liver function and decreased progression of risk factors in NAFLD patients. Although there are no studies regarding the probiotics' specific mechanism of action in NAFLD, the ability of probiotic bacteria to modulate the microbiota in the gastrointestinal tract can lead to improvement in patients with NAFLD. Despite some limitations in the studies, probiotics can be a promising therapy against NAFLD.

Conflict of interest

There is no conflict of interest from any party.

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REVIEW ARTICLE

Application of X-ray photoelectron spectroscopy (XPS) and a functional near infrared spectroscopy (fNIRS) in medicine

Marek Szklarczyk[1,2]

[1] Laboratory of Electrochemistry, University of Warsaw, Poland
[2] Shim-Pol A.M. Borzymowski, Warsaw, Poland

Abstract

The development of modern clinical medicine and the truthful medical diagnosis require hard physicochemical data. Furthermore, the social demands for more accurate and collected data, which refer to human health in real conditions and in real time, force the quest for techniques which would be possible to be applied in a broadly understood clinical field. In this article two techniques are described. A high vacuum technique which is an X-ray photoelectron spectroscopy (XPS), and a functional near infrared spectroscopy (fNIRS), which might be applied *in vivo* condition. For the both techniques, the results pointing their applicability in the clinical and modern biochemical research are described.

Keywords: X-ray photoelectron spectroscopy · XPS · functional near infrared spectroscopy · fNIRS · brain activity

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Introduction

With the development in science, technique and technology, and above all people's anticipation and hopes for information about the quality of available products and information about the surrounding environment, as well as the knowledge of our own health condition, a strong demand has arisen for chemical imaging techniques at the end of the 20th century. Imaging techniques permit not only excellent structural research, qualitative and quantitative markings, but they also allow for determination of spatial placement of different substances in the tested samples. Furthermore, due to the eldering of the population and the increasing intensity of brain diseases, there is a call for techniques that allow real-time brain testing, *in vivo*.

At the turn of the nineteenth and twentieth centuries there was a period of a stormy development in physics and chemistry. At the time many theories were formulated,

Corresponding author:
Marek Szklarczyk, Laboratory of Electrochemistry, University of Warsaw, Poland
e-mail: mareks@shim-pol.pl
Available online: www.ejtcm.gumed.edu.pl

which have been the basis of modern physicochemical analytical methods. Thanks to the progress in science in the first half of the 20th century, it became possible to design many new solutions in the field of spectroscopy, chromatography and microscopy. The second half of the 20th century appeared as an avalanche development in the production of analytical apparatus enabling the analysis of a chemical composition at an unprecedented level of concentrations even to attmo mol (10^{-18} – MALDI-TOF spectroscopy). During this period the introduction of the photoelectron spectroscopy method (XPS – X-ray Photoelectron Spectroscopy was a milestone. XPS is a vacuum technique (10^{-13} Bar), which enables the simultaneous determination of both the qualitative and quantitative elemental chemical composition and the type of bonds between the elements in the samples; both on their surface and in their bulk without having to dissolve them [1]. Another significant achievement at the end of the 20th century was to prove that the non-invasive technique of near-infrared spectroscopy (NIRS –Near InfraRed Spectroscopy) might be used to study the brain activity. It can be applied to study depression, dementia and Alzheimer's disease (atherosclerotic dementia). This technique used in real conditions is called functional technique, i.e. fNIRS [2-3].

The development of medicine, science, technology and environmental protection possibilities in the 21st century depends on the availability of physicochemical spatial information. The development of modern medicine is increasingly dependent on the availability of information concerning the site of changes and the site of accumulation of a given metabolite in the body, the type of organ and the place where some chemicals accumulate. The knowledge of change of chemical identification and a spatial location of proteins in the human body may enable the development of a method of spatially targeted therapy as well as the preparation for appropriate drugs.

In this article two methods are presented, XPS and fNIRS which for several years become increasingly popular in a widely understood medical field. The first one is a classic *in vitro* vacuum technique, while the second one is an *in vivo* technique. These both techniques are discussed in this paper to point out that a similar physical phenomenon might be used in different areas and in different way. This phenomenon is an interaction of incoming radiation with matter. In the case of XPS technique it is X-ray radiation, while in the case of fNIRS technique it is near IR radiation. In both cases, as the result of these interactions, the radiation from the studied sample is outgoing either corpuscular one (XPS) or wave one (fNIRS). The common denominator for both techniques is therefore the applicability of the universal Lambert-Beer equation [4-7] given in the general form as

$$I/I_0 = exp(-axl)$$

where I is intensity of the radiation coming out from the sample, I_0 is intensity of incident radiation, a is so called linear (napierian) absorption coefficient of the radiation and l is the way in which radiation interacts with matter of the sample. The proper description of a coefficient is the most important and difficult in both techniques.

X-ray photoelectron spectroscopy (XPS)

The XPS method uses the effect of electrons ejected from the material during exposure to X-ray radiation. The X-ray radiation transmits its energy to the electrons in the sample during the interaction with the sample. As a result of this process, the electron is kicked out of the sample. The electron obtained in this way is called a photoelectron. Irradiation of the material by X-rays might result in ejected photoelectrons from both; high-energy internal electronic levels as well as outer low-energy valence levels [8-9] (Fig. 1A).

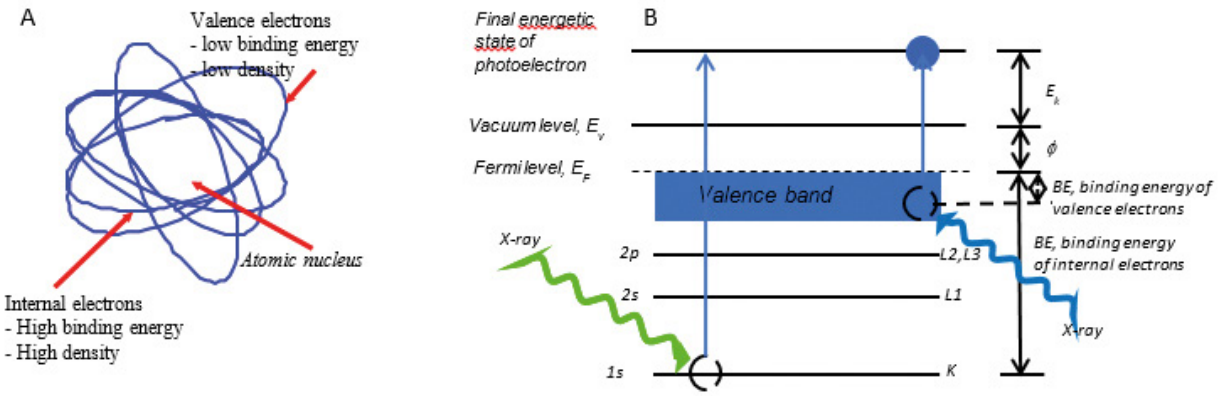

Fig. 1A-B.

A. Schematic representation of the structure of the atom.

B. Energy scheme of the process of striking the photoelectron from the inner shell (left side of the drawing) and valence band (right side of the drawing). Description: E_v –vacuum level, E_F – Fermi level, E_k – electron kinetic energy, ϕ – electronic work function, BE – electronic binding energy, 1s / K, 2s / L1, 2p / L2, L3 – electron shell description

Because the number of internal (core) electrons is usually much higher than valence electrons, the XPS technique of internal electrons is characterized by much a greater sensitivity than the XPS technique of valence electrons. The sensitivity that can be achieved is even tenths of an atomic percentage. On the other hand, valence electrons have more specific energy, i.e. they carry more information about the sample material so that the XPS valence electron technique can be used even to distinguish isomers. The energy of the recorded radiation of photoelectrons enables the chemical identification of atoms and bonds, while its intensity allows the determination of a chemical concentration.

When considering the applicability of the XPS method, it should be taken into account that the phenomenon of ejecting a photoelectron is a one-stage phenomenon (Fig. 1B). The equation of the energy balance of the process of striking of photoelectron by X-ray radiation of *hv* energy, for example from the 1s shell (K shell) can be represented as follows:

$$E_k = hv - (E_{B,K} + \phi)$$

The $E_{B,K}$ symbol means the electron binding energy at the *1s* shell. The experimentally measured kinetic energy of the electron, E_k, is therefore determined by the energy of one electron level, which allows both; an accurate experimental measurement and a theoretical calculation.

The XPS technique is extremely attractive for a chemical analysis and an imaging of sample surfaces. Although the X-ray ionizing sample penetrates the tested material relatively deeply, but the measured objects are photoelectrons, and their so-called free exit way, often called the escape way, due to their much bigger absorbance factor comparing to X-ay radiation penetrates much narrowed layer of studied material. Most often it is below 2 nm, thanks to which the chemical state of even mono layers can be tested with a proper measurement design.

The energy of ejected photoelectrons depends on such physicochemical factors as the absorption coefficient of X radiation and photoelectron, the interaction of photoelectron with other electrons and atoms, the entropy of elemental composition, or the surface roughness. As a result of these dependencies, the energy of the tested photoelectrons coming out of the real sample differs from the energy of those coming out of the pure elemental or calculated sample. These energy differences are called chemical shifts and allow to obtain some information about chemical bonds, concentration and composition dependence on distance from the surface.

The importance of an accurate determination of the chemical energy shift increases with the decreasing of a chemical composition and a spatial distribution diversity variation of individual components in the studied sample. For inorganic samples of large atomic diversity, so also large differences in energy of ejected photoelectrons, it is less important than for organic samples (medicine, biology, biochemistry, biophysics) which mainly consists of carbon and hydrogen, and low atomic concentrations of such elements like oxygen, nitrogen, sulfur and sometimes a limited type of metal. In this case, the ability to determine the energy shifts of the photoelectron accurately is crucial to determine the composition of the sample.

In Fig. 2 the chemical formula and the PET polymer sample spectra is presented [9]. The PET molecule consists of

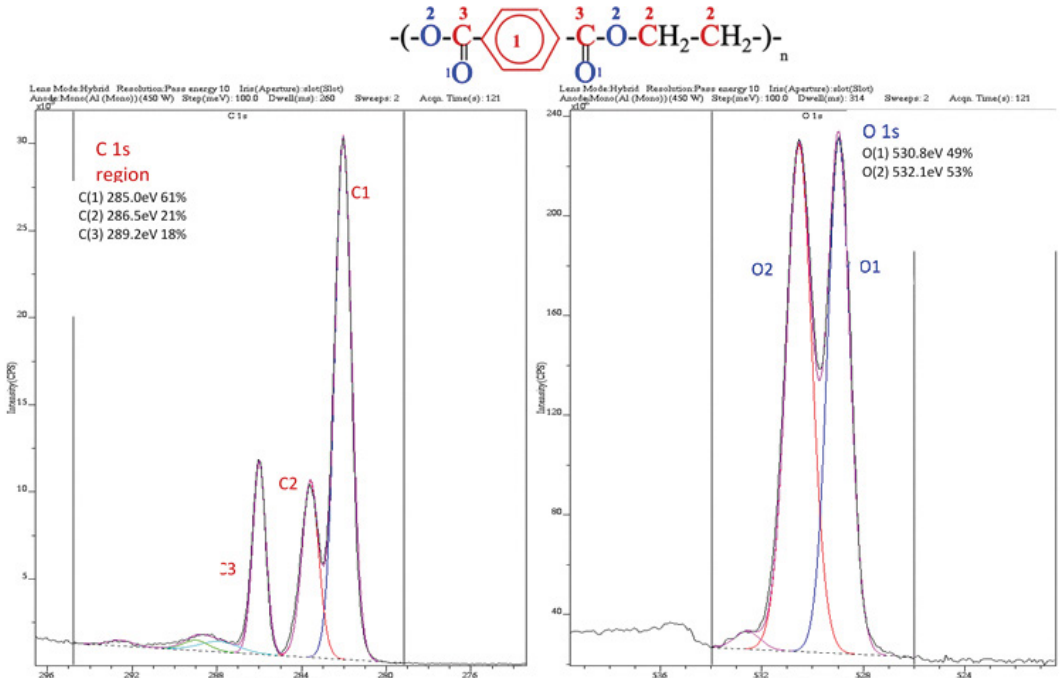

Fig. 2. Chemical formula and PET polymer sample spectra. Numeric energy values for individual types of labeled elements as well as their atomic concentrations are given

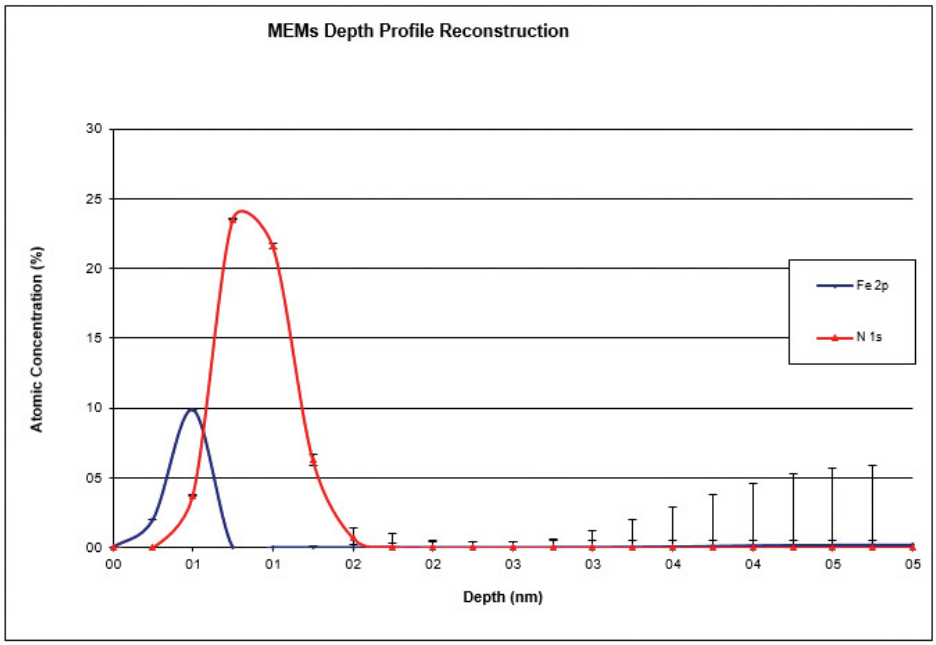

Fig. 3. Example of determining the position of atoms in the Z direction for molecule shown at left side of the figure

three types of carbon atoms and two types of oxygen atoms, which are perfectly distinguished on the attached spectra due to the possibility of using a high-class XPS spectrometer. The energy differences between the individual peaks are in the order of 1 eV, i.e. 1.602×10^{-19} J, so these are extremely small differences, but thanks to the use of a suitable detection system in this case they are clear.

The high accuracy of determining the energy of currently produced XPS spectrometers allows, with the help of an appropriate software, to determine even the position of atoms in the Z direction (vertically to the surface of the sample) [10]. In Fig 3. the spectrum in the Z axis direction is shown for the mono layer of the self-organizing film (SAM) containing iron and nitrogen. These results show that the spatial accuracy in the Z axis can be of the order of parts of the nanometer.

The great possibilities of the XPS method technique have enabled its application to one of the most difficult samples, i.e. the characteristics of medical samples.

XPS analysis of drug-coated surgical stents

One of the biochemical problems in the use of tubes/stents in cardiac surgery is the pathological response of the human immunological system, which results in the occurrence of thrombosis and hypertrophy, especially in the case of steel stents. Currently, polymer stents made of lactic acid polymer (polylactic acid [PLA]) strengthened with $C_{51}H_xNO_{13}$ compound, are increasingly used and protected by applying special layers about 1.8 μm thick on drugs surface. The key to determining the state of stents is determining the C: O: N ratio indicating their durability or biochemical resistance. In Fig. 4 the results of such tests carried out by means the XPS technique are shown.

XPS analysis of polymer surgical meshes

Woven fabrics of polymer meshes, made of polypropylene (PP) and polyester (PE), are used in a hernia surgery as well as in a surgery for other soft tissues to secure their positioning. The problem which often is faced up is infections and inflammation. The use of plasma functionalization of the applied fabrics as well as the deposition of various adsorbates on the surface of polymer fabrics are tested and used due to the possibility of improving patient's safety. This type of the procedure results in the preparation of the surface

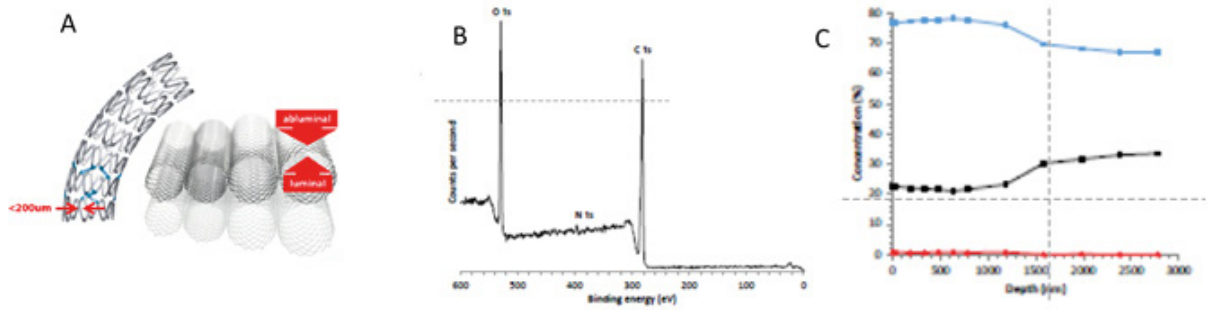

Fig. 4A-C. A. Schematic appearance of the B stent. XPS spectrum of the stent sample. C. Dependence of the concentration of elements C, O and N from the distance (C –blue, O –black and N – red). With permission of Kratos Analytical, Manchester, England

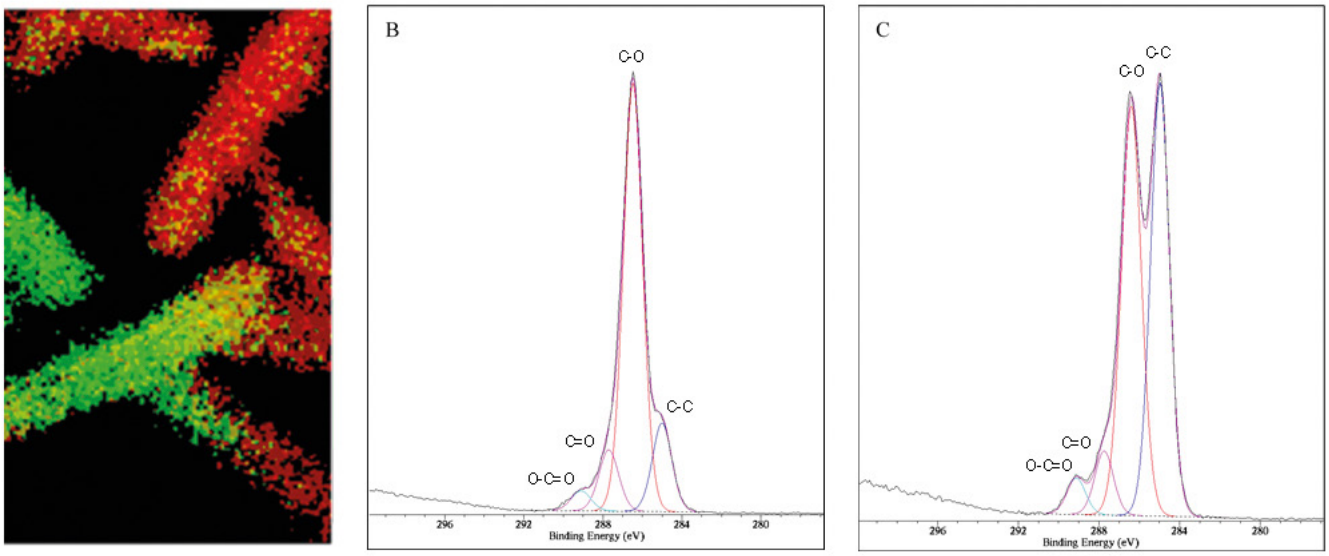

Fig. 5A-C. XPS map of C 1s signals of C-C and C-O bonds for PEG polymer embedded on PP polymer for surgeon mesh. Green regions: good coverage by PEG (high C-O concentration) showed in spectrum B, red regions: poor coverage by PEG (high C-C concentration) showed in spectrum C, respectively. Image 0.8 x 0.8 mm

that reduces the adhesion of bacterial biofilms. One possible coating is a polyethylene glycol (PEG) film.

In Fig. 5 results of XPS analysis of a medical fabric covered with PEG are presented [11].

The chemical map shown in Fig. 5 was constructed on the basis of testing C-O bonds concentrations, binding in PEG and C-C bonds, binding in PP. A patient's safety is likely to increase with PEG concentration, i.e. such tests may be extremely useful for manufacturers of medical fabrics as control tests and in research and the development of the field.

XPS analysis of caffeine-containing tablets

The XPS technique can also be widely used in pharmacy and the control of manufactured drugs. Fig. 6 shows the three-dimensional distribution of substances which contained in the caffeine tablet.

Functional near infrared spectroscopy method, fNIRS

The 21st century is said to be the age of the brain research. Experimental neurological tests and advanced analytical interdisciplinary techniques will allow enormous progress in knowledge of the mechanism of the brain action under both; static and dynamic conditions. It is anticipated that this progress will influence both; basic and clinical knowledge. This progress may push ahead the preparation of therapy for brain's and thus nervous system diseases treatment, which will result in psychological, psychiatric, neurological and rehabilitation assistance for hundreds of millions of people. It will be possible to study the processes of thinking, remembering, recognizing, learning, feeling emotions, fatigue or making decisions.

Methods traditionally used to study brain activity processes are electroencephalography (EEG), functional magnetic resonance imaging (fMRI), positron tomography (PET), magneto encephalography (MEG), computed tomography (CAT), by cranial magnetic stimulation (TMS). The functional near infrared (fNIRS) method is new and rapidly gains popularity.

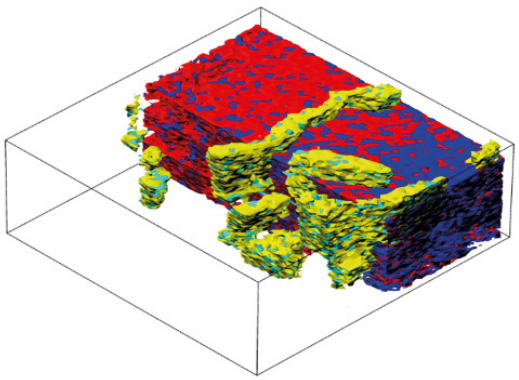

Fig. 6. Three-dimensional distribution of elements in a tablet containing caffeine. Red, blue and yellow indicate the presence of carbon, oxygen and nitrogen, respectively. Nitrogen is recognized as an indicator of caffeine. With permission of Kratos Analytical, Manchester, England

The human body consisting of both soft and hard tissues, is avery good absorber of electromagnetic radiation so it does not pass radiation. However, it turns out that there is a so-called optical window in the range of about 650-950 nm, thanks to which it is possible to examine the light absorption by compounds in the depth of the body when electron transitions are in this energy range. This phenomenon was observed in 1977 by Franz Jobsis during tissue tests [2]. The optical window is formed by radiation absorption on the short wavelength side by oxygenated hemoglobin (deoxy-Hb), and by water (H_2O) on the long wavelength side, Fig. 7.

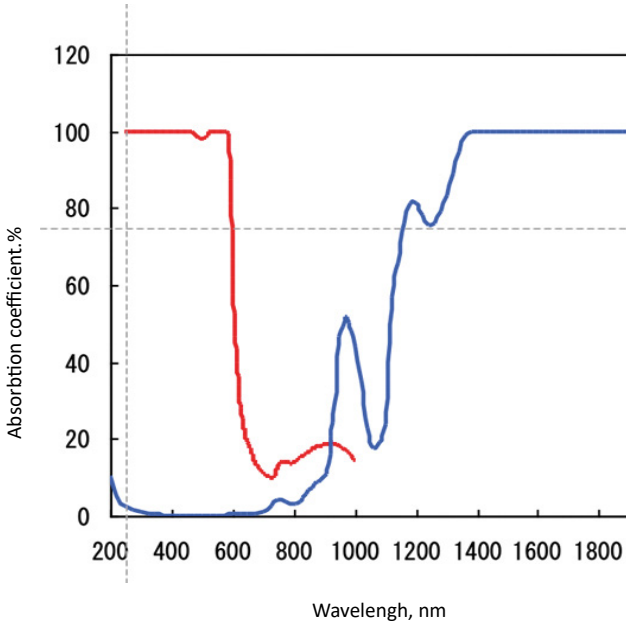

Fig. 7. Spectrophotometric window permeability for infrared radiation of human skull

Measurements using the fNIRS technique involve monitoring the amount of absorbance which results from deoxy-Hb concentration, at the wavelength of about 780 nm, oxy-Hb, at the wavelength of about 830 nm, and for so-called. isobestic point, at 805 nm. At this wavelength, deoxy-Hb and oxy-Hb absorbance should be equal and this point is the reference point (Fig. 7). The knowledge of oxy-Hb and deoxy-Hb absorption coefficients makes it possible to calculate their concentrations and their changes, depending on the object activity. Studying changes in concentration over time allows to determine the effect of mental or physical activity on processes taking place in the brain [12].

The fNIRS method is a non-invasive method, which uses harmless infrared radiation and has significantly less restrictions than other brain imaging methods. This method can be used for both; stationary and mobile objects, for example for children playing, people undergoing rehabilitation, drivers, pilots or athletes. Among brain imaging methods, it is also a cheap method that is easy to be used simultaneously with other methods, such as EEG, so that the two types of tests can be done at the same time. The results obtained using the fNIRS method could also be easily correlated with the results of other methods such as fMRI or PET.

Fig. 8 schematically presents the principle of fNIRS measurement. With the help of optical fibers, light with adequate energy is brought to the head and discharged after interaction with brain tissue.

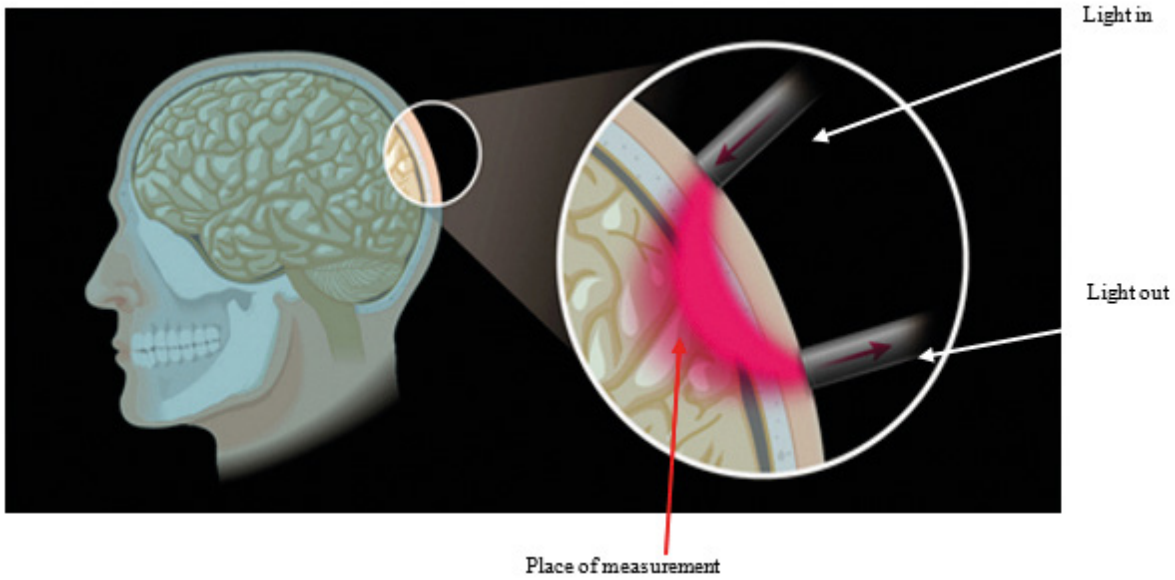

Fig. 8. Graphic representation of the principle of fNIRS measurement of brain activity. With permission of Shimadzu Europe, Duisburg, Germany

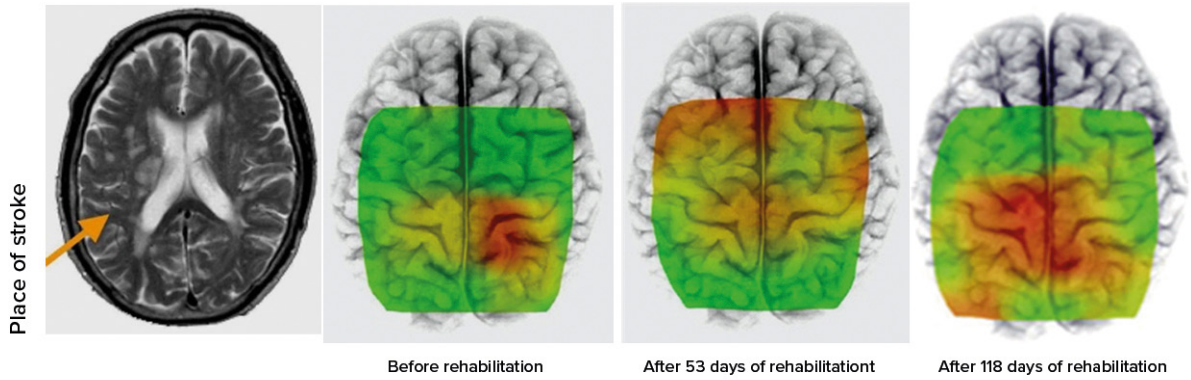

Fig. 9. Studies of influence of time of physical activity on activity of brain

Physical activity is one of the methods of monitoring objects after a stroke. In Fig. 9 an example of changes in the brain activity which depends on the time of rehabilitation involving a walk on a treadmill is given [13]. The increasing the area of the activity dependent on exercise time is clearly visible.

The fNIRS technique can be used practically in all areas, when the brain activity changes, sensitivity to smells, noise, fatigue, impact of security systems on the condition of drivers, or even in studies of differences between diseases such as depression, dementia, or atherosclerotic dementia. Therefore, the technique will definitely become an important tool in psychology and psychiatry.

In Fig. 10 there is given an example presenting the results of the fNIRS studies on distinguishing between objects with depression (D) and Alzheimer's disease (AD). The VFT (verbal fluency task) test shows significant differences between the healthy controls (HC) and patients with depression and Alzheimer's disease particularly for frontal cortex studies [14]. Cortical activation for the D and AD group is decreasing in comparison with healthy controls (Fig. 10A, upper row). In the case of parietal cortex, the differences are much smaller (Fig. 10A, lower row). The results of VT (visuospatial task) test shows that the differences in brain activity between healthy and depressed objects are rather small, while between these two groups and AD objects, they are much more significant (Fig. 10B). Summarizing these results, it is clear that fNIRS technique is capable of distinguishing differences in brain activation between healthy, depressed and Alzheimer's objects.

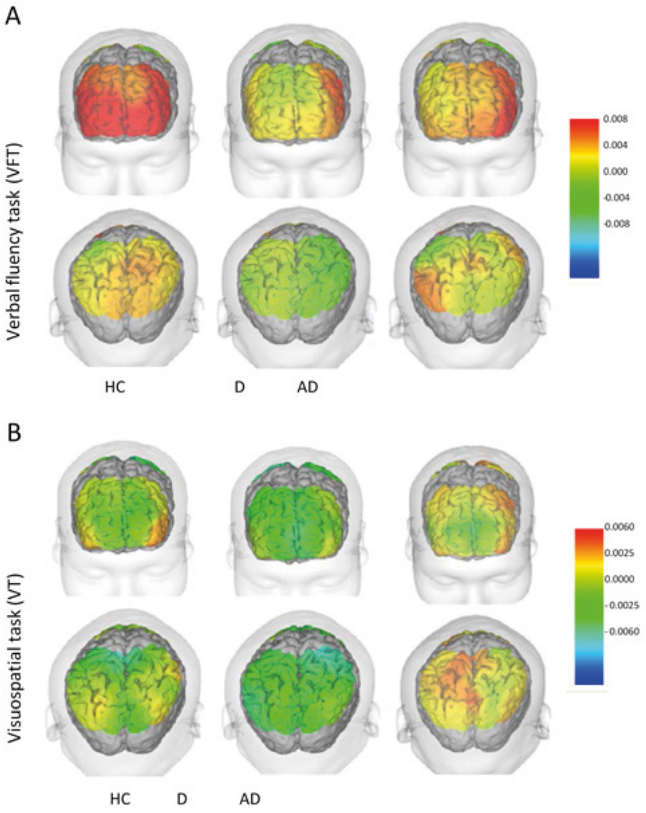

Fig. 10A-B. Topography of cortical activation in the three tested participant groups; HC – healthy controls; D – depression; AD – Alzheimer's disease. Superimposed fNIRS images on 3-D MRI represent cortical activation in the verbal fluency task (A) and visuospatial task (B). The upper row shows an activation of the frontal cortex, and the lower row shows the activation of the parietal cortex. The color bar indicates concentration of [oxy-Hb] (mM·cm). The scale differs between figures (A) and (B)

Summary

In the presented material in this article the possibility of application of two extremely different methods are discussed, namely photoelectron spectroscopy, a high vacuum technique, XPS, and technique for brain activity determination under in vivo conditions, i.e. functional near infrared spectroscopy, fNIRS. It has been shown by reporting chosen data that

both techniques are suitable for the application to the testing clinical samples and objects and that the results of their application might help solve important problems in the clinical medicine as well as in the fundamental medical research.

XPS technique can be used to determine the chemical consistence from the simple substance to the studies of the phenomenon of a bacteria influence of artificial objects placed in our body; like implants and stents and degradation of bioactive molecules and antibacterial coatings. fNIRS may be used to study the processes taking place in the real time and in the real objects. It is used to monitor an influence on our brain activity of such factors like smell or noise up to such diseases like autism, depression and dementia, or atherosclerotic Alzheimer's disease.

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REVIEW ARTICLE

Diagnostic challenges of maxillofacial sarcomas – literature review and case reports

Mateusz Nowicki[1], Łukasz Garbacewicz[1], Adam Polcyn[1], Barbara Drogoszewska[1]

[1] Oro-Maxillo-Facial Surgery Clinic, Medical University of Gdańsk, Gdańsk, Poland

Abstract

Sarcomas are malignant tumors of mesenchymal origin. They comprise of < 1% adult malignancies and about 15-20% of pediatric malignancies. These tumors are subdivided into bone and cartilage sarcomas and soft tissue type. Sarcomas of the maxillofacial region comprise 5-10% of all sarcomas and < 1% of all malignancies of this part of the body. Usually sarcomas do not originate from benign tumors. Among the factors known to induce sarcoma growth are: genetic defects, exposure to pesticides and herbicides, exposure to ionizing radiation and immunosuppression. Treatment of sarcomas is based on complete tumor resection with a safety margin. Early and correct diagnosis greatly increases the therapeutic options and improves the prognosis.

Keywords: Sarcoma · maxillofacial · rhabdomyosarcoma · Ewing sarcoma

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Introduction

Vast majority (about 80%) of all tumors in the maxillofacial region of adults consists of planoepithelial cell carcinoma of medium and high grade. Other malignancies of this region are: adenocarcinomas, cystadenocarcinoma, muco-epidermal carcinoma, osteosarcoma, chondrosarcoma, lymphomas and myelomas [1]. Furthermore, metastases from tumors of the kidney, testicle, breast, bronchi and lungs can also be found in the maxillofacial region [1-2]. Tumors growing in the oral cavity and facial region may have non-specific signs and symptoms which sometimes leads to wrong diagnoses and wrong treatment.

Corresponding author:
Mateusz Tadeusz Nowicki, Oro-Maxillo-Facial Surgery Clinic, Medical University of Gdańsk, Poland
e-mail: mateusznowickim@gmail.com

Available online: www.ejtcm.gumed.edu.pl

Material and methods

We conducted a narrative type of review of the available literature published in the English language. Using the keywords "sarcoma", "maxillofacial", "rhabdomyosarcoma", "Ewing sarcoma", "etiology", "histopathology", "treatment", "presentation", "radiology" we searched the Scopus, Google Scholar and PubMed databases. The exclusion criteria were: animal studies, publishing date before 1980. We also screened the references of each included article for any additional relevant articles. No statistical analysis was performed. In addition, to illustrate the diagnostic challenges of sarcomas, we included brief case reports of 2 patients referred to the Orofacial Surgery Clinic of the University Clinical Centre in Gdańsk (Poland) from outpatient dental clinics in the years 2019-2021.

Results

Our search retrieved a total of 92 abstracts, of which 76 were included in further analysis. A total of 42 full-text articles were included in the review.

Case 1

A 57-year old female with history of allergy to analgesics was referred to our Outpatient Clinic in January 2019 due to right maxilla oral cavity vestible shallowing. The patient reported pain radiating to the ear and cranium that began 1 month after she noticed the tumor. Her dentist diagnosed her with edema due to allergy and did not initiate any treatment. The patient's history was also positive for sensory deficits in the region innervated by the maxillary branch of the right trigeminal nerve, whereas physical examination revealed facial asymmetry due to the tumor of the right cheek. Skin above the tumor was normal. Oral cavity examination revealed shallowing of oral cavity vestible and mobility of teeth 12-17. Mucosa covering the tumor was normal. The volumetric computer tommography (CT) scan of the maxillofacial region revealed a pathological infiltration of the right cheek, adhering to the alveolar process of the maxilla, 26 x 15 mm in cross-section and a segmental cavity of the cortical layer of the alveolar process of the maxilla at the level of the infiltration.

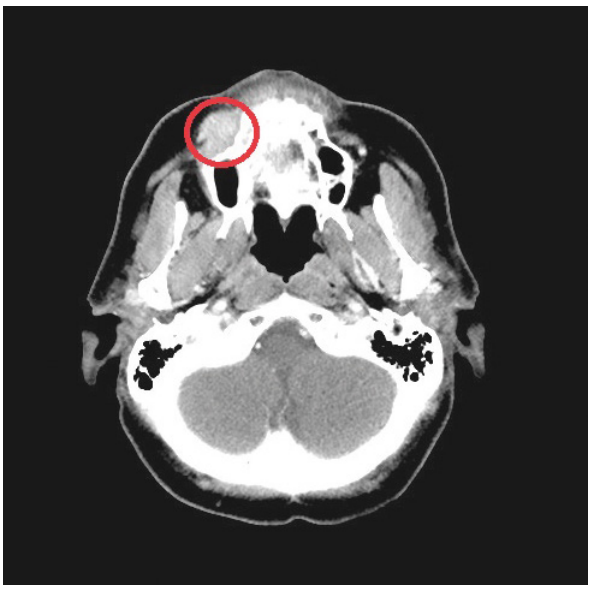

Fig. 1. Patient 1's computer tommography scan revealed a tumor of her maxilla

The tumor was biopsied under anesthesia. Histopathological examination revealed small round blue cell tumor with the following immunophenotype: CK AE1/AE3 -, LCA -, TTF-1 -, Melan A -, SOX10 -, p40 -, INI1 +, FLI1 -, CD99 -, PAS -, chromogranin A focally +, synaptophysin focally +, desmin +, Myf4 +, Myod1 +. The clinical picture and the above-described additional tests suggested the diagnosis of rhabdomyosarcoma. In addition, this patient was diagnosed with metastases to the thoracic and lumbar segments of the vertebral column with soft-tissue infiltration. She was treated with radiotherapy of the maxillary sinus region and regional lymph nodes, paliative chemotherapy and paliative radiotherapy of the thoracic and lumbar vertebrae.

Case 2

An 81-year old female with history of Alzheimer's deasease, hepatosplenomegaly and hearing loss was referred to our Outpatient Clinic due to the suspicion of persistent inflammation of the left maxilla. Prior to that she was unsuccessfully treated at an outpatient dental clinic where she was diagnosed with abscess of the left cheek. Oral cavity examination revealed extensive ulceration of the hard palate, the oral cavity vestibule and the alveolar process of maxilla on the left side. The abnormal tissue was biopsied and the histopathological examination confirmed the diagnosis of Ewing sarcoma with the following immunophenotype: CD99 (+), CKAE1/AE3 (focally +), CK7 (-), CK20 (-), CD3 (-), CD20 (-), p40 (-), synaptophysin (-),chromogranin (-), S100 (-), SOX10 (-), TTF-1 (-), FLI-1 (-), Ki67 (> 80%) and PAS (+). In addition, it was determined that the EWSR1 gene was rearranged. Volumetric CT scan revealed extensive pathological infiltration (53 x 73 x 64mm) of the left side of the maxilla, including the left maxillary sinus, palate (without crossing the midline) and left nasal cavity. The infiltration penetrated the ethmoid bone, left frontal sinus, left sphenoid sinus, left orbit and left pterygopalatine fossa, reaching the cavernous sinus and the superior orbital fissure. Due to the extensiveness of the tumor, the patient was treated with paliative radiotherapy and was transferred to a hospice where she died within 3 months of making the diagnosis.

Discussion

Epidemiology

Sarcomas are diverse group of mesenchymal tumors [3]. They comprise of < 1% of adult malignancies and 15-20% of pediatric malignancies [3-4]. Maxillofacial sarcomas comprise 5-10% of all sarcoma cases [5-12] and < 1% of all malignant maxillofacial tumors [5, 10, 13-16]. Over 70 subtypes of sarcomas were desribed so far [3, 17]. They can be subdivided into two groups: soft-tissue sarcomas (STS, 80% of cases) and osteo/chondrosarcomas (20%) [15-19]. Both types can occur in the maxillofacial region, as noted in the case reports above.

STS have a tendency to form in the muscle, adipose and nerve tissues, joints, blood vessels and deep layers of the skin [4]. They usually occur in the limbs, most often in the soft tissues of the thigh [17, 20], slightly more frequently in men (1,4:1) and the average age at the time of diagnosis is 59 years of age [17, 20]. Among the tumors of this type are liposarcomas, leiomyosarcomas, rhabdomyosarcomas and fiborsarcomas.

Osteosarcomas are less frequent and usually affect a much younger population (< 20 years of age) [17, 20]. Besides bones, they can occur in cartilage as well [4]. The most frequent tumor from this group, osteosarcoma, occurs significantly more often during puberty than in adulthood [17, 21]. Ewing sarcoma is more common in childhood and puberty, though it can occur also in adulthood [17, 21]. Whereas the most common osteosarcoma subtype among adults, chondrosarcoma, usually occurs in patients 30-60 years of age [17, 21].

Ewing sarcomas occur significantly more often in Caucasian patients [4, 22], whereas STS have a slight predilection for patients of African origin (5.1/100 000 people) compared to Caucasian (4.5/100 000) or Asian (2.8/100 000) [4, 23].

Etiology

Sarcomas usually do not grow from benign tumors but occur de novo [24]. In majority of cases the etiology of sarcomas is not known, however external and internal factors were identified as predisposing to sarcoma development [17]. One of the external factors is exposure to ionizing radiation [3]. Sarcomas induced by radiotherapy of breast, lung, testicular or prostate cancer comprise 0.5-5.5% of all sarcomas [3, 25-26]. In addition, research from Japan suggests increased affinity for sarcomas of bones (mainly osteosarcoma) and STS (leiomyosarcoma in particular) among the survivors of atomic blasts in Hiroshima and Nagasaki [17, 27-28]. Another external factor is exposure to chemicals such as polyvinyl chloride (PVC), dioxins, nonorganic arsenic, copper, nitrotoluene, chlorophenol and anabolic steroids [3]. Increased risk of osteosarcoma has been noted among

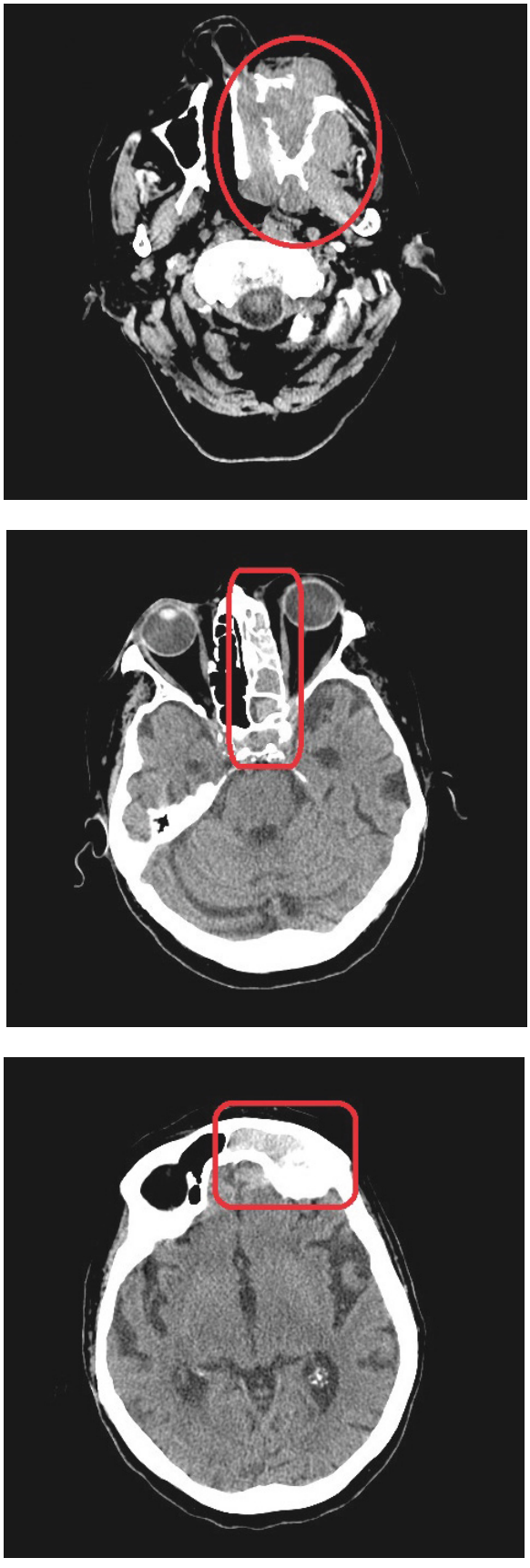

Fig. 2, 3, 4. Patient 2's volumetric computer tommography scans revealing tumor infiltrating maxillofacial structures

people working with metals, carpenters, bricklayers/masons and blacksmiths [17, 29]. Exposure to PVC, arsenic and treatment with anabolic steroids are related to the incidence of hepatis angiosarcoma (HAS) [30].

The leading internal risk factors are genetic defects and chronic immunosuppression (e.g. in the course of AIDS or after organ transplantation). Chronic immunosuppression in the course of AIDS is favorable to the development of Kaposi Sarcoma (KS), which is associated with a human herpesvirus 8 (HHV8) infection [3]. Patients who are immunosuppressed after transplantation of internal organs also have an increased incidence of Kaposi Sarcoma, particularly in the regions of the world that are endemic for HHV8 [3, 31-32]. In the pediatric population, a co-infection with the Epstein-Barr virus (EBV) in the course of AIDS is favorable to leiomyosarcomas [33].

Among the genetic defects predisposing to sarcomas are: Li-Fraumeni syndrome (TP53 gene), retinoblastoma (RB1 gene), von Recklinghausen disease (NF1 gene), familial adenomatous polyposis of the colon (APC gene), gastrointestinal stromal tumors (GIST), Bloom syndrome, Werner syndrome, Carney-Stratakis syndrome and the Rothmund-Thomson syndrome [17].

Physical examination

Swelling of the adjacent tissues is the main sign of sarcomas [5]. Pain is significantly more common in patients with osteo- or chondrosacocomas [5]. Another sign is redness of the skin, ulceration of oral cavity, paresthesias and in case of maxillofacal sarcomas: mobility of teeth adjacent to the tumor [5].

Imaging

CT and MRI scans of the affected area are the key elements of the diagnostic process. If distant metastases are suspected, then imaging of the thorax, abdomen and pelvis are indicated. Positron emission tomography (PET) or bone scintigraphy are needed sporadically [34]. Sarcomas have a variety of radiological presentations: they can be translucent or opaque [5], without distinct borders or homogenous structure, with cystic components and periosteal reaction [35]. Imaging of osteo- and chondrosarcomas may destruction of bone and frequent infiltration of soft tissues [35]. The sunburst sign is pathognomonic for osteosarcoma [5].

Histopathology

The histopathological examination of sarcomas reveals atypical neoplastic cells (in case of chondrosarcoma they are similar to chondrocytes, whereas in case of osteosarcoma the are osteoid-like) with loss of normal architecture. Additional microscopic features are multiple foci of bleeding and necrosis or in the case of osteosarcomas: foci of calcification and ossification [36]. In majority of cases, to make the diagnosis of sarcoma it is necessary to use immunohistochemical or molecular techniques [5, 37].

Treatment

Surgery is the treatment of choice in sarcomas and tumor resection with margin of > 5 mm in soft tissues and >2 cm in bone [16]. Radiotherapy has its place in adjuvant treatment of STS in case of lack of safe margins or paliative treatment [16]. The role of chemotherapy as induction (pre-operative), adjuvant or paliative treatment is equivocal [34]. It was reported effective in the treatment of long bone osteosarcomas, whereas its effectiveness in maxillo-facial sarcomas is the subject of discussion [18, 38-39].

Compared to sarcomas of other body parts, the maxillo-facial sarcomas have worse prognosis in terms of recurrences and survival, which is partly due to the difficulty in performing complete resection (numerous adjacent structures that are critical to patient's functioning) [16, 40]. Tumor stage at the time of diagnosis is the most significant prognostic factor of oral cavity neoplasms. Early diagnosis significantly improves the prognosis and therapeutic options [41-42]. Both the patient and the dentist have a role in delaying diagnosis and initiation of treatment. Patients frequently delay consulting with a dentist, hoping that the change in the oral cavity will heal spontaneously. Whereas the dentists do not connect the presenting complaints with a potential neoplasm and prescribe analgesics, antibiotics or perform the previously planned procedures without focusing on diagnosing the problem [41].

Clinical pearls

Every patient of a dental clinic and general practitioner (family doctor) should at each visit undergo examination of the entire oral cavity as well as the temporo-mandibular joints, maxillo-facial soft tissues and palpation of cervical lymph nodes. Diagnostic investigation of any pathological change in the oral cavity should also begin at the dental clinic. Patients with suspected neoplasm of the maxillofacial region should be urgently referred to a specialist in order to verify the tumor using imaging and histopathological investigation.

Conclusions

Dentists and general practitioners (family doctors) treat patients who might have oral cavity sarcomas. In the differential diagnosis of tooth-related inflammation, allergic edema, maxillary sinusitis and benign neoplasm of the oral cavity it is important to always consider the likelihood of

malignant tumor. During each patient visit it is worth remembering that neoplastic processes of the oral cavity and maxillofacial region may present with a diverse and non-specific set of symptoms. It is very important to take a detailed history from the patient because this helps to confirm or exclude neoplastic disease. Early diagnosis is the key to successful treatment.

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REVIEW ARTICLE

Adverse events related to herbal products used by patients presenting at emergency departments

Sunanta Tangnitipong[1], Supat Jiranusornkul[2],

Pathomwat Wongrattanakamon[2]

[1] School of Public Health, University of Phayao, Thailand
[2] Faculty of Pharmacy, Chiang Mai University, Thailand

Abstract

Herbal medications are gaining popularity in many countries. Although most can be used without any problem, serious toxicities do occur. Adverse events can be anticipated when herbal medications are used at excessive dose, long-term, for non-approved indications or by patients who are using multiple medications. Adverse events should be anticipated when these herbal medications with identified pharmacological effects or toxic ingredients are used. Healthcare professionals need to discuss or advise patients regarding their use. Physician-obtained medication histories towards specific herbal product use could provide relevant pharmacologic information and uncover cases of adverse events or toxicity from the used herbal products.

Keywords: Adverse events · Emergency · Herbs · Toxicity · Traditional use

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Introduction

Herbal medicines, also called phytomedicines, consist in the use of plants or plant extracts for medicinal purposes. Medicinal plant products include raw or processed plant parts such as roots, stems, leaves, flowers, and seeds [1]. Many Asian, African, and Pacific people utilize traditional medicine as primary health care [2-5]. In Thailand, particular in rural areas, where access to medical treatments with a Western approach may be expensive or simply not

Corresponding author:
Pathomwat Wongrattanakamon, Faculty of Pharmacy, Chiang Mai University, Thailand
e-mail: pathomwat.w@cmu.ac.th
Available online: www.ejtcm.gumed.edu.pl

available. Thai herbs are available for collection from many areas. Preparation of the medicinal plants for remedy such as tea, tincture or filtrate is inexpensive and can be practiced with a minimal amount of training. Therefore, for utilization in primary health care, a herbal medicine may hold great promise [6].

Many Thai people receive Thai traditional medicine including herbal medicines, herbal steam bath, acupuncture, and traditional massage. Traditional healers, including herbal, massage, and spiritual healers as well as traditional midwives, are distributed all over Thailand [7]. The Thai government has been supporting an endeavor to systematically develop Thai herbal medicines and medicinal plant products. As part of a vigorous attempt to advocate the utilize of herbal medicines/medicinal plant products and serve diversity of alternatives for primary health care or to Western medicines, some medicinal plant products have been placed on the National List of Essential Medicines [8].

All of these Thai medicinal plants provide a lot of pharmacological effects. Even though many of these products are used safely, but not all of them have adequate clinical research to advocate the use [8]. Despite extensive uses of medicinal plant formulations along with their benefits, these formulations are not fully safe. The indiscriminate or non-regulated utilize of the medicinal plant products may put consumers' health at risk of toxicity. Moreover, there is limited scientific evidence regarding evaluation of clinical safety and effectiveness of medicine plant formulas. Adverse reactions to medicinal plants have been noted when utilized alone or in combination with conventional medicines. The aim of this study is to summarize the literature regarding the adverse events related to specific herbal medications requiring emergency department visits.

Materials and methods

This is a narrative type of review. Independent English-language literature search was done by all authors during December 2020, using PubMed, Web of Science, Scopus and Google Scholar with no limitation on the year of publish. We used the keywords "emergency department," in addition with the following condition-specific terms: botanicals, complementary and alternative medicine, diseases, herbal medicine, medicinal plants, patients, admitted, traditional use, treatment, side effects, and adverse effects/events. A detailed cross-review of references was also carried out. Exclusion criteria were: reports of non-emergency cases, conventional medications, and adverse events not involving herbal medications. No statistical calculations were performed due to the narrative format of this review.

Results

Over 100 articles were retrieved in the search, 37 of the articles met the criteria and 13 of those were included in this analysis. The adverse effects which were most commonly noticed in the analysis were neurological adverse effects including delirium, encephalopathy and neuropathy and seizures.

Photosensitivity rash

Photosensitivity is an occasional adverse event related to herbal medicine utilization [9]. St. John's wort was most regularly implicated case reports in the literature. One case report was found describing emergency department treatment of hypertension due to use of a multi-herbal formula. Palanisamy et al. [10], reported a case of pruritic, erythematous rash on the sunexposed surfaces of the patient's neck and extremities after taking a dietary supplement product containing various herbs including ginseng, goldenseal and bee pollen. Although cases of photosensitivity due to the individual components in this supplement have not been reported, it is possible that this toxic reaction may caused by interactions among the ingredients. Therefore, the combining of multiple herbs/ingredients into one single formulation should be cautioned [10].

Hypertension

With long-term use, ginseng may cause hypertension [9, 11]. Siegel [12] described emergency department treatment of hypertension due to excess use of ginseng herb. The author also described the major stimulant effects of ginseng and indicated that long-term use resulted in ginseng abuse syndrome which can be associated with hypertension. Symptoms of central nervous system excitation and arousal occurred with most subjects. Such effects are probably due to the dammarenetriol glycosides found in ginseng that have strong central nervous system excitatory actions. Moreover, some of ginseng's components have a molecular structure similar to the digitalis glycosides [12].

Hypokalemia

Liquorice is a particular example of the herbal product that causes hypokalemia [9, 13]. Liquorice is a medicinal plant product produce from the dried roots and rhizomes of the plants, such as, from Glycyrrhiza species; G. inflate, G. uralensis, and G. glabra are regularly utilized as traditional medicines for cough, eliminating phlegm, tonifying the spleen and stomach, supplement the vital energy and alleviating pain. Moreover, it is also utilized as a flavoring agent, health food and snack [14]. If used long-term and in large quantities, liquorice exhibits a mineralocorticoid effect that

may result in severe depletion of potassium stores in the entire body [15]. Two case reports (in 2 articles) were found describing emergency department treatment of hypokalemia due to excess use of liquorice. The first report described a patient with symptoms of bradycardia and hypotension [15]. Systematic investigation revealed that the patient suffered from constipation for a number of months, therefore on a daily basis she patient took large quantities of liquorice for its laxative effect. Hypokalemia secondary to liquorice overindulgence was diagnosed this patient. Such cause of hypokalemia is rare, however every emergency health care provider should be mindful of herbal product use as a possible cause of hypokalemic cardiac arrest [15]. The second report concerned an emergency department patient with ventricular fibrillation and medical history of liquorice intake for some medical proposes [16]. After stopping liquorice intake, potassium replacement was given resulting in a progressive change of the Brugada-like electrocardiographic pattern. This case report shows the importance of medical history-taking at emergency departments regarding herbal product use in patients with electrolyte abnormalities. Moreover, emergency care providers should be aware of the potential role of hypokalemia in inducing a life-threatening arrhythmia in patients with Brugada syndrome [16].

Neurological adverse effects

Some serious neurological adverse effects can be caused by herbal medications. Numerous case reports reveal various central nervous system adverse effects including cerebral arteritis, cerebral edema, coma, confusion, delirium, encephalopathy, hallucinations, intracerebral hemorrhage or mood disturbances. In addition, other cerebrovascular disorders and peripheral neurological disorders including movement disorders, muscle weakness, paresthesiae as well as seizures. They result from improper utilization, toxicity of herbal constituents, adulteration or contamination of herbal preparations as well as herb-drug interactions [17].

Delirium

Traditional medicine adulterated or contaminated with toxic plants, conventional drugs as well as heavy metals are major health hazards for herbal product consumers. One case of delirium due to contaminated *Strobilanthes forrestii* Diels was reported by Shea et al. [18]. One hour after taking the herbal tea, a mixup between *Cyathula officinalis* Kuan (extensively utilized in a traditional medicine for the treatment of inflammatory disorders [19]) and *S. forrestii* (commonly used as an adulterant [20]), the patient presented slurred speech, disorientation, visual hallucination (central anticholinergic toxicity), clumsiness in all four limbs and generalized weakness (peripheral anticholinergic toxicity). It was found that *S. forrestii* was incorrectly prescribed (due to the similar local nomenclature of both plants) and contaminated with tropane alkaloids including atropine, scopolamine and anisodamine during the manufacturing process. The clinical presentation pointed out the anti-cholinergic toxicity of tropane alkaloids [18]. Frequent monitoring and surveying of medicinal plant products for undeclared ingredients or potentially toxic adulterants are very necessary and the use of these substances should be strictly legislated.

Encephalopathy and neuropathy

Two case reports from two different studies were found describing emergency department treatment of encephalopathy and neuropathy due to the use of some herbs. The first case regarding traditional medicine contaminated with a toxic plant have also been reported by Ng et al. [21]. The two patients developed intoxication caused by podophyllin after intake of a broth of guijiu, the root preparation of the plant *Podophyllum emodi* Wall. The neurological manifestations in both patients as well as the pathology of peripheral neuropathy were seen. The decoction prepared from the herbal formulation longdancao was intended to have been prepared for both patients. The roots of *Gentiana species; G. manschurica, G. scabra, G. triflora,* and *G. rigescens* are officially accepted as plant parts used for this herbal formulation. Many importers and retailers have been mistaking guijiu for longdancao due to their apparent similarity in morphology. Guijiu is prepared from the root of *P. emodi*, (also known as *P. emodi* Wall var. *chinensis* Sprague and *Sinopodophyllum emodi* (Wall). Ying) which contains podophyllotoxin, demethylpodophyllotoxin, demethyldeoxypodophyllotoxin, deoxypodophyllotoxin, diphyllin, sikkimotoxin, as well as flavonoids kaempferol and quercetin. Use of Guijiu as an adulterant of longdancao should be considered as a safety issue. Regarding the clinical manifestation of podophyllin neurotoxicity, cerebral involvement leads to acute alteration of sensorium, which ranges from mild confusion to frank coma and higher mental function impairment which is permanent. The described patients suffered severe encephalopathy. Podophyllotoxin, a lipid-soluble molecule that crosses cell membranes with ease, was speculated to be the toxic compound responsible. Colchicine and podophyllotoxin are structurally related resulting in colchicine-like effect (similar clinical effects regarding arresting the mitotic spindle) of this compound and its derivatives. *In vitro* ability to bind to microtubular protein and inhibit axoplasmic flow may lead to neurotoxicity of podophyllotoxin [21].

The second set of cases was reported by Yang et al. and involved peripheral neuropathy, probably induced by tartarian buckwheat products that have been using as a hypoglycemic health food [22]. Tartarian buckwheat (*Fagopyrum ta-*

taricum (L.) Gaertn.) is used to prepare the buckwheat black tea composite tablets. Its seed contains high-quality proteins which provide essential amino acids. Flavonoids are the major active components of this medicinal plant and they are considered to possess hypoglycemic, antioxidant, antiaging, lipid-lowering, anti-atherosclerosis, immunity-enhancing, sterilizing, anti-mutagenic and anticancer activities. Although various forms of neurotoxicity caused by herbs were documented, case reports regarding tartarian buckwheat poisoning are rare. Yang et al reported new-onset polyneuropathy with dyskinesia in five male patients prospectively induced by recent use of *F. tataricum* products as a diabetes remedy. All the cases indicated that products prepared from *F. tataricum* may lead to toxic peripheral nerve lesion. Therefore, better regulation and close monitoring of consumption of tartarian buckwheat products are needed [22].

Seizures

Camphor has been used for centuries for multiple purposes. Currently it is freely available widely used in many Asian countries. In Ayurvedic traditions, camphor is used orally for sinusitis, flatulence, circulatory problems, joint pain, painful menstruation, epilepsy, gout, as a lactation suppressant and as an analeptic. Externally, it is used for chapped lips and cold sores, as muscle and joint liniment and as an inhalant for respiratory disorders. MacKinney et al reported the case of the patient who intentionally ingested approximately 10 g of pure camphor for purported medical purposes has been reported by [23]. This patient had a history of nasal allergies and consequently used locally a small amount of produced pure camphor crystals to clear the nasal passages. Moreover, the patient consumed a heaping teaspoon of loose camphor crystals (on the day of admission) for purpose of digestion improvement. A few hours later, the patient developed abdominal pain and nausea, palpitations and confusion, headache, followed by two grand mal seizures and admission to an emergency department. Camphor is rapidly absorbed in the gastrointestinal tract (found in the blood within minutes) as well as through the skin and via inhalation. Large ingestions generally result in nausea, vomiting and mucosal irritation within 5 to 15 minutes, along with tonic-clonic convulsions, which often are the first sign of intoxication. Finding the cause of new-onset seizures is often challenging for emergency health care providers and neurologists, therefore careful history taking is essential, including questions about the use of herbal medications and supplements [23].

Discussion

As herbal product use has become more common, patients are likely to be using these products alongside conventional medication or even instead of it. Healthcare professionals need to be aware of herbal products' adverse effects and interactions with conventional medication. Many studies showed that patients often do not wish to inform their healthcare professionals regarding herbal product use, therefore such information is not available in their medical records [24]. The reports of photosensitivity rash, hypertension, hypokalemia, and neurological adverse effects related to concurrent multi-herbal formula, ginseng, liquorice, adulterants, tartarian buckwheat and camphor use are a reminder of the importance of investigation of patients' herbal product use.

Conclusion

Herbal medications play an important role in the general health care of people in many countries worldwide. Most of herbal medications can be used safely and healthcare professionals need to discuss or advise patients regarding their use. Adverse effects or toxicities can arise from the herbal products. A rational approach to such situations should focus on good resuscitation, symptomatic and supportive care.

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